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(54) Title: ANTI-STAPHYLOCOCCUS AUREUS ANTIBODY RIFAMYCIN CONJUGATES AND USES THEREOF

(57) Abstract: The invention provides rF1 antibody antibiotic conjugates and methods of using same.

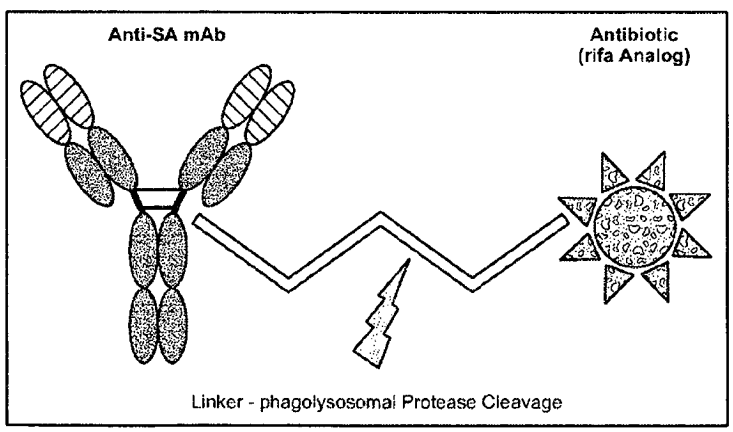

FIG. 2

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AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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ANTI-STAPHYLOCOCCUS AUREUS ANTIBODY RIFAMYCIN CONJUGATES AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 62/087,213, filed December 3, 2014, which is incorporated herein by reference in its entirety for all purposes.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on December 1, 2015, is named P32350WO_PCTSequenceListing.txt and is 26,747 bytes in size.

TECHNICAL FIELD

The invention relates to anti-Staphylococcus antibodies conjugated to rifamycin-type antibiotics and to use of the resultant antibody-antibiotic conjugates in the treatment of Staphylococcus infections.

BACKGROUND OF THE INVENTION

Staphylococcus aureus and *S. epidermidis* are successful human commensals that primarily colonize the nares and skin. *Staphylococcus aureus* (*S. aureus*; SA) can also invade a variety of tissues, leading to life-threatening infections; it is the leading cause of bacterial infections in humans worldwide. Recently emerged strains of *S. aureus* show increased virulence and enhanced ability to cause disease in otherwise healthy individuals. Over the last several decades, infection with *S. aureus* has become increasingly difficult to treat due to the emergence and rapid spread of methicillin-resistant *S. aureus* (MRSA) that is resistant to all known beta-lactam antibiotics (Boucher, H.W., *et al.* (2009) *Clin Infect Dis* 48, 1-12). Currently, the most prevalent and most virulent clinical strain of methicillin resistant *S. aureus* (MRSA) is USA300, which has the capacity to produce a large number of virulence factors and cause mortality in infected individuals (Chambers, HF and Deleo FR (2009) Nature Reviews Microbiology 7:629-641). The most serious infections such as endocarditis, osteomyelitis, necrotizing pneumonia and sepsis occur following dissemination of the bacteria into the bloodstream (Lowy, F.D. (1998) *N ENGL J MED* **339**, 520-532). *S. epidermidis*, which is

closely related to *S. aureus*, is often associated with hospital-acquired infections, and represents the most common source of infections on indwelling medical devices.

Important for staphylococcal adhesion to and successful colonization of host tissues, is a family of cell wall proteins, characterized by a large stretch of serine-aspartate dipeptide (SDR) repeats adjacent to an adhesive A-domain, that is present in staphylococci (Foster TJ, Hook M (1998) Trends Microbiol 6: 484-488). Such proteins important for adherence include clumping factor (Clf)A and ClfB (Foster TJ, supra). In addition to ClfA and ClfB, *S. aureus* also expresses three SDR-proteins, SdrC, SdrD and SdrE, which are organized in tandem in the genome. These proteins are also thought to be involved in tissue colonization, and elimination of any of them decreases bacterial virulence (Cheng AG, et al. (2009) FASEB Journal 23: 3393-3404). Three additional members of this family, SrdF, SdrG and SdrH, are present in most *S. epidermidis* strains (McCrea KW, et al. (2000) The serine-aspartate repeat (Sdr) protein family in *Staphylococcus epidermidis*. Microbiology 146 (Pt 7): 1535-1546). In each of these proteins, the SDR-region, which contains between 25 and 275 SD-dipeptide repeats (SEQ ID NO: 24), is located between the N-terminal ligand-binding A-domain and a C-terminal LPXTG-motif (SEQ ID NO: 25), which mediates anchoring to the cell wall by the transpeptidase sortase A. The function of the SDR-domain remains unknown, although it has been proposed to act as a cell wall spanning domain allowing exposure of the N terminal ligand binding sites of these proteins (Hartford O, et al. (1997) Mol Microbiol 25: 1065-1076).

It was found that the SDR-domains of all SDR-proteins of *S. aureus* and *S. epidermidis* are heavily glycosylated by two novel glycosyltransferases, SdgA and SdgB, which are responsible for glycosylation in two steps (Hazenbos et al. (2013) PLOS Pathogens 9 (10):1-18). These glycosylation events prevent degradation of these proteins by host proteases, thereby preserving bacterial host tissue interactions. Hazenbos et al. (2013) also showed that the SdgB-mediated glycosylation creates an immunodominant epitope for highly opsonic antibodies in humans. These antibodies account for a significant proportion of the total anti-staphylococcal IgG response.

Invasive MRSA infections are hard to treat, with a mortality rate of ~20% and are the leading cause of death by an infectious agent in the USA. Vancomycin, linezolid and daptomycin have thus become the few antibiotics of choice for treating invasive MRSA infections (Boucher, H., Miller, L.G. & Razonable, R.R. (2010) *Clin Infect Dis* 51 Suppl 2, S183-197). However, reduced susceptibility to vancomycin and cross-resistance to linezolid and daptomycin have already been reported in MRSA clinical strains (Nannini, E., Murray, B.E. & Arias, C.A. (2010) *Curr Opin Pharmacol* **10**, 516-521). Over time, the vancomycin dose

necessary to overcome resistance has crept upward to levels where nephrotoxicity occurs. Thus, mortality and morbidity from invasive MRSA infections remains high despite these antibiotics.

Investigations have revealed that *S. aureus* is able to invade and survive inside mammalian cells including the phagocytic cells that are responsible for bacterial clearance (Thwaites, G.E. & Gant, V. (2011) *Nat Rev Microbiol* **9**, 215-222); Rogers, D.E., Tompsett, R. (1952) *J. Exp. Med* **95**, 209-230); Gresham, H.D., *et al.* (2000) *J Immunol* **164**, 3713-3722); Kapral, F.A. & Shayegani, M.G. (1959) *J Exp Med* **110**, 123-138; Anwar, S., et al. (2009) *Clin Exp Immunol* **157**, 216-224); Fraunholz, M. & Sinha, B. (2012) *Front Cell Infect Microbiol* **2**, 43); Garzoni, C. & Kelley, W.L. (2011) *EMBO Mol Med* **3**, 115-117). *S. aureus* is taken up by host phagocytic cells, primarily neutrophils and macrophages, within minutes following intravenous infection (Rogers, D.E. (1956) *JEM* **103**, 713). While the majority of the bacteria are effectively killed by these cells, incomplete clearance of *S. aureus* inside blood borne phagocytes can allow these infected cells to act as "Trojan horses" for dissemination of the bacteria away from the initial site of infection. Indeed, patients with normal neutrophil counts may be more prone to disseminated disease than those with reduced neutrophil counts (Thwaites, G.E. & Gant, V. (2011) supra). Once delivered to the tissues, *S. aureus* can invade various non-phagocytic cell types, and intracellular *S. aureus* in tissues is associated with chronic or recurrent infections. Furthermore, exposure of intracellular bacteria to suboptimal antibiotic concentrations may encourage the emergence of antibiotic resistant strains, thus making this clinical problem more acute. Consistent with these observations, treatment of patients with invasive MRSA infections such as bacteremia or endocarditis with vancomycin or daptomycin was associated with failure rates greater than 50% (Kullar, R., Davis, S.L., Levine, D.P. & Rybak, M.J. Impact of vancomycin exposure on outcomes in patients with methicillin-resistant Staphylococcus aureus bacteremia: support for consensus guidelines suggested targets. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 52, 975-981 (2011); Fowler, V.G., Jr. et al. Daptomycin versus standard therapy for bacteremia and endocarditis caused by Staphylococcus aureus. *The New England journal of medicine* 355, 653-665 (2006); Yoon, Y.K., Kim, J.Y., Park, D.W., Sohn, J.W. & Kim, M.J. Predictors of persistent methicillin-resistant Staphylococcus aureus bacteraemia in patients treated with vancomycin. *The Journal of antimicrobial chemotherapy* 65:1015-1018 (2010)). Therefore, a more successful anti-staphylococcal therapy should include the elimination of intracellular bacteria.

Ansamycins are a class of antibiotics, including rifamycin, rifampin, rifampicin, rifabutin, rifapentine, rifalazil, ABI-1657, and analogs thereof, that inhibit bacterial RNA

polymerase and have exceptional potency against gram-positive and selective gram-negative bacteria (Rothstein, D.M., et al (2003) Expert Opin. Invest. Drugs 12(2):255-271; US 7342011; US 7271165).

Immunotherapies have been reported for preventing and treating *S. aureus* (including MRSA) infections. US2011/0262477 concerns uses of bacterial adhesion proteins Eap, Emp and AdsA as vaccines to stimulate immune response against MRSA. WO2000071585 describes isolated monoclonal antibodies reactive to specific *S. aureus* strain isolates. US20110059085A1 suggests an Ab-based strategy utilizing IgM Abs specific for one or more SA capsular antigens, although no actual antibodies were described.

Antibody-drug conjugates (ADC), also known as immunoconjugates, are targeted chemotherapeutic molecules which combine ideal properties of both antibodies and cytotoxic drugs by targeting potent cytotoxic drugs to antigen-expressing tumor cells (Teicher, B.A. (2009) *Curr. Cancer Drug Targets* 9:982-1004), thereby enhancing the therapeutic index by maximizing efficacy and minimizing off-target toxicity (Carter, P.J. and Senter P.D. (2008) *The Cancer J.*. 14(3):154-169; Chari, R.V. (2008) *Acc. Chem. Res.* 41:98-107. ADC comprise a targeting antibody covalently attached through a linker unit to a cytotoxic drug moiety. Immunoconjugates allow for the targeted delivery of a drug moiety to a tumor, and intracellular accumulation therein, where systemic administration of unconjugated drugs may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated (Polakis P. (2005) *Curr. Opin. Pharmacol.* 5:382-387).

Non-specific immunoglobulin-antibiotic conjugates are described that bind to the surface of target bacteria via the antibiotic for treating sepsis (US 5545721; US 6660267). Antibiotic-conjugated antibodies are described that have an antigen-binding portion specific for a bacterial antigen (such as SA capsular polysaccharide), but lack a constant region that reacts with a bacterial Fc-binding protein, e.g., staphylococcal protein A (US 7569677).

In view of the alarming rate of resistance of MRSA to conventional antibiotics and the resultant mortality and morbidity from invasive MRSA infections, there is a high unmet need for new therapeutics to treat S. aureus infections. The present invention satisfies this need and by providing compositions and methods that overcome the limitations of current therapeutic compositions as well as offer additional advantages that will be apparent from the detailed description below.

SUMMARY OF THE INVENTION

The present invention provides a unique therapeutic that includes the elimination of intracellular bacteria. The present invention demonstrates that such a therapeutic is efficacious in-vivo where conventional antibiotics like vancomycin fail.

The invention provides compositions referred to as "antibody-antibiotic conjugates," or "AAC") comprising an antibody conjugated by a covalent attachment to one or more rifamycin-type antibiotic moieties.

An aspect of the invention is an antibody-antibiotic conjugate compound comprising an rF1 antibody, covalently attached by a protease-cleavable, non-peptide linker to a rifamycin-type antibiotic.

An exemplary embodiment of the invention is an antibody-antibiotic conjugate having the formula:

$$Ab–(PML–abx)_p$$

wherein:

Ab is the rF1 antibody;

PML is the protease-cleavable, non-peptide linker having the formula:

$$–Str–PM–Y–$$

where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit;

abx is the rifamycin-type antibiotic; and

p is an integer from 1 to 8.

The antibody-antibiotic conjugate compounds of any of the preceding embodiments can comprise any one of the anti-SDR Abs and specifically rF1 antibodies described herein. These rF1 antibodies bind to Staphylococcus aureus. In exemplary rF1 antibodies, the Ab is a monoclonal antibody comprising a light (L) chain and a heavy (H) chain, the L chain comprising CDR L1, CDR L2, and CDR L3 and the H chain comprising CDR H1, CDR H2 and CDR H3, wherein the CDR H1, CDR H2 and CDR H3 and the CDR L1, CDR L2, and CDR L3 and comprise the amino acid sequences of the CDRs of each of Abs F1 (SEQ ID NO. 1-6), rF1 (SEQ ID NO. 1-5,7), rF1.v1 (SEQ ID NO. 1,8,3,4-6), respectively, as indicated in Tables 4A and 4B.

In some embodiments, the rF1 antibody comprises a heavy chain variable region (VH), wherein the VH comprises at least 95% sequence identity over the length of the VH region selected from the VH sequence of SEQ ID NO.13. The antibodies may further comprise a L chain variable region (VL) wherein the VL comprises at least 95% sequence identity over the length of the VL region selected from the VL sequence of SEQ ID NO.14 and SEQ ID NO.15, of antibodies rF1 and rF1.v6, respectively.

In specific embodiments, the rF1 antibody comprises L and H chain pairs as follows: a L chain comprising the sequence of SEQ ID NO. 9 paired with a H chain comprising the sequence of SEQ ID NO.10; L chain comprising the sequence of SEQ ID NO. 11paired with a H chain comprising the sequence of SEQ ID NO.10; a L chain comprising the sequence of SEQ ID NO. 11 paired with a H chain comprising the sequence of SEQ ID NO.12.

In any one of the preceding embodiments, the antibody may be an antigen-binding fragment lacking a Fc region. In some embodiments, the antibody is a F(ab) or $F(ab')_2$. In some embodiments, the antibody further comprises a heavy chain constant region and/or a light chain constant region, wherein the heavy chain constant region and/or the light chain constant region comprise one or more amino acids that are substituted with cysteine residues. In some embodiments, the heavy chain constant region comprises amino acid substitution A118C and/or S400C, and/or the light chain constant region comprises amino acid substitution V205C, wherein the numbering system is according to EU numbering.

In some embodiments of any of the antibodies described above, the antibody is not an IgM isotype. In some embodiments of any of the antibodies described above, the antibody is an IgG (e.g., IgG1, IgG2, IgG3, IgG4), IgE, IgD, or IgA (e.g., IgA1 or IgA2) isotype.

An exemplary embodiment of the invention is a pharmaceutical composition comprising the antibody-antibiotic conjugate compound, and a pharmaceutically acceptable carrier, glidant, diluent, or excipient.

Another aspect of the invention is a method of treating a bacterial infection comprising administering to an infected patient a therapeutically-effective amount of the antibody-antibiotic conjugate of any of the preceding embodiments. Another aspect of the invention is a method of treating a Staphylococcal infection in a patient comprising administering to the patient a therapeutically-effective amount of an antibody-antibiotic conjugate of the invention. In one embodiment, the patient is a human. In one embodiment the patient is infected with a *Staphylococcus aureus* and/or a *Staphylococcus epidermidis* infection. In some embodiments,

the patient has been diagnosed with a S. aureus infection. In some embodiments, treating the bacterial infection comprises reducing the bacterial load or counts.

Another aspect of the invention is a method of treating a Staphylococcal infection in an infected patient comprising administering to the patient a therapeutically-effective amount of an antibody-antibiotic conjugate of any one of the preceding embodiments. In one embodiment, the patient is a human. In one embodiment the bacterial infection is a *Staphylococcus aureus* infection. In some embodiments, the patient has been diagnosed with a S. aureus infection. In some embodiments, treating the bacterial infection comprises reducing the bacterial load or counts.

In one embodiment of any of the preceding methods of treatment, the is administered to patients where the bacterial infection including S. aureus has led to bacteremia. In specific embodiments the method is used to treat Staphylococcal endocarditis or osteomyelitis. In one embodiment, the antibody-antibiotic conjugate compound is administered to the infected patient at a dose in the range of about 50mg/kg to 100mg/kg.

Also provided is method of killing intracellular *S. aureus* in the cells of a *S. aureus* infected patient without killing the host cells by administering a rF1 antibiotic conjugate compound of any of the above embodiments. Another method is provided for killing persister Staphylococcal bacterial cells (e.g, *S. aureus*) in vivo by contacting the persister bacteria with an AAC of any of the preceding embodiments.

In another embodiment, the method of treatment further comprises administering a second therapeutic agent. In a further embodiment, the second therapeutic agent is an antibiotic including an antibiotic against Staph aureus in general or MRSA in particular.

In one embodiment, the second antibiotic administered in combination with the antibody-antibiotic conjugate compound of the invention is selected from the structural classes: (i) aminoglycosides; (ii) beta-lactams; (iii) macrolides/cyclic peptides; (iv) tetracyclines; (v) fluoroquinolines/fluoroquinolones; (vi) and oxazolidinones.

In one embodiment, the second antibiotic administered in combination with the antibody-antibiotic conjugate compound of the invention is selected from clindamycin, novobiocin, retapamulin, daptomycin, GSK-2140944, CG-400549, sitafloxacin, teicoplanin, triclosan, napthyridone, radezolid, doxorubicin, ampicillin, vancomycin, imipenem, doripenem, gemcitabine, dalbavancin, and azithromycin.

In some embodiments herein, the bacterial load in the infected patient has been reduced to an undetectable level after the treatment. In one embodiment, the patient's blood culture is negative after treatment as compared to a positive blood culture before treatment. In some

embodiments herein, the bacterial resistance in the subject is undetectable or low. In some embodiments herein, the patient is not responsive to treatment with methicillin or vancomycin.

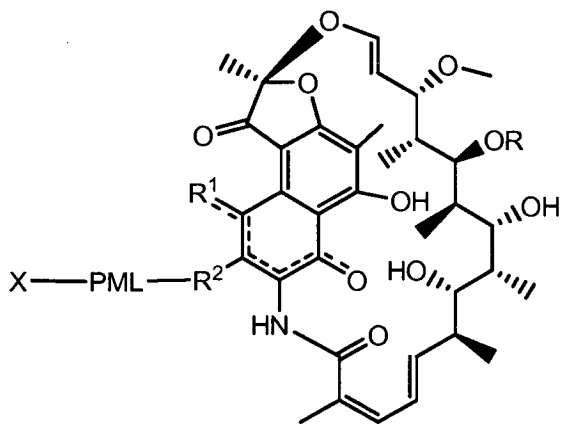
An exemplary embodiment of the invention is a process for making the antibody-antibiotic conjugate comprising conjugating a rifamycin-type antibiotic to an rF1 antibody.

An exemplary embodiment of the invention is a kit for treating a bacterial infection, comprising:

a) the pharmaceutical composition comprising the antibody-antibiotic conjugate compound, and a pharmaceutically acceptable carrier, glidant, diluent, or excipient; and

b) instructions for use.

An aspect of the invention is an antibiotic-linker intermediate having Formula II:



II

wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

R^1 is OH;

R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH;

PML is a protease-cleavable, non-peptide linker attached to R^2 or the fused heteroaryl or heterocyclyl formed by R^1 and R^2; and having the formula:

–Str–PM–Y–

where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit; and

X is a reactive functional group selected from maleimide, thiol, amino, bromide, bromoacetamido, iodoacetamido, p-toluenesulfonate, iodide, hydroxyl, carboxyl, pyridyl disulfide, and N-hydroxysuccinimide.

It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present invention. These and other aspects of the invention will become apparent to one of skill in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1F: Intracellular stores of MRSA are protected from vancomycin in vivo and in vitro. Figure 1A shows a schematic of the experimental design for generating free bacteria (planktonic) vs. intracellular bacteria. Four cohorts of mice were infected by intravenous injection with roughly equivalent doses of viable free bacteria or intracellular bacteria and selected groups were treated with vancomycin immediately after infection and then once per day (see Example 2). Figure 1B and Figure 1C show bacterial loads in kidney and brain, respectively of infected mice 4 days post infection. The dashed line indicates the limit of detection for the assay. Figure 1D shows that MRSA is protected from vancomycin when cultured on a monolayer of infectable cells. (ND = none detected). Figure 1E and Figure 1F show that MRSA is able to grow in the presence of vancomyicn when cultured on a monolayer of infectable cells. MRSA (free bacteria) was seeded in media, media + vancomycin, or media + vancomycin and plated on a monolayer of MG63 osteoblasts (Figure 1E) or Human Brain Microvascular Endothelial Cells (HBMEC, Figure 1F). Extracellular bacteria (free bacteria) grew well in media alone, but were killed by vancomycin. In wells containing a monolayer of mammalian cells (Intracellular + vanco) a fraction of the bacteria were protected from vancomycin during the first 8 hours after infection and were able to expand within the intracellular compartment over 24 hours. Error bars show standard deviation for triplicate wells.

Figure 2: shows the concept of an Antibody Antibiotic Conjugate (AAC). In one example, the AAC consists of an antibody directed against an epitope on the surface of S. aureus linked to a potent rifamycin-type antibiotic (e.g. Rifalog) via a linker that is cleaved by lysosomal proteases.

Figure 3 shows a possible mechanism of drug activation for antibody-antibiotic conjugates (AAC). AACs bind to extracellular bacteria via the antigen binding domain (Fab) of the antibody and promote uptake of the opsonized bacteria via Fc-mediated phagocytosis. The

linker is cleaved by lysosomal proteases such as cathepsin B. Following cleavage of the linker, the linker is hydrolyzed releasing free antibiotic inside the phagolysosome. The free antibiotic kills the opsonized and phagocytosed bacteria along with any previously internalized bacteria residing in the same compartment.

Figures 4A and 4B show aspects of serine-aspartate (SDR) proteins. Figure 4A shows alignment of SDR proteins revealed by mass-spectrometry from *S. aureus* and *S. epidermidis*. SDR-regions are indicated by hatches. The rF1 epitope is expressed in abundance since there are multiple SDR proteins on S. aureus and multiple epitopes per protein. Figure 4A discloses 'SDSDSDSD' as SEQ ID NO: 27. Figure 4B is a model showing the step-wise glycosylation of SDR proteins by SdgA and SdgB. First, SdgB appends GlcNAc moieties onto the SD-region on SDR proteins, followed by additional GlcNAc modification by SdgA. The epitope for mAb rF1 includes the SdgB-dependent GlcNAc moieties. Data suggests that rF1 binds to GlcNac and parts of the SD backbone. Figure 4B discloses 'SDSDSD' as SEQ ID NO: 28.

Figures 5A, 5B and 5C show mAb rF1 exhibits robust binding to and killing of *S. aureus* bacteria. (Figures A-C) Bacteria were preopsonized with huIgG1 mAbs rF1 (squares), 4675 anti-ClfA (triangles), or anti-herpes virus gD (circles). (Figure 5A): Binding of mAbs to WT (USA300-Δ*spa*) bacteria was assessed by flow cytometry, and expressed as mean fluorescent intensity (MFI). (Figure 5B): CFSE-labeled, preopsonized WT (USA300-Δ*spa*) bacteria were incubated with human PMN. Bacterial uptake was expressed as % of CFSE-positive PMN, after gating for CD11b-positive cells by flow cytometry. (Figure 5C): Preopsonized WT (USA300-Δ*spa*) bacteria were incubated with PMN to assess bacterial killing. Numbers of viable CFU per mL are representative of at least three experiments.

Figure 6 shows flow cytometry analysis of binding of rF1 to *S. aureus* from various infected tissues. Homogenized tissues were double stained with mAb rF1 (X-axis), and with anti-peptidoglycan mAb 702 to distinguish bacteria from tissue debris (Y-axis) (left panel; gate indicated by arrow), followed by gating of bacteria to generate histogram figures (see also, Hazenbos et al. (2013) PLOS Pathogens 9 (10):1-18, Fig. 1D).

Figure 7 shows binding of rF1 to various staphylococcal and non-staphylococcal Gram-positive bacterial species by flow cytometry (see also, Hazenbos et al. (2013) PLOS Pathogens 9 (10):1-18, Fig. 1E).

Figure 8 shows selection of a potent rifamycin-type antibiotic (rifalog) dimethylpipBOR for its ability to kill non-replicating MRSA.

Figure 9: Growth inhibition assay demonstrating that intact TAC (a form of AAC) does not kill planktonic bacteria unless the antibiotic is released by treatment with cathepsin B. TAC was incubated in buffer alone (open circles) or treated with cathepsin B (closed circles). The intact TAC was not able to prevent bacterial growth after overnight incubation. Pretreatment of the TAC with cathepsin B released sufficient antibiotic activity to prevent bacterial growth at .6 ug/mL of TAC, which is predicted to contain .006 ug/mL of antibiotic.

Figure 10 shows efficacy of the rF1-AACs in an *in vitro* macrophage assay, as described in Example 19.

Figures 11A and 11B show the efficacy of the rF1-AACs *in vivo* as described in Example 20. Treatment of S. aureus infected mice with rF1-AACs greatly reduced or eradicated bacterial counts in infected organs as compared to naked antibody. Figure 11A shows treatment with AAC containing 2 antibiotic molecules per antibody (AAR2) reduced bacterial load in the kidneys by approximately 30-fold and treatment with the AAC containing 4 antibiotic molecules per antibody (AAR4) reduced bacterial burdens by more than 30,000-fold. Figure 11B shows that treatment with AAC AAR2 reduced bacterial burdens in the heart by approximately 70-fold with 6 out of 8 mice having undetectable level of bacteria in hearts; treatment with the AAC AAR4 completely eradicated infection in hearts resulting in 8 out of 8 mice having undetectable levels of bacteria.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

Reference will now be made in detail to certain embodiments of the invention, examples of which are illustrated in the accompanying structures and formulas. While the invention will be described in conjunction with the enumerated embodiments, including methods, materials and examples, such description is non-limiting and the invention is intended to cover all alternatives, modifications, and equivalents, whether they are generally known, or incorporated herein. In the event that one or more of the incorporated literature, patents, and similar materials differs from or contradicts this application, including but not limited to defined terms, term usage, described techniques, or the like, this application controls. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. One skilled in the art will recognize many

methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. The present invention is in no way limited to the methods and materials described.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

I. GENERAL TECHNIQUES

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* 3d edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; *Current Protocols in Molecular Biology* (F.M. Ausubel, et al. eds., (2003)); the series *Methods in Enzymology* (Academic Press, Inc.): *PCR 2: A Practical Approach* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) *Antibodies, A Laboratory Manual*, and *Animal Cell Culture* (R.I. Freshney, ed. (1987)); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., 1998) Academic Press; *Animal Cell Culture* (R.I. Freshney), ed., 1987); *Introduction to Cell and Tissue Culture* (J.P. Mather and P.E. Roberts, 1998) Plenum Press; *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-8) J. Wiley and Sons; *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991); *Short Protocols in Molecular Biology* (Wiley and Sons, 1999); *Immunobiology* (C.A. Janeway and P. Travers, 1997); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V.T. DeVita et al., eds., J.B. Lippincott Company, 1993).

The nomenclature used in this Application is based on IUPAC systematic nomenclature, unless indicated otherwise. Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs, and are consistent with: Singleton et al (1994) *Dictionary of Microbiology*

and Molecular Biology, 2nd Ed., J. Wiley & Sons, New York, NY; and Janeway, C., Travers, P., Walport, M., Shlomchik (2001) *Immunobiology*, 5th Ed., Garland Publishing, New York.

II. Definitions

Staphylococcus aureus is also referred to herein as Staph A or *S. aureus* in short. Likewise, *Staphylococcus epidermidis* is also referred herein as Staph E or *S. epidermidis*.

"Antibody Antibiotic Conjugate" or AAC is a compound composed of an antibody that is chemically linked to an antibiotic by a linker. The antibody binds an antigen or epitope on a bacterial surface, for example, a bacterial cell wall component. As used in this invention, the linker is a protease-cleavable, non-peptide linker that is designed to be cleaved by proteases, including cathepsin B, a lysosomal protease found in most mammalian cell types (Dubowchik et al (2002) Bioconj. Chem. 13:855-869). A diagram of the AAC with its 3 components is depicted in Figure 2. "THIOMAB™ Antibiotic Conjugate" or "TAC" is a form of AAC in which the antibody is chemically conjugated to a linker-antibiotic unit via one or more cysteines, generally a cysteine that is recombinantly engineered into the antibody at specific site(s) on the antibody to not interfere with the antigen binding function.

When indicating the number of substituents, the term "*one or more*" refers to the range from one substituent to the highest possible number of substitution, i.e. replacement of one hydrogen up to replacement of all hydrogens by substituents. The term "*substituent*" denotes an atom or a group of atoms replacing a hydrogen atom on the parent molecule. The term "*substituted*" denotes that a specified group bears one or more substituents. Where any group may carry multiple substituents and a variety of possible substituents is provided, the substituents are independently selected and need not to be the same. The term "*unsubstituted*" means that the specified group bears no substituents. The term "*optionally substituted*" means that the specified group is unsubstituted or substituted by one or more substituents, independently chosen from the group of possible substituents. When indicating the number of substituents, the term "*one or more*" means from one substituent to the highest possible number of substitution, i.e. replacement of one hydrogen up to replacement of all hydrogens by substituents.

The term "antibiotic" (abx or Abx) includes any molecule that specifically inhibits the growth of or kill micro-organisms, such as bacteria, but is non-lethal to the host at the concentration and dosing interval administered. In a specific aspect, an antibiotic is non-toxic to the host at the administered concentration and dosing intervals. Antibiotics effective against bacteria can be broadly classified as either bactericidal (*i.e.*, directly kills) or bacteriostatic (*i.e.*,

prevents division). Anti-bactericidal antibiotics can be further subclassified as narrow-spectrum or broad-spectrum. A broad-spectrum antibiotic is one effective against a broad range of bacteria including both Gram-positive and Gram-negative bacteria, in contrast to a narrow-spectrum antibiotic, which is effective against a smaller range or specific families of bacteria. Examples of antibiotics include: (i) aminoglycosides, *e.g.*, amikacin, gentamicin, kanamycin, neomycin, netilmicin, streptomycin, tobramycin, paromycin, (ii) ansamycins, e.g., geldanamycin, herbimycin, (iii) carbacephems, e.g., loracarbef, (iv), carbapenems, *e.g.,* ertapenum, doripenem, imipenem/cilastatin, meropenem, (v) cephalosporins (first generation), *e.g.,* cefadroxil, cefazolin, cefalotin, cefalexin, (vi) cephalosporins (second generation), e.g., ceflaclor, cefamandole, cefoxitin, cefprozil, cefuroxime, (vi) cephalosporins (third generation), e.g., cefixime, cefdinir, cefditoren, cefoperazone, cefotaxime, cefpodoxime, ceftazidime, ceftibuten, ceftizoxime, ceftriaxone, (vii) cephalosporins (fourth generation), e.g., cefepime, (viii), cephalosporins (fifth generation), *e.g.*, ceftobiprole, (ix) glycopeptides, e.g., teicoplanin, vancomycin, (x) macrolides, *e.g.*, axithromycin, clarithromycin, dirithromycine, erythromycin, roxithromycin, troleandomycin, telithromycin, spectinomycin, (xi) monobactams, *e.g.*, axtreonam, (xii) penicilins, *e.g.*, amoxicillin, ampicillin, axlocillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, meticillin, nafcilin, oxacillin, penicillin, peperacillin, ticarcillin, (xiii) antibiotic polypeptides, *e.g.*, bacitracin, colistin, polymyxin B, (xiv) quinolones, e.g., ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, lemefloxacin, moxifloxacin, norfloxacin, orfloxacin, trovafloxacin, (xv) sulfonamides, *e.g.*, mafenide, prontosil, sulfacetamide, sulfamethizole, sulfanilamide, sulfasalazine, sulfisoxazole, trimethoprim, trimethoprim-sulfamethoxazole (TMP-SMX), (xvi) tetracyclines, *e.g.*, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline and (xvii) others such as arspenamine, chloramphenicol, clindamycin, lincomycin, ethambutol, fosfomycin, fusidic acid, furazolidone, isoniazid, linezolid, metronidazole, mupirocin, nitrofurantoin, platensimycin, pyrazinamide, quinupristin/dalfopristin, rifampin/rifampicin or tinidazole.

The term "methicillin-resistant *Staphylococcus aureus"* (MRSA), alternatively known as multidrug resistant *Staphylococcus aureus* or *oxacillin-resistant Staphylococcus aureus* (ORSA), refers to any strain of *Staphylococcus aureus* that is resistant to beta-lactam antibiotics, which in include the penicillins (*e.g.*, methicillin, dicloxacillin, nafcillin, oxacillin, *etc.*) and the cephalosporins. "Methicillin-sensitive *Staphylococcus aureus"(MSSA)* refers to any strain of *Staphylococcus aureus* that is sensitive to beta-lactam antibiotics.

The term "minimum inhibitory concentration" ("MIC") refers to the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight

incubation. Assay for determining MIC are known. One method is as described in the Example section below.

The terms "anti-Staph a antibody" and "an antibody that binds to Staph a" refer to an antibody that is capable of binding an antigen on *Staphylococcus aureus* ("S. aureus") with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting S. aureus. In one embodiment, the extent of binding of an anti-Staph a antibody to an unrelated, non-Staph a protein is less than about 10% of the binding of the antibody to MRSA as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, an antibody that binds to Staph a has a dissociation constant (Kd) of $\leq$ 1μM, $\leq$ 100 nM, $\leq$ 10 nM, , $\leq$ 5 Nm, , $\leq$ 4 nM, , $\leq$ 3 nM, , $\leq$ 2 nM, $\leq$ 1 nM, $\leq$ 0.1 nM, $\leq$ 0.01 nM, or $\leq$ 0.001 nM (e.g., 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-13} M, e.g., from 10^{-9} M to 10^{-13} M). In certain embodiments, an anti-Staph a antibody binds to an epitope of Staph a that is conserved among Staph from different species. An anti-Staph antibody herein will refer to an antibody that binds to at least one more Staphylococcal species in addition S. Aureus.

"SDR" refers to serine-aspartate repeat; SDRs are are present in a family of cell wall proteins, characterized by a large stretch of serine-aspartate dipeptide repeats adjacent to an adhesive A-domain, that is present in staphylococci (Foster TJ, Hook M (1998) Trends Microbiol 6: 484-488). Such proteins involved in adherence include clumping factor (Clf)A and ClfB. In addition to ClfA and ClfB, *S. aureus* also expresses three SDR-proteins, SdrC, SdrD and SdrE, Three additional members of this family, SrdF, SdrG and SdrH, are present in most *S. epidermidis* strains (McCrea KW, et al. (2000) The serine-aspartate repeat (Sdr) protein family in *Staphylococcus epidermidis*. Microbiology 146 (Pt 7): 1535-1546). In each of these proteins, the SDR-region, which contains between 25 and 275 SD-dipeptide repeats (SEQ ID NO: 24), is located between the N-terminal ligand-binding A-domain and a C-terminal LPXTG-motif (SEQ ID NO: 25),

The antibody designated "F1" has heavy chain and light chain variable domain sequences as depicted in Figure 1 of US 8,617,556, which is incorporated herein by reference in its entirety. The CDR sequences of F1, which in particular contribute to the antigen-binding properties of F1, are also depicted in Figure 1. Antibody F1 is fully human, is capable of specifically binding *Staphylococcus* species such as *S. aureus* and *S. epidermidis* . Importantly, antibody F1 is capable of binding whole bacteria *in vivo* as well as *in vitro*. Furthermore, antibody F1 is capable of binding to bacteria that have been grown in infected tissue of, for example, an animal. Recombinantly produced F1 is herein also called "rF1". rF1 (and F1) antibody is an anti-SDR monoclonal Ab. The epitope for mAb rF1 includes the SdgB-dependent GlcNAc moieties. Data suggests that rF1 binds to

GlcNac and parts of the SD backbone. "rF1 antibody" as used herein encompasses the F1 antibody, the rF1 antibody as well as all variants of rF1containing amino acid alterations relative to rF1. The amino acid sequences of the rF1 and variant antibodies are provided below.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, dimers, multimers, multispecific antibodies (e.g., bispecific antibodies), and antigen binding antibody fragments thereof, (Miller et al (2003) J. of Immunology 170:4854-4861). Antibodies may be murine, human, humanized, chimeric, or derived from other species. An antibody is a protein generated by the immune system that is capable of recognizing and binding to a specific antigen (Janeway, C., Travers, P., Walport, M., Shlomchik (2001) Immuno Biology, 5th Ed., Garland Publishing, New York). A target antigen generally has numerous binding sites, also called epitopes, recognized by CDRs on multiple antibodies. Each antibody that specifically binds to a different epitope has a different structure. Thus, one antigen may be recognized and bound by more than one corresponding antibody. An antibody includes a full-length immunoglobulin molecule or an immunologically active portion of a full-length immunoglobulin molecule, i.e., a molecule that contains an antigen binding site that immunospecifically binds an antigen of a target of interest or part thereof, such targets including but not limited to, cancer cell or cells that produce autoimmune antibodies associated with an autoimmune disease, an infected cell or a microorganism such as a bacterium. The immunoglobulin (Ig) disclosed herein can be of any isotype except IgM (e.g., IgG, IgE, IgD, and IgA) and subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The immunoglobulins can be derived from any species. In one aspect, the Ig is of human, murine, or rabbit origin. In a specific embodiment, the Ig is of human origin.

The "class" of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively.

"Native antibodies" refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant

domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

The terms "full length antibody," "intact antibody," and "whole antibody" are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc region as defined herein.

An "antigen-binding fragment" of an antibody refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, $F(ab')_2$; diabodies; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation (e.g., natural variation in glycosylation), such variants generally being present in minor amounts. One such possible variant for IgG1 antibodies is the cleavage of the C-terminal lysine (K) of the heavy chain constant region. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies.

The term "chimeric antibody" refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

A "humanized antibody" refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A "humanized form" of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization.

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). (See, e.g., Kindt et al. Kuby Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007).) A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. See, e.g., Portolano et al., J. Immunol. 150:880-887 (1993); Clarkson et al., Nature 352:624-628 (1991).

The term "hypervariable region," "HVR," or "HV," when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence ("complementarity determining regions" or "CDRs") and/or form structurally defined loops and/or contain the antigen-contacting residues ("antigen contacts"). Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, e.g., Xu et al., Immunity 13:37-45 (2000); Johnson and Wu, in Methods in Molecular Biology 248:1-25 (Lo, ed., Human Press, Totowa,

NJ, 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain (Hamers-Casterman et al., (1993) Nature 363:446-448; Sheriff et al., (1996) Nature Struct. Biol. 3:733-736).

A number of HVR delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk, (1987) J. Mol. Biol. 196:901-917). For antigen contacts, refer to MacCallum et al. *J. Mol. Biol.* 262: 732-745 (1996). The AbM HVRs represent a compromise between the Kabat HVRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" HVRs are based on an analysis of the available complex crystal structures. The residues from each of these HVRs are noted below.

Loop	Kabat	AbM	Chothia	Contact
L1	L24-L34	L24-L34	L26-L32	L30-L36
L2	L50-L56	L50-L56	L50-L52	L46-L55
L3	L89-L97	L89-L97	L91-L96	L89-L96
H1	H31-H35B	H26-H35B	H26-H32	H30-H35B (Kabat numbering)
H1	H31-H35	H26-H35	H26-H32	H30-H35 (Chothia numbering)
H2	H50-H65	H50-H58	H53-H55	H47-H58
H3	H95-H102	H95-H102	H96-H101	H93-H101

HVRs may comprise "extended HVRs" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 or 89-96 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102, or 95-102 (H3) in the VH. Unless otherwise indicated, HVR residues, CDR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al., *supra*.

The expression "variable-domain residue-numbering as in Kabat" or "amino-acid-position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy-chain variable domains or light-chain variable domains of the compilation of antibodies in Kabat et al., supra. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or HVR of the variable domain. For example, a heavy-chain variable domain may include a

single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc. according to Kabat) after heavy-chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

"Framework" or "FR" refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

An "acceptor human framework" for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al., supra. In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al., supra.

The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain. The term includes native-sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy-chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system - also called the EU index, as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National

Institutes of Health, Bethesda, MD, 1991) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue. The term "Fc receptor" or "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus. Guyer et al., J. Immunol. 117: 587 (1976) and Kim et al., J. Immunol. 24: 249 (1994). Methods of measuring binding to FcRn are known (see, e.g., Ghetie and Ward, Immunol. Today 18: (12): 592-8 (1997); Ghetie et al., Nature Biotechnology 15 (7): 637-40 (1997); Hinton et al., J. Biol. Chem. 279(8): 6213-6 (2004); WO 2004/92219 (Hinton et al.). Binding to FcRn in vivo and serum half-life of human FcRn high-affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides having a variant Fc region are administered. WO 2004/42072 (Presta) describes antibody variants which improved or diminished binding to FcRs. See also, e.g., Shields et al., J. Biol. Chem. 9(2): 6591-6604 (2001).

An "affinity matured" antibody refers to an antibody with one or more alterations in one or more hypervariable regions (HVRs), compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for antigen.

The term "epitope" refers to the particular site on an antigen molecule to which an antibody binds.

An "antibody that binds to the same epitope" as a reference antibody refers to an antibody that blocks binding of the reference antibody to its antigen in a competition assay by 50% or more, and conversely, the reference antibody blocks binding of the antibody to its antigen in a competition assay by 50% or more. An exemplary competition assay is provided herein.

A "naked antibody" refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.

"Effector functions" refer to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding;

antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

"Antibody-dependent cell-mediated cytotoxicity" or ADCC refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., natural killer (NK) cells, neutrophils and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are required for killing of the target cell by this mechanism. The primary cells for mediating ADCC, NK cells, express Fcγ(gamma)RIII only, whereas monocytes express Fcγ(gamma)RI, Fcγ(gamma)RII and Fcγ(gamma)RIII. Fc expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol. 9: 457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in US 5,500,362 or US 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and natural killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes et al., PNAS USA 95:652-656 (1998).

"Phagocytosis" refers to a process by which a pathogen is engulfed or internalized by a host cell (e.g., macrophage or neutrophil). Phagocytes mediate phagocytosis by three pathways: (i) direct cell surface receptors (for example, lectins, integrins and scavenger receptors) (ii) complement enhanced - using complement receptors (including CRI, receptor for C3b, CR3 and CR4) to bind and ingest complement opsonized pathogens, and (iii) antibody enhanced - using Fc Receptors (including FcγgammaRI, FcγgammaRIIA and FcγgammaRIIIA) to bind antibody opsonized particles which then become internalized and fuse with lysosomes to become phagolysosomes. In the present invention, it is believed that pathway (iii) plays a significant role in the delivery of the anti-MRSA AAC therapeutics to infected leukocytes, e.g., neutrophils and macrophages (Phagocytosis of Microbes: complexity in Action by D. Underhill and A Ozinsky. (2002) Annual Review of Immunology, Vol 20:825).

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g., as described in Gazzano-Santoro et al., J. Immunol. Methods 202: 163 (1996), may be performed.

The carbohydrate attached to the Fc region may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, e.g., Wright et al. (1997) TIBTECH 15:26-32. The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GIcNAc), galactose, and sialic acid, as well as a fucose attached to a GIcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an IgG may be made in order to create IgGs with certain additionally improved properties. For example, antibody modifications are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. Such modifications may have improved ADCC function. See, e.g. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to "defucosylated" or "fucose-deficient" antibody modifications include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031140; Okazaki et al., J. Mol. Biol. 336: 1239-1249 (2004); Yamane-Ohnuki et al. Biotech. Bioeng. 87: 614 (2004). Examples of cell lines capable of producing defucosylated antibodies include Lee 13 CHO cells deficient in protein fucosylation (Ripka et al. Arch. Biochem. Biophys. 249:533-545 (1986); US Pat. Appl. Pub. No. 2003/0157108 Al, Presta, L; and WO 2004/056312 Al, Adams et al., especially at Example 11), and knockout cell lines, such as alpha- 1,6-fucosyltransferase gene, FUT8, knockout CHO cells (see, e.g., Yamane-Ohnuki et al., Biotech. Bioeng. 87: 614 (2004); Kanda, Y. et al, Biotechnol. Bioeng., 94(4):680-688 (2006); and WO2003/085107).

An "isolated antibody" is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman et al., J. Chromatogr. B 848:79-87 (2007).

An "isolated nucleic acid" refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

"Isolated nucleic acid encoding a rF1antibody" refers to one or more nucleic acid molecules encoding antibody heavy and light chains, including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

As use herein, the term "specifically binds to" or is "specific for" refers to measurable and reproducible interactions such as binding between a target and an antibody, which is determinative of the presence of the target in the presence of a heterogeneous population of molecules including biological molecules. For example, an antibody that specifically binds to a target (which can be an epitope) is an antibody that binds this target with greater affinity, avidity, more readily, and/or with greater duration than it binds to other targets. In one embodiment, the extent of binding of an antibody to a target unrelated to rF1 is less than about 10% of the binding of the antibody to the target as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, an antibody that specifically binds to rF1 has a dissociation constant (Kd) of $\leq$ 1μM, $\leq$ 100 nM, $\leq$ 10 nM, $\leq$ 1 nM, or $\leq$ 0.1 nM. In certain embodiments, an antibody specifically binds to an epitope on that is conserved from different species. In another embodiment, specific binding can include, but does not require exclusive binding.

"Binding affinity" generally refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity that reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

In one embodiment, the "Kd" or "Kd value" according to this invention is measured by a radiolabeled antigen-binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as described by the following assay. Solution-binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, e.g., Chen et al., (1999) J. Mol. Biol. 293:865-881). To

establish conditions for the assay, microtiter plates (DYNEX Technologies, Inc.) are coated overnight with 5 μg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbent plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (e.g., consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta et al., Cancer Res. 57:4593-4599 (1997)). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (e.g., about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (e.g., for one hour). The solution is then removed and the plate washed eight times with 0.1% TWEEN-20™ surfactant in PBS. When the plates have dried, 150 μl/well of scintillant (MICROSCINT-20™; Packard) is added, and the plates are counted on a TOPCOUNT™ gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

According to another embodiment, the Kd is measured by using surface-plasmon resonance assays using a BIACORE®-2000 or a BIACORE®-3000 instrument (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIAcore Inc.) are activated with N-ethyl-N'- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 μg/ml (~0.2 μM) before injection at a flow rate of 5 μl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% TWEEN 20™ surfactant (PBST) at 25°C at a flow rate of approximately 25 μl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIAcore® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on}. See, e.g., Chen et al., J. Mol. Biol. 293:865-881 (1999). If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface-plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence-emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25 °C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow-equipped spectrophotometer (Aviv

Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

An "on-rate," "rate of association," "association rate," or "k_{on}" according to this invention can also be determined as described above using a BIACORE®-2000 or a BIACORE®-3000 system (BIAcore, Inc., Piscataway, NJ).

The terms "host cell," "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

The term "vector," as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors".

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the

source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y, where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described.

The term "rifamycin-type antibiotic" means the class or group of antibiotics having the structure of, or similar structure to, rifamycin.

The term "rifalazil-type antibiotic" means the class or group of antibiotics having the structure of, or similar structure to, rifalazil.

When indicating the number of substituents, the term “one or more” refers to the range from one substituent to the highest possible number of substitution, i.e. replacement of one hydrogen up to replacement of all hydrogens by substituents. The term “substituent” denotes an atom or a group of atoms replacing a hydrogen atom on the parent molecule. The term “substituted” denotes that a specified group bears one or more substituents. Where any group may carry multiple substituents and a variety of possible substituents is provided, the substituents are independently selected and need not to be the same. The term “unsubstituted” means that the specified group bears no substituents. The term “optionally substituted” means that the specified group is unsubstituted or substituted by one or more substituents, independently chosen from the group of possible substituents. When indicating the number of substituents, the term “one or more” means from one substituent to the highest possible number of substitution, i.e. replacement of one hydrogen up to replacement of all hydrogens by substituents.

The term “alkyl” as used herein refers to a saturated linear or branched-chain monovalent hydrocarbon radical of one to twelve carbon atoms (C_1–C_{12}), wherein the alkyl radical may be

optionally substituted independently with one or more substituents described below. In another embodiment, an alkyl radical is one to eight carbon atoms (C_1–C_8), or one to six carbon atoms (C_1–C_6). Examples of alkyl groups include, but are not limited to, methyl (Me, -CH_3), ethyl (Et, -CH_2CH_3), 1-propyl (n-Pr, n-propyl, -$CH_2CH_2CH_3$), 2-propyl (i-Pr, i-propyl, -$CH(CH_3)_2$), 1-butyl (n-Bu, n-butyl, -$CH_2CH_2CH_2CH_3$), 2-methyl-1-propyl (i-Bu, i-butyl, -$CH_2CH(CH_3)_2$), 2-butyl (s-Bu, s-butyl, -$CH(CH_3)CH_2CH_3$), 2-methyl-2-propyl (t-Bu, t-butyl, -$C(CH_3)_3$), 1-pentyl (n-pentyl, -$CH_2CH_2CH_2CH_2CH_3$), 2-pentyl (-$CH(CH_3)CH_2CH_2CH_3$), 3-pentyl (-$CH(CH_2CH_3)_2$), 2-methyl-2-butyl (-$C(CH_3)_2CH_2CH_3$), 3-methyl-2-butyl (-$CH(CH_3)CH(CH_3)_2$), 3-methyl-1-butyl (-$CH_2CH_2CH(CH_3)_2$), 2-methyl-1-butyl (-$CH_2CH(CH_3)CH_2CH_3$), 1-hexyl (-$CH_2CH_2CH_2CH_2CH_2CH_3$), 2-hexyl (-$CH(CH_3)CH_2CH_2CH_2CH_3$), 3-hexyl (-$CH(CH_2CH_3)(CH_2CH_2CH_3)$), 2-methyl-2-pentyl (-$C(CH_3)_2CH_2CH_2CH_3$), 3-methyl-2-pentyl (-$CH(CH_3)CH(CH_3)CH_2CH_3$), 4-methyl-2-pentyl (-$CH(CH_3)CH_2CH(CH_3)_2$), 3-methyl-3-pentyl (-$C(CH_3)(CH_2CH_3)_2$), 2-methyl-3-pentyl (-$CH(CH_2CH_3)CH(CH_3)_2$), 2,3-dimethyl-2-butyl (-$C(CH_3)_2CH(CH_3)_2$), 3,3-dimethyl-2-butyl (-$CH(CH_3)C(CH_3)_3$, 1-heptyl, 1-octyl, and the like.

The term "alkylene" as used herein refers to a saturated linear or branched-chain divalent hydrocarbon radical of one to twelve carbon atoms (C_1–C_{12}), wherein the alkylene radical may be optionally substituted independently with one or more substituents described below. In another embodiment, an alkylene radical is one to eight carbon atoms (C_1–C_8), or one to six carbon atoms (C_1–C_6). Examples of alkylene groups include, but are not limited to, methylene (-CH_2-), ethylene (–CH_2CH_2–), propylene (–$CH_2CH_2CH_2$–), and the like.

The term "alkenyl" refers to linear or branched-chain monovalent hydrocarbon radical of two to eight carbon atoms (C_2–C_8) with at least one site of unsaturation, i.e., a carbon-carbon, sp^2 double bond, wherein the alkenyl radical may be optionally substituted independently with one or more substituents described herein, and includes radicals having "cis" and "trans" orientations, or alternatively, "E" and "Z" orientations. Examples include, but are not limited to, ethylenyl or vinyl (–CH=CH_2), allyl (–CH_2CH=CH_2), and the like.

The term "alkenylene" refers to linear or branched-chain divalent hydrocarbon radical of two to eight carbon atoms (C_2–C_8) with at least one site of unsaturation, i.e., a carbon-carbon, sp^2 double bond, wherein the alkenylene radical may be optionally substituted independently with one or more substituents described herein, and includes radicals having "cis" and "trans" orientations, or alternatively, "E" and "Z" orientations. Examples include, but are not limited to, ethylenylene or vinylene (–CH=CH–), allyl (–CH_2CH=CH–), and the like.

The term "alkynyl" refers to a linear or branched monovalent hydrocarbon radical of two to eight carbon atoms (C_2–C_8) with at least one site of unsaturation, i.e., a carbon-carbon, sp triple bond, wherein the alkynyl radical may be optionally substituted independently with one or more substituents described herein. Examples include, but are not limited to, ethynyl (-C≡CH), propynyl (propargyl, -CH_2C≡CH), and the like.

The term "alkynylene" refers to a linear or branched divalent hydrocarbon radical of two to eight carbon atoms (C_2–C_8) with at least one site of unsaturation, i.e., a carbon-carbon, sp triple bond, wherein the alkynylene radical may be optionally substituted independently with one or more substituents described herein. Examples include, but are not limited to, ethynylene (-C≡C-), propynylene (propargylene, -CH_2C≡C-), and the like.

The terms "carbocycle", "carbocyclyl", "carbocyclic ring" and "cycloalkyl" refer to a monovalent non-aromatic, saturated or partially unsaturated ring having 3 to 12 carbon atoms (C_3–C_{12}) as a monocyclic ring or 7 to 12 carbon atoms as a bicyclic ring. Bicyclic carbocycles having 7 to 12 atoms can be arranged, for example, as a bicyclo [4,5], [5,5], [5,6] or [6,6] system, and bicyclic carbocycles having 9 or 10 ring atoms can be arranged as a bicyclo [5,6] or [6,6] system, or as bridged systems such as bicyclo[2.2.1]heptane, bicyclo[2.2.2]octane and bicyclo[3.2.2]nonane. Spiro moieties are also included within the scope of this definition. Examples of monocyclic carbocycles include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, 1-cyclopent-1-enyl, 1-cyclopent-2-enyl, 1-cyclopent-3-enyl, cyclohexyl, 1-cyclohex-1-enyl, 1-cyclohex-2-enyl, 1-cyclohex-3-enyl, cyclohexadienyl, cycloheptyl, cyclooctyl, cyclononyl, cyclodecyl, cycloundecyl, cyclododecyl, and the like. Carbocyclyl groups are optionally substituted independently with one or more substituents described herein.

"Aryl" means a monovalent aromatic hydrocarbon radical of 6-20 carbon atoms (C_6–C_{20}) derived by the removal of one hydrogen atom from a single carbon atom of a parent aromatic ring system. Some aryl groups are represented in the exemplary structures as "Ar". Aryl includes bicyclic radicals comprising an aromatic ring fused to a saturated, partially unsaturated ring, or aromatic carbocyclic ring. Typical aryl groups include, but are not limited to, radicals derived from benzene (phenyl), substituted benzenes, naphthalene, anthracene, biphenyl, indenyl, indanyl, 1,2-dihydronaphthalene, 1,2,3,4-tetrahydronaphthyl, and the like. Aryl groups are optionally substituted independently with one or more substituents described herein.

"Arylene" means a divalent aromatic hydrocarbon radical of 6-20 carbon atoms (C_6–C_{20}) derived by the removal of two hydrogen atom from a two carbon atoms of a parent aromatic ring system. Some arylene groups are represented in the exemplary structures as "Ar". Arylene

includes bicyclic radicals comprising an aromatic ring fused to a saturated, partially unsaturated ring, or aromatic carbocyclic ring. Typical arylene groups include, but are not limited to, radicals derived from benzene (phenylene), substituted benzenes, naphthalene, anthracene, biphenylene, indenylene, indanylene, 1,2-dihydronaphthalene, 1,2,3,4-tetrahydronaphthyl, and the like. Arylene groups are optionally substituted with one or more substituents described herein.

The terms "heterocycle," "heterocyclyl" and "heterocyclic ring" are used interchangeably herein and refer to a saturated or a partially unsaturated (i.e., having one or more double and/or triple bonds within the ring) carbocyclic radical of 3 to about 20 ring atoms in which at least one ring atom is a heteroatom selected from nitrogen, oxygen, phosphorus and sulfur, the remaining ring atoms being C, where one or more ring atoms is optionally substituted independently with one or more substituents described below. A heterocycle may be a monocycle having 3 to 7 ring members (2 to 6 carbon atoms and 1 to 4 heteroatoms selected from N, O, P, and S) or a bicycle having 7 to 10 ring members (4 to 9 carbon atoms and 1 to 6 heteroatoms selected from N, O, P, and S), for example: a bicyclo [4,5], [5,5], [5,6], or [6,6] system. Heterocycles are described in Paquette, Leo A.; "Principles of Modern Heterocyclic Chemistry" (W.A. Benjamin, New York, 1968), particularly Chapters 1, 3, 4, 6, 7, and 9; "The Chemistry of Heterocyclic Compounds, A series of Monographs" (John Wiley & Sons, New York, 1950 to present), in particular Volumes 13, 14, 16, 19, and 28; and J. Am. Chem. Soc. (1960) 82:5566. "Heterocyclyl" also includes radicals where heterocycle radicals are fused with a saturated, partially unsaturated ring, or aromatic carbocyclic or heterocyclic ring. Examples of heterocyclic rings include, but are not limited to, morpholin-4-yl, piperidin-1-yl, piperazinyl, piperazin-4-yl-2-one, piperazin-4-yl-3-one, pyrrolidin-1-yl, thiomorpholin-4-yl, S-dioxothiomorpholin-4-yl, azocan-1-yl, azetidin-1-yl, octahydropyrido[1,2-a]pyrazin-2-yl, [1,4]diazepan-1-yl, pyrrolidinyl, tetrahydrofuranyl, dihydrofuranyl, tetrahydrothienyl, tetrahydropyranyl, dihydropyranyl, tetrahydrothiopyranyl, piperidino, morpholino, thiomorpholino, thioxanyl, piperazinyl, homopiperazinyl, azetidinyl, oxetanyl, thietanyl, homopiperidinyl, oxepanyl, thiepanyl, oxazepinyl, diazepinyl, thiazepinyl, 2-pyrrolinyl, 3-pyrrolinyl, indolinyl, 2H-pyranyl, 4H-pyranyl, dioxanyl, 1,3-dioxolanyl, pyrazolinyl, dithianyl, dithiolanyl, dihydropyranyl, dihydrothienyl, dihydrofuranyl, pyrazolidinylimidazolinyl, imidazolidinyl, 3-azabicyco[3.1.0]hexanyl, 3-azabicyclo[4.1.0]heptanyl, azabicyclo[2.2.2]hexanyl, 3H-indolyl quinolizinyl and N-pyridyl ureas. Spiro moieties are also included within the scope of this definition. Examples of a heterocyclic group wherein 2 ring atoms are substituted with oxo (=O) moieties are

pyrimidinonyl and 1,1-dioxo-thiomorpholinyl. The heterocycle groups herein are optionally substituted independently with one or more substituents described herein.

The term "heteroaryl" refers to a monovalent aromatic radical of 5-, 6-, or 7-membered rings, and includes fused ring systems (at least one of which is aromatic) of 5-20 atoms, containing one or more heteroatoms independently selected from nitrogen, oxygen, and sulfur. Examples of heteroaryl groups are pyridinyl (including, for example, 2-hydroxypyridinyl), imidazolyl, imidazopyridinyl, pyrimidinyl (including, for example, 4-hydroxypyrimidinyl), pyrazolyl, triazolyl, pyrazinyl, tetrazolyl, furyl, thienyl, isoxazolyl, thiazolyl, oxadiazolyl, oxazolyl, isothiazolyl, pyrrolyl, quinolinyl, isoquinolinyl, tetrahydroisoquinolinyl, indolyl, benzimidazolyl, benzofuranyl, cinnolinyl, indazolyl, indolizinyl, phthalazinyl, pyridazinyl, triazinyl, isoindolyl, pteridinyl, purinyl, oxadiazolyl, triazolyl, thiadiazolyl, thiadiazolyl, furazanyl, benzofurazanyl, benzothiophenyl, benzothiazolyl, benzoxazolyl, quinazolinyl, quinoxalinyl, naphthyridinyl, and furopyridinyl. Heteroaryl groups are optionally substituted independently with one or more substituents described herein.

The heterocycle or heteroaryl groups may be carbon (carbon-linked), or nitrogen (nitrogen-linked) bonded where such is possible. By way of example and not limitation, carbon bonded heterocycles or heteroaryls are bonded at position 2, 3, 4, 5, or 6 of a pyridine, position 3, 4, 5, or 6 of a pyridazine, position 2, 4, 5, or 6 of a pyrimidine, position 2, 3, 5, or 6 of a pyrazine, position 2, 3, 4, or 5 of a furan, tetrahydrofuran, thiofuran, thiophene, pyrrole or tetrahydropyrrole, position 2, 4, or 5 of an oxazole, imidazole or thiazole, position 3, 4, or 5 of an isoxazole, pyrazole, or isothiazole, position 2 or 3 of an aziridine, position 2, 3, or 4 of an azetidine, position 2, 3, 4, 5, 6, 7, or 8 of a quinoline or position 1, 3, 4, 5, 6, 7, or 8 of an isoquinoline.

By way of example and not limitation, nitrogen bonded heterocycles or heteroaryls are bonded at position 1 of an aziridine, azetidine, pyrrole, pyrrolidine, 2-pyrroline, 3-pyrroline, imidazole, imidazolidine, 2-imidazoline, 3-imidazoline, pyrazole, pyrazoline, 2-pyrazoline, 3-pyrazoline, piperidine, piperazine, indole, indoline, 1H-indazole, position 2 of a isoindole, or isoindoline, position 4 of a morpholine, and position 9 of a carbazole, or β-carboline.

A "metabolite" is a product produced through metabolism in the body of a specified compound or salt thereof. Metabolites of a compound may be identified using routine techniques known in the art and their activities determined using tests such as those described herein. Such products may result for example from the oxidation, reduction, hydrolysis, amidation, deamidation, esterification, deesterification, enzymatic cleavage, and the like, of the administered compound. Accordingly, the invention includes metabolites of compounds of the

invention, including compounds produced by a process comprising contacting a Formula I compound of this invention with a mammal for a period of time sufficient to yield a metabolic product thereof.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

A "sterile" formulation is aseptic or free from all living microorganisms and their spores.

A "stable" formulation is one in which the protein therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, New York, Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For rapid screening, the formulation may be kept at 40 °C for 2 weeks to 1 month, at which time stability is measured. Where the formulation is to be stored at 2-8 °C, generally the formulation should be stable at 30 °C or 40 °C for at least 1 month and/or stable at 2-8°C for at least 2 years. Where the formulation is to be stored at 30 °C, generally the formulation should be stable for at least 2 years at 30 °C and/or stable at 40 °C for at least 6 months. For example, the extent of aggregation during storage can be used as an indicator of protein stability. Thus, a "stable" formulation may be one wherein less than about 10% and preferably less than about 5% of the protein are present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation during storage of the formulation can be determined.

An "isotonic" formulation is one which has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. The term "hypotonic" describes a formulation with an osmotic pressure below that of human blood. Correspondingly, the term "hypertonic" is used to describe a formulation with an osmotic pressure above that of human blood. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example. The formulations of the present invention are hypertonic as a result of the addition of salt and/or buffer.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH

buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONICS™.

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative. A "pharmaceutically acceptable acid" includes inorganic and organic acids which are nontoxic at the concentration and manner in which they are formulated. For example, suitable inorganic acids include hydrochloric, perchloric, hydrobromic, hydroiodic, nitric, sulfuric, sulfonic, sulfinic, sulfanilic, phosphoric, carbonic, etc. Suitable organic acids include straight and branched-chain alkyl, aromatic, cyclic, cycloaliphatic, arylaliphatic, heterocyclic, saturated, unsaturated, mono, di- and tri-carboxylic, including for example, formic, acetic, 2-hydroxyacetic, trifluoroacetic, phenylacetic, trimethylacetic, t-butyl acetic, anthranilic, propanoic, 2-hydroxypropanoic, 2-oxopropanoic, propandioic, cyclopentanepropionic, cyclopentane propionic, 3-phenylpropionic, butanoic, butandioic, benzoic, 3-(4-hydroxybenzoyl)benzoic, 2-acetoxy-benzoic, ascorbic, cinnamic, lauryl sulfuric, stearic, muconic, mandelic, succinic, embonic, fumaric, malic, maleic, hydroxymaleic, malonic, lactic, citric, tartaric, glycolic, glyconic, gluconic, pyruvic, glyoxalic, oxalic, mesylic, succinic, salicylic, phthalic, palmoic, palmeic, thiocyanic, methanesulphonic, ethanesulphonic, 1,2-ethanedisulfonic, 2-hydroxyethanesulfonic, benzenesulphonic, 4-chorobenzenesulfonic, napthalene-2-sulphonic, p-toluenesulphonic, camphorsulphonic, 4-methylbicyclo[2.2.2]-oct-2-ene-1-carboxylic, glucoheptonic, 4,4'-methylenebis-3-(hydroxy-2-ene-1-carboxylic acid), hydroxynapthoic.

"Pharmaceutically-acceptable bases" include inorganic and organic bases which are non-toxic at the concentration and manner in which they are formulated. For example, suitable bases include those formed from inorganic base forming metals such as lithium, sodium, potassium, magnesium, calcium, ammonium, iron, zinc, copper, manganese, aluminum, N-methylglucamine, morpholine, piperidine and organic nontoxic bases including, primary, secondary and tertiary amines, substituted amines, cyclic amines and basic ion exchange resins,

[e.g., $N(R')_4^+$ (where R' is independently H or C_{1-4} alkyl, e.g., ammonium, Tris)], for example, isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, ethanolamine, 2-diethylaminoethanol, trimethamine, dicyclohexylamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, methylglucamine, theobromine, purines, piperazine, piperidine, N-ethylpiperidine, polyamine resins and the like. Particularly preferred organic non-toxic bases are isopropylamine, diethylamine, ethanolamine, trimethamine, dicyclohexylamine, choline, and caffeine.

Additional pharmaceutically acceptable acids and bases useable with the present invention include those which are derived from the amino acids, for example, histidine, glycine, phenylalanine, aspartic acid, glutamic acid, lysine and asparagine.

"Pharmaceutically acceptable" buffers and salts include those derived from both acid and base addition salts of the above indicated acids and bases. Specific buffers and/ or salts include histidine, succinate and acetate.

A "pharmaceutically acceptable sugar" is a molecule which, when combined with a protein of interest, significantly prevents or reduces chemical and/or physical instability of the protein upon storage. When the formulation is intended to be lyophilized and then reconstituted, "pharmaceutically acceptable sugars" may also be known as a "lyoprotectant". Exemplary sugars and their corresponding sugar alcohols include: an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher molecular weight sugar alcohols, e.g. glycerin, dextran, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; PLURONICS®; and combinations thereof. Additional exemplary lyoprotectants include glycerin and gelatin, and the sugars mellibiose, melezitose, raffinose, mannotriose and stachyose. Examples of reducing sugars include glucose, maltose, lactose, maltulose, iso-maltulose and lactulose. Examples of non-reducing sugars include non-reducing glycosides of polyhydroxy compounds selected from sugar alcohols and other straight chain polyalcohols. Preferred sugar alcohols are monoglycosides, especially those compounds obtained by reduction of disaccharides such as lactose, maltose, lactulose and maltulose. The glycosidic side group can be either glucosidic or galactosidic. Additional examples of sugar alcohols are glucitol, maltitol, lactitol and iso-maltulose. The preferred pharmaceutically-acceptable sugars are the non-reducing sugars trehalose or sucrose. Pharmaceutically acceptable sugars are added to the formulation in a "protecting amount" (e.g. pre-lyophilization) which means that the protein essentially retains its physical and chemical stability and integrity during storage (e.g., after reconstitution and storage).

The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a liquid formulation, such as a formulation reconstituted after lyophilization. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. In an alternative embodiment, diluents can include aqueous solutions of salts and/or buffers.

A "preservative" is a compound which can be added to the formulations herein to reduce bacterial activity. The addition of a preservative may, for example, facilitate the production of a multi-use (multiple-dose) formulation. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. The most preferred preservative herein is benzyl alcohol.

An "individual" or "subject" or "patient" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human.

As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention designed to alter the natural course of the individual, tissue or cell being treated during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, decreasing the rate of disease progression, ameliorating or palliating the disease state, and remission or improved prognosis, all measurable by one of skill in the art such as a physician. In one embodiment, treatment can mean alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, decreasing the rate of infectious disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, the AACs and TACs of the invention are used to delay development of a disease or to slow the progression of an infectious disease or reduce the bacterial load in the blood stream and/or in infected tissues and organs.

As used herein, "in conjunction with" refers to administration of one treatment modality in addition to another treatment modality. As such, "in conjunction with" refers to administration of one treatment modality before, during or after administration of the other treatment modality to the individual.

The term "bacteremia" refers to the presence of bacteria in the bloodstream which is most commonly detected through a blood culture. Bacteria can enter the bloodstream as a severe complication of infections (like pneumonia or meningitis), during surgery (especially when involving mucous membranes such as the gastrointestinal tract), or due to catheters and other foreign bodies entering the arteries or veins. Bacteremia can have several consequences. The immune response to the bacteria can cause sepsis and septic shock, which has a relatively high mortality rate. Bacteria can also use the blood to spread to other parts of the body, causing infections away from the original site of infection. Examples include endocarditis or osteomyelitis.

A "therapeutically effective amount" is the minimum concentration required to effect a measurable improvement of a particular disorder. A therapeutically effective amount herein may vary according to factors such as the disease state, age, sex, and weight of the patient, and the ability of the antibody to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody are outweighed by the therapeutically beneficial effects. In one embodiment, a therapeutically effective amount is an amount effective to reduce bacteremia in an *in vivo* infection. In one aspect, a "therapeutically effective amount" is at least the amount effective to reduce the bacterial load or colony forming units (CFU) isolated from a patient sample such as blood by at least one log relative to prior to drug administration. In a more specific aspect, the reduction is at least 2 logs. In another aspect, the reduction is at least 3, 4, 5 logs. In yet another aspect, the reduction is to below detectable levels using assays known in the art including assays exemplified herein. In another embodiment, a therapeutically effective amount is the amount of an AAC in one or more doses given over the course of the treatment period, that achieves a negative blood culture (i.e., does not grow out the bacteria that is the target of the AAC) as compared to the positive blood culture before or at the start of treatment of the infected patient.

A "prophylactically effective amount" refers to an amount effective, at the dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to, at the earlier stage of disease, or even prior to exposure to conditions where the risk of infection is elevated, the prophylactically effective amount can be less than the therapeutically effective amount. In one embodiment, a prophylactically effective amount is at least an amount effective to reduce, prevent the occurrence of or spread of infection from one cell to another.

"Chronic" administration refers to administration of the medicament(s) in a continuous as opposed to acute mode, so as to maintain the initial therapeutic effect (activity) for an

extended period of time. "Intermittent" administration is treatment that is not consecutively done without interruption, but rather is cyclic in nature.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

The term "chiral" refers to molecules which have the property of non-superimposability of the mirror image partner, while the term "achiral" refers to molecules which are superimposable on their mirror image partner.

The term "stereoisomers" refers to compounds which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space.

"Diastereomer" refers to a stereoisomer with two or more centers of chirality and whose molecules are not mirror images of one another. Diastereomers have different physical properties, e.g. melting points, boiling points, spectral properties, and reactivities. Mixtures of diastereomers may separate under high resolution analytical procedures such as electrophoresis and chromatography.

"Enantiomers" refer to two stereoisomers of a compound which are non-superimposable mirror images of one another.

Stereochemical definitions and conventions used herein generally follow S. P. Parker, Ed., McGraw-Hill Dictionary of Chemical Terms (1984) McGraw-Hill Book Company, New York; and Eliel, E. and Wilen, S., Stereochemistry of Organic Compounds (1994) John Wiley & Sons, Inc., New York. Many organic compounds exist in optically active forms, i.e., they have the ability to rotate the plane of plane-polarized light. In describing an optically active compound, the prefixes D and L, or R and S, are used to denote the absolute configuration of the molecule about its chiral center(s). The prefixes d and l or (+) and (-) are employed to designate the sign of rotation of plane-polarized light by the compound, with (-) or 1 meaning that the compound is levorotatory. A compound prefixed with (+) or d is dextrorotatory. For a given chemical structure, these stereoisomers are identical except that they are mirror images of one another. A specific stereoisomer may also be referred to as an enantiomer, and a mixture of such isomers is often called an enantiomeric mixture. A 50:50 mixture of enantiomers is referred to as a racemic mixture or a racemate, which may occur where there has been no stereoselection or stereospecificity in a chemical reaction or process. The terms "racemic mixture" and "racemate" refer to an equimolar mixture of two enantiomeric species, devoid of optical activity.

The term "protecting group" refers to a substituent that is commonly employed to block or protect a particular functionality while other functional groups react on the compound. For example, an "amino-protecting group" is a substituent attached to an amino group that blocks or protects the amino functionality in the compound. Suitable amino-protecting groups include, but are not limited to, acetyl, trifluoroacetyl, t-butoxycarbonyl (BOC), benzyloxycarbonyl (CBZ) and 9-fluorenylmethylenoxycarbonyl (Fmoc). For a general description of protecting groups and their use, see T. W. Greene, Protective Groups in Organic Synthesis, John Wiley & Sons, New York, 1991, or a later edition.

The term "about" as used herein refers to the usual error range for the respective value readily known to the skilled person in this technical field. Reference to "about" a value or parameter herein includes (ad describes) embodiments that are directed to that value or parameter per se.

As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly indicates otherwise. For example, reference to an "antibody" is a reference to from one to many antibodies, such as molar amounts, and includes equivalents thereof known to those skilled in the art, and so forth.

III. Compositions and Methods

ANTIBODY-ANTIBIOTIC CONJUGATES (AAC)

The experimental results herein are a strong indication that therapies aimed at eliminating intracellular bacteria will improve clinical success. Towards this aim, the present invention provides a unique therapeutic that selectively kills *S. aureus* organisms that have invaded intracellular compartments of host cells. The present invention demonstrates that such a therapeutic is efficacious in in-vivo models where conventional antibiotics like vancomycin fail.

The invention provides an antibacterial therapy that aims to prevent antibiotic escape by targeting populations of bacteria that evade conventional antibiotic therapy. The novel antibacterial therapy is achieved with an Antibody Antibiotic Conjugate (AAC) in which an rF1 antibody specific for cell wall components found on *S. aureus* (including MRSA) is chemically linked to a potent rifamycin-type antibiotic (a derivative of rifamycin). The rifamycin-type antibiotic is joined to the antibody via a protease-cleavable, non-peptide linker that is designed to be cleaved by proteases, including cathepsin B, a lysosomal protease found in most mammalian cell types (Dubowchik et al (2002) Bioconj. Chem. 13:855-869). A diagram of the AAC with its 3 components is depicted in FIG. 2. Not to be limited by any one theory, one

mechanism of action of the AAC is schematized in FIG. 3. The AAC acts as a pro-drug in that the rifamycin-type antibiotic is inactive (due to the large size of the antibody) until the linker is cleaved. Since a significant proportion of *S. aureus* found in a natural infection is taken up by host cells, primarily neutrophils and macrophages, at some point during the course of infection in the host, the time spent inside host cells provides a significant opportunity for the bacterium to evade antibiotic activity. The AACs of the invention are designed to bind to the Staph bacteria and release the antibiotic inside the phagolysosome after bacteria are taken up by host cells. By this mechanism, AAC are able to concentrate the active antibiotic specifically in a location where S. aureus is poorly treated by conventional antibiotics. While the invention is not limited or defined by an particular mechanism of action, the AAC improve antibiotic activity via three potential mechanisms: (1) The AAC delivers antibiotic inside mammalian cells that take up the bacteria, thereby increasing the potency of antibiotics that diffuse poorly into the phagolysosomes where bacteria are sequestered. (2) AAC opsonize bacteria thereby increasing uptake of free bacteria by phagocytic cells, and release the antibiotic locally to kill the bacteria while they are sequestered in the phagolysosome. Since thousands of AACs can bind to a single bacterium, this platform releases sufficient antibiotics in these intracellular niches to sustain maximal antimicrobial killing. Furthermore, as more bacteria are released from pre-existing intracellular reservoirs, the fast on-rate of this antibody-based therapy ensures immediate "tagging" of these bacteria before they can escape to neighboring or distant cells, thus mitigating further spread of the infection. (3) AAC improve the half-life of antibiotics *in vivo* (improved pharmacokinetics) by linking the antibiotic to an antibody, as compared to antibiotics which are cleared rapidly from serum. Improved pharmacokinetics of AAC enable delivery of sufficient antibiotic in regions where *S. aureus* is concentrated while limiting the overall dose of antibiotic that needs to be administered systemically. This property should permit long-term therapy with AAC to target persistent infection with minimal antibiotic side effects.

An antibody-antibiotic conjugate compound of the invention comprises an anti-SDR antibody covalently attached by a protease-cleavable, non-peptide linker via a recombinantly introduced cysteine, to a rifamycin-type antibiotic.

In an exemplary embodiment, the anti-SDR antibody (e.g. rF1 antibody) is a cysteine-engineered antibody comprising a recombinantly introduced cysteine amino acid.

In an exemplary embodiment, the protease-cleavable, non-peptide linker is covalently attached via a recombinantly introduced cysteine on the rF1, anti-SDR antibody, to the rifamycin-type antibiotic

An exemplary embodiment is the antibody-antibiotic conjugate having the formula:

$$Ab–(PML–abx)_p$$

wherein:

Ab is the rF1 antibody;

PML is the protease-cleavable, non-peptide linker having the formula:

$$–Str–PM–Y–$$

where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit;

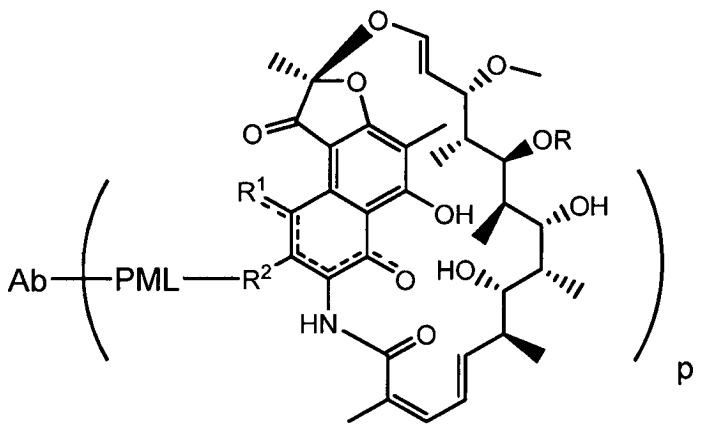
abx is the rifamycin-type antibiotic; and

p is an integer from 1 to 8.

The rifamycin-type antibiotic may be a rifalazil-type antibiotic.

The rifamycin-type antibiotic may comprise a quaternary amine attached to the protease-cleavable, non-peptide linker.

An exemplary embodiment of the antibody-antibiotic conjugate has Formula I:



I

wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

R^1 is OH;

R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH;

PML is the protease-cleavable, non-peptide linker attached to R^2 or the fused heteroaryl or heterocyclyl formed by R^1 and R^2; and

Ab is the rF1 antibody.

The number of antibiotic moieties which may be conjugated via a reactive linker moiety to an antibody molecule may be limited by the number of free cysteine residues, which are introduced by the methods described herein. Exemplary AAC comprise antibodies which have 1, 2, 3, or 4 engineered cysteine amino acids (Lyon, R. et al (2012) *Methods in Enzym.* 502:123-138).

To be effective target on MRSA, the epitope is preferably highly abundant, stably expressed during infection and highly conserved in all clinical MRSA strains. The rF1 antibody fulfills these requirements and additionally, also binds to Staph epidermidis as well.

ANTI-SDR AND rF1 ANTIBODIES

Anti-SDR antibodies can be produced as described below for the generation of F1 antibody. Several examples of anti-SDR antibodies are provided herein including rF1, SD2, SD3 and SD4.

The rF1 Abs will be described in detail here.

rF1 antibody is a fully human is capable of specifically binding *Staphylococcus* species such as *S. aureus* and *S. epidermidis*. Importantly, rF1 is capable of binding whole bacteria *in vivo* as well as *in vitro*. Furthermore, antibody rF1 is capable of binding to bacteria that have been grown in infected tissue of, for example, an animal. The rF1 Abs provided herein or functional equivalents thereof are capable of binding to *S. aureus* surface proteins ClfA, ClfB, SdrC, SdrD and SdrE.

Table 4A and Table 4B show an alignment of the H chain and L chain CDR sequences of the parent antibody F1, rF1 antibody and its variants. F1 and rF1 differ in sequence in FW1 and LC CDR3 (QHYXRFPYT, where X can be I or M (SEQ ID NO: 26); F1 is I (SEQ ID NO: 6) and rF1 is M (SEQ ID NO: 7)).

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Table 4A: Heavy chain CDR sequences

Antibody	HC CDR1	HC CDR2	HC CDR3
F1	RFAMS (SEQ ID NO:1)	SINNGNNPYYARSVQY (SEQ ID NO: 2)	DHPSSGWPTFDS (SEQ ID NO: 3)
rF1	RFAMS (SEQ ID NO:1)	SINNGNNPYYARSVQY (SEQ ID NO: 2)	DHPSSGWPTFDS (SEQ ID NO: 3)
rF1.v1	RFAMS (SEQ ID NO:1)	SINSGNNPYYARSVQY (SEQ ID NO: 8)	DHPSSGWPTFDS (SEQ ID NO: 3)

Table 4B Light chain CDR sequences

Antibody	LC CDR1	LC CDR2	LC CDR3
F1	RASENVGDWLA (SEQ ID NO: 4)	KTSILES (SEQ ID NO:5)	QHYIRFPYT (SEQ ID NO:6)
rF1	RASENVGDWLA (SEQ ID NO: 4)	KTSILES (SEQ ID NO:5)	QHYMRFPYT (SEQ ID NO:7)
rF1.v6	RASENVGDWLA (SEQ ID NO: 4)	KTSILES (SEQ ID NO:5)	QHYIRFPYT (SEQ ID NO:6)

In one embodiment, the H and L chain Framework (FR) sequences are as follows:

HC FW1 EVQLVESGGGLVQPGGSLRLSCAASGFTLS (SEQ ID NO. 16)

HC FW2 WVRQAPGRGLEWVA (SEQ ID NO. 17)

HC FW3 RFTVSRDVSQNTVSLQMNNLRAEDSATYFCAK (SEQ ID NO. 18)

HC FW4 WGPGTLVTVSS (SEQ ID NO. 19)

LC FW1 DIQLTQSPSALPASVGDRVSITC (SEQ ID NO. 20)

LC FW2 WYRQKPGKAPNLLIY (SEQ ID NO. 21)

LC FW3 GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC (SEQ ID NO. 22)

LC FW4 FGQGTKVEIKRTV (SEQ ID NO. 23)

Various amino acid modifications were made to rF1 to improve stability and function. In the HC CDR2, the NG deamindation site was eliminated by changing the 4th residue N to S, thus improving the stability of the antibody. A repair of TV was made to the LC backbone to eliminate the severe antibody aggregation present in rF1.

For conjugation to form the therapeutic AACs of the invention, the following pairings of H and L chain can be made to form the full tetrameric antibody. Boxed are the CDR1, CDR2, CDR3 sequences. The introduced Cysteine (C) is underlined. Residues in bold are amino acid changes over the parent F1. In the Lchain, the A after the bolded "RTV" is the first residue of the Constant region. The underlined C at Kabat position 114 in the H chain starts the Constant region.

In 1A and 2A, the full length (FL) L chain of SEQ ID NO. 9 with an engineered Cys at aa 205 near the end of C kappa is paired with the FL IgG1 H chain of SEQ ID NO. 10 (no Cys). This antibody will have 2 Cys sites, one on each L chain, for conjugation to the linker-antibiotic unit to form the AAC.

1A. rF1-V205C FL Light chain

DIQLTQSPSALPASVGDRVSITC[RASENVGDWLA]WYRQKPGKAPNLLIY[KTSILES]GVPSRFSG
SGSGTEFTLTISSLQPDDFATYYC[QHY*M*RFPYT]FGQGTK**V**EIK**RTV**AAPSVFIFPPSDEQLKSG
TASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVY
ACEVTHQGLSSPCTKSFNRGEC (SEQ ID NO.9)

2A. rF1.v1 FL Heavy chain (No Cys), pair of rF1-V205C Light Chain with Cys205

EVQL**V**ESGGGLVQPGGSLRLSCAASGFTLS[RFAMS]WVRQAPGRGLEWVA[SIN**S**GNNPYYARSVQ
[Y]RFTVSRDVSQNTVSLQMNNLRAEDSATYFCAK[DHPSSGWPTFDS]WGPGTLVTVSSASTKGPSV
FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI
SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK

EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G (SEQ ID NO: 10)

In 1B with 2A, rF1.v6 L chain of SEQ ID NO. 11 with an engineered Cys 205 is paired with the FL IgG1 H chain of SEQ ID NO. 10 (no Cys). This antibody will have 2 Cys sites, one on each L chain, for conjugation to the linker-antibiotic unit.

1B. rF1.v6-V205C Light chain

DIQLTQSPSALPASVGDRVSITCRASENVGDWLAWYRQKPGKAPNLLIYKTSILESGVPSRFSG
SGSGTEFTLTISSLQPDDFATYYCQHYIRFPYTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSG
TASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVY
ACEVTHQGLSSPCTKSFNRGEC (FL SEQ ID NO. 11)

In 1B with 2B, each of L and H chains has an engineered Cys, thus the tetramer antibody can have up to 4 AAR (Antibiotic: antibody ratio).

2B. rF1.v1 FL Heavy chain, with Cys114 (114 Kabat numbering, or 118 -Eu numbering)

EVQLVESGGGLVQPGGSLRLSCAASGFTLSRFAMSWVRQAPGRGLEWVASINSGNNPYYARSVQ
YRFTVSRDVSQNTVSLQMNNLRAEDSATYFCAKDHPSSGWPTFDSWGPGTLVTVSS
CSTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS
LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT
QKSLSLSPG (SEQ ID NO. 12)

rF1.v1 H chain Variable region

EVQL**V**ESGGGLVQPGGSLRLSCAASGFTLSRFAMSWVRQAPGRGLEWVASIN**S**GNNPYY
ARSVQYRFTVSRDVSQNTVSLQMNNLRAEDSATYFCAKDHPSSGWPTFDSWGPGTLVTVSS
(SEQ ID NO. 13)

rF1 L chain Variable region

DIQLTQSPSALPASVGDRVSITCRASENVGDWLAWYRQKPGKAPNLLIYKTSILESGVP SRFSGSGSGTEFTLTISSLQPDDFATYYCQHYMRFPYTFGQGTKVEIKRTV (SEQ ID NO. 14)

rF1.v6 L chain Variable region

DIQLTQSPSALPASVGDRVSITCRASENVGDWLAWYRQKPGKAPNLLIYKTSILESGVP SRFSGSGSGTEFTLTISSLQPDDFATYYCQHYIRFPYTFGQGTKVEIKRTV (SEQ ID NO. 15)

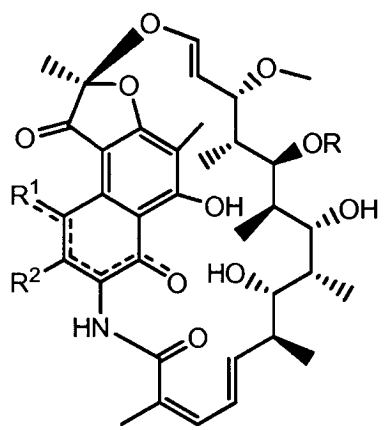
The anti-SDR Abs including rF1 may comprise at least one amino acid other than cysteine has been replaced with cysteine. In some embodiments, the at least one amino acid other than cysteine is valine at light chain position 205 and/or valine at light chain position 110, and/or alanine at heavy chain position 114, whereby the amino acid numbering is according to Kabat (1991), which is the same as position 118 according to the Eu numbering convention.

RIFAMYCIN-TYPE ANTIBIOTIC MOIETIES

The antibiotic moiety (abx) of the antibody-antibiotic conjugates (AAC) of the invention is a rifamycin-type antibiotic or group that has a cytotoxic or cytostatic effect. The rifamycins are a group of antibiotics that are obtained either naturally by the bacterium, *Nocardia mediterranei, Amycolatopsis mediterranei* or artificially. They are a subclass of the larger Ansamycin family which inhibit bacterial RNA polymerase (Fujii et al (1995) Antimicrob. Agents Chemother. 39:1489-1492; Feklistov, et al (2008) Proc Natl Acad Sci USA, 105(39): 14820-5) and have potency against gram-positive and selective gram-negative bacteria. Rifamycins are particularly effective against mycobacteria, and are therefore used to treat tuberculosis, leprosy, and mycobacterium avium complex (MAC) infections. The rifamycin-type group includes the "classic" rifamycin drugs as well as the rifamycin derivatives rifampicin (rifampin, CA Reg. No. 13292-46-1), rifabutin (CA Reg. No. 72559-06-9; US 2011/0178001), rifapentine and rifalazil (CA Reg. No. 129791-92-0, Rothstein et al (2003) Expert Opin. Investig. Drugs 12(2):255-271; Fujii et al (1994) Antimicrob. Agents Chemother. 38:1118-1122. Many rifamycin-type antibiotics share the detrimental property of resistance development (Wichelhaus et al (2001) J. Antimicrob. Chemother. 47:153-156). Rifamycins were first isolated in 1957 from a fermentation culture of *Streptomyces mediterranei*. About seven rifamycins

were discovered, named Rifamycin A, B, C, D, E, S, and SV (US 3150046). Rifamycin B was the first introduced commercially and was useful in treating drug-resistant tuberculosis in the 1960s. Rifamycins have been used for the treatment of many diseases, the most important one being HIV-related Tuberculosis. Due to the large number of available analogues and derivatives, rifamycins have been widely utilized in the elimination of pathogenic bacteria that have become resistant to commonly used antibiotics. For instance, Rifampicin is known for its potent effect and ability to prevent drug resistance. It rapidly kills fast-dividing bacilli strains as well as "persisters" cells, which remain biologically inactive for long periods of time that allow them to evade antibiotic activity. In addition, rifabutin and rifapentine have both been used against tuberculosis acquired in HIV-positive patients.

Antibiotic moieties (abx) of the Formula I antibody-antibiotic conjugates are rifamycin-type moieties having the structure:



wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

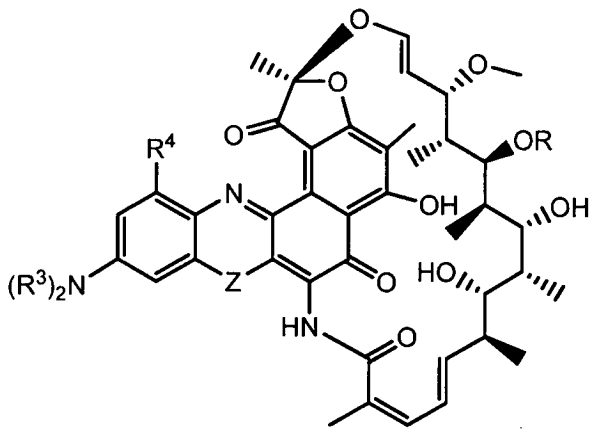
R^1 is OH;

R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH; and

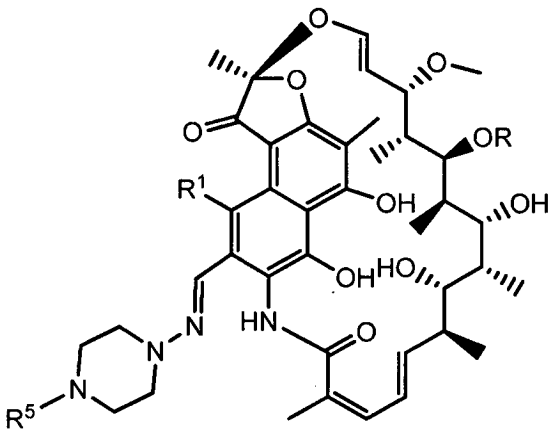
where the non-peptide linker PML is covalently attached to R^2.

An embodiment of a rifamycin-type moiety is:



wherein R^3 is independently selected from H and C_1–C_{12} alkyl; R^4 is selected from H, F, Cl, Br, I, C_1–C_{12} alkyl, and OH; and Z is selected from NH, N(C_1–C_{12} alkyl), O and S; and where the non-peptide linker PML is covalently attached to the nitrogen atom of $N(R^3)_2$.

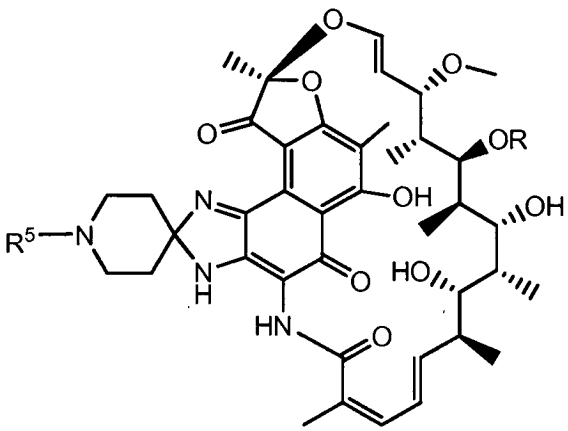
An embodiment of a rifampicin-type moiety is:



wherein

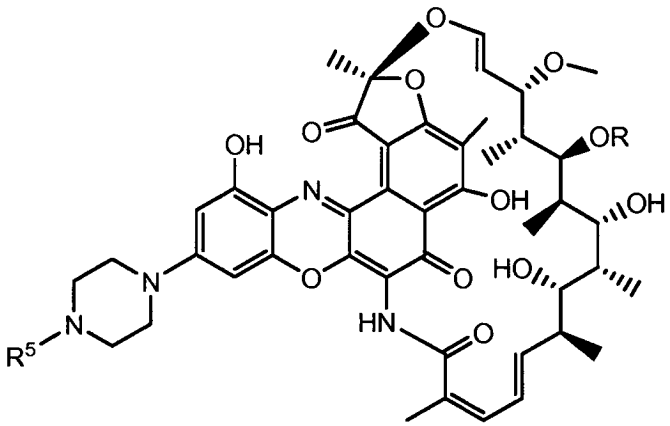
R^5 is selected from H and C_1–C_{12} alkyl; and where the non-peptide linker PML is covalently attached to the nitrogen atom of NR^5.

An embodiment of a rifabutin-type moiety is:



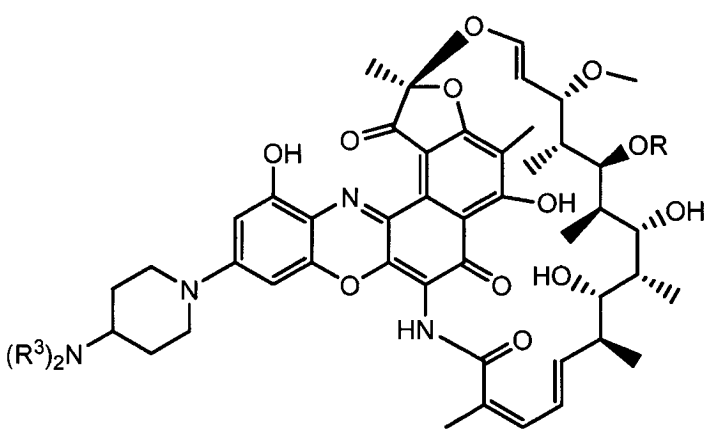
wherein R^5 is selected from H and C_1–C_{12} alkyl; and where the non-peptide linker PML is covalently attached to the nitrogen atom of NR^5.

An embodiment of a benzoxazinorifamycin-type moiety is:



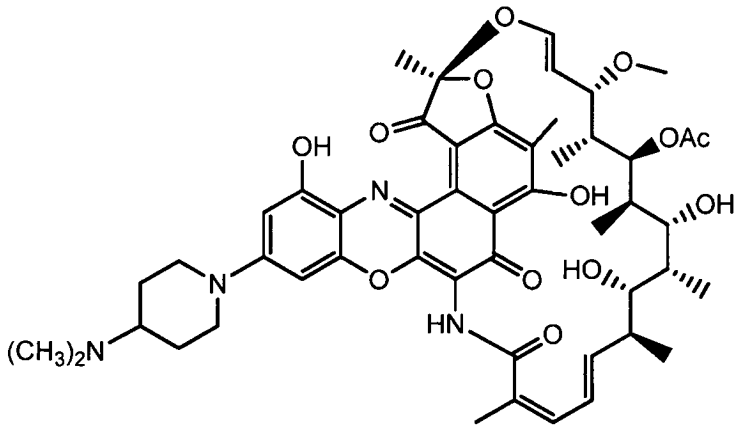
wherein R^5 is selected from H and C_1–C_{12} alkyl; and where the non-peptide linker PML is covalently attached to the nitrogen atom of NR^5.

An embodiment of a benzoxazinorifamycin-type moiety, referred to herein as pipBOR, is:



wherein R^3 is independently selected from H and C_1–C_{12} alkyl; and where the non-peptide linker PML is covalently attached to the nitrogen atom of $N(R^3)_2$.

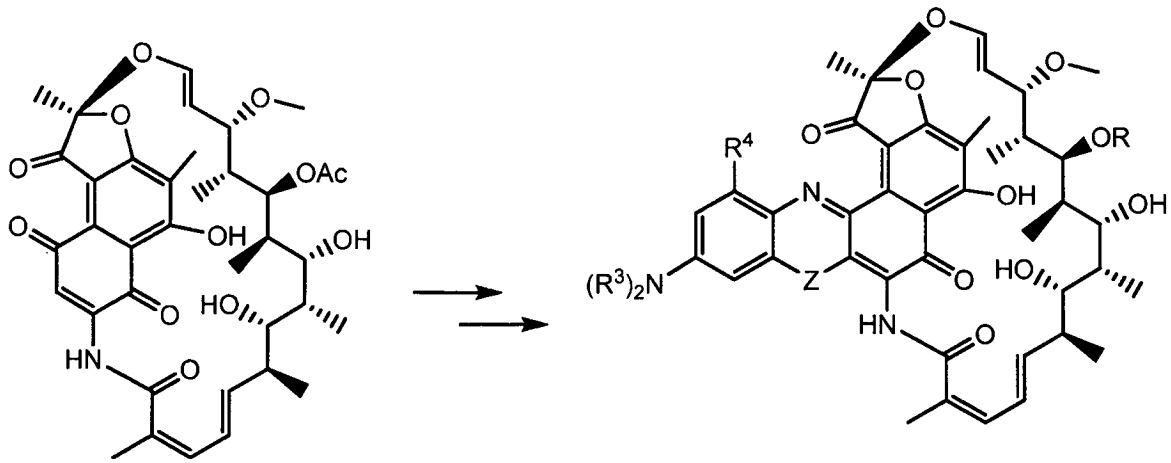
An embodiment of a benzoxazinorifamycin-type moiety, referred to herein as dimethylpipBOR, is:



where the non-peptide linker PML is covalently attached to the nitrogen atom of $N(CH_3)_2$.

The semi-synthetic derivative rifamycin S, or the reduced, sodium salt form rifamycin SV, can be converted to Rifalazil-type antibiotics in several steps, where R is H, or Ac, R^3 is independently selected from H and C_1–C_{12} alkyl; R^4 is selected from H, F, Cl, Br, I, C_1–C_{12} alkyl, and OH; and Z is selected from NH, N(C_1–C_{12} alkyl), O and S (see, e.g., Fig. 23A and B, and Fig. 25A and B in WO 2014/194247). Benzoxazino (Z = O), benzthiazino (Z = S), benzdiazino (Z = NH, N(C_1–C_{12} alkyl) rifamycins may be prepared (US 7271165). Benzoxazinorifamycin (BOR), benzthiazinorifamycin (BTR), and benzdiazinorifamycin (BDR) analogs that contain substituents are numbered according to the numbering scheme provided in formula A at column 28 in US 7271165, which is incorporated by reference for this purpose. By "25-O-deacetyl" rifamycin is meant a rifamycin analog in which the acetyl group at the 25-position has been removed. Analogs in which this position is further derivatized are referred to as a "25-O-deacetyl-25-(substituent) rifamycin", in which the nomenclature for the derivatizing group replaces "substituent" in the complete compound name.

Rifamycin-type antibiotic moieties can be synthesized by methods analogous to those disclosed in US 4610919; US 4983602; US 5786349; US5981522; US 4859661; US 7271165; US 2011/0178001; Seligson, et al., (2001) Anti-Cancer Drugs 12:305-13; Chem. Pharm. Bull., (1993) 41:148, and in WO 2014/194247, each of which is hereby incorporated by reference). Rifamycin-type antibiotic moieties can be screened for antimicrobial activity by measuring their minimum inhibitory concentration (MIC), using standard MIC in vitro assays (Tomioka et al., (1993) Antimicrob. Agents Chemother. 37:67).



rifamycin-S benzoxazinorifamycin

PROTEASE-CLEAVABLE NON-PEPTIDE LINKERS

A "protease-cleavable, non-peptide linker" (PML) is a bifunctional or multifunctional moiety which is covalently attached to one or more antibiotic moieties (abx) and an antibody unit (Ab) to form antibody-antibiotic conjugates (AAC) of Formula I. Protease-cleavable, non-peptide linkers in AAC are substrates for cleavage by intracellular proteases, including under lysosomal conditions. Proteases includes various cathepsins and caspases. Cleavage of the non-peptide linker of an AAC inside a cell may release the rifamycin-type antibiotic with anti-bacterial effects.

Antibody-antibiotic conjugates (AAC) can be conveniently prepared using a linker reagent or linker-antibiotic intermediate having reactive functionality for binding to the antibiotic (abx) and to the antibody (Ab). In one exemplary embodiment, a cysteine thiol of a cysteine engineered antibody (Ab) can form a bond with a functional group of a linker reagent, an antibiotic moiety or antibiotic-linker intermediate.

The PML moiety of an AAC may comprise one amino acid residue.

The PML moiety of an AAC comprises a peptidomimetic unit.

In one aspect, a linker reagent or linker-antibiotic intermediate has a reactive site which has an electrophilic group that is reactive to a nucleophilic cysteine present on an antibody. The cysteine thiol of the antibody is reactive with an electrophilic group on a linker reagent or linker-antibiotic, forming a covalent bond. Useful electrophilic groups include, but are not limited to, maleimide and haloacetamide groups.

Cysteine engineered antibodies react with linker reagents or linker-antibiotic intermediates, with electrophilic functional groups such as maleimide or α-halo carbonyl,

according to the conjugation method at page 766 of Klussman, et al (2004), *Bioconjugate Chemistry* 15(4):765-773, and according to the protocol of Example 18.

In another embodiment, the reactive group of a linker reagent or linker-antibiotic intermediate contains a thiol-reactive functional group that can form a bond with a free cysteine thiol of an antibody. Examples of thiol-reaction functional groups include, but are not limited to, maleimide, α-haloacetyl, activated esters such as succinimide esters, 4-nitrophenyl esters, pentafluorophenyl esters, tetrafluorophenyl esters, anhydrides, acid chlorides, sulfonyl chlorides, isocyanates and isothiocyanates.

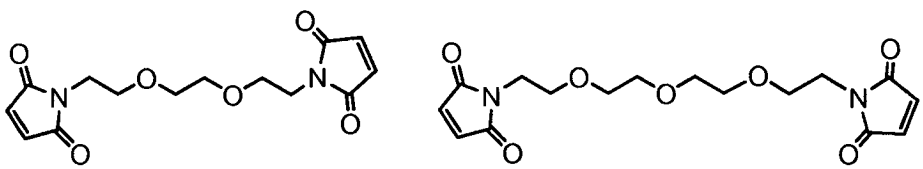
In another embodiment, a linker reagent or antibiotic-linker intermediate has a reactive functional group which has a nucleophilic group that is reactive to an electrophilic group present on an antibody. Useful electrophilic groups on an antibody include, but are not limited to, pyridyl disulfide, aldehyde and ketone carbonyl groups. The heteroatom of a nucleophilic group of a linker reagent or antibiotic-linker intermediate can react with an electrophilic group on an antibody and form a covalent bond to an antibody unit. Useful nucleophilic groups on a linker reagent or antibiotic-linker intermediate include, but are not limited to, hydrazide, oxime, amino, thiol, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide. The electrophilic group on an antibody provides a convenient site for attachment to a linker reagent or antibiotic-linker intermediate.

A PML moiety may comprise one or more linker components. Exemplary linker components include a single amino acid such as citrulline ("cit"), 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), and p-aminobenzyloxycarbonyl ("PAB"), N-succinimidyl 4-(2-pyridylthio) pentanoate ("SPP"), and 4-(N-maleimidomethyl) cyclohexane-1 carboxylate ("MCC"). Various linker components are known in the art, some of which are described below.

In another embodiment, the linker may be substituted with groups that modulate solubility or reactivity. For example, a charged substituent such as sulfonate ($-SO_3^-$) or ammonium, may increase water solubility of the reagent and facilitate the coupling reaction of the linker reagent with the antibody or the antibiotic moiety, or facilitate the coupling reaction of Ab-L (antibody-linker intermediate) with abx, or abx-L (antibiotic-linker intermediate) with Ab, depending on the synthetic route employed to prepare the AAC.

The AAC of the invention expressly contemplate, but are not limited to, those prepared with linker reagents: BMPEO, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, sulfo-SMPB, SVSB (succinimidyl-(4-vinylsulfone)benzoate),

and bis-maleimide reagents such as DTME, BMB, BMDB, BMH, BMOE, $BM(PEG)_2$, and $BM(PEG)_3$. Bis-maleimide reagents allow the attachment of the thiol group of a cysteine engineered antibody to a thiol-containing antibiotic moiety, label, or linker intermediate, in a sequential or convergent fashion. Other functional groups besides maleimide, which are reactive with a thiol group of a cysteine engineered antibody, antibiotic moiety, or linker-antibiotic intermediate include iodoacetamide, bromoacetamide, vinyl pyridine, disulfide, pyridyl disulfide, isocyanate, and isothiocyanate.



$BM(PEG)_2$ $BM(PEG)_3$

Useful linker reagents can also be obtained via other commercial sources, such as Molecular Biosciences Inc.(Boulder, CO), or synthesized in accordance with procedures described in Toki et al (2002) J. Org. Chem. 67:1866-1872; Dubowchik, et al. (1997) Tetrahedron Letters, 38:5257-60; Walker, M.A. (1995) J. Org. Chem. 60:5352-5355; Frisch et al (1996) Bioconjugate Chem. 7:180-186; US 6214345; WO 02/088172; US 2003130189; US2003096743; WO 03/026577; WO 03/043583; and WO 04/032828.

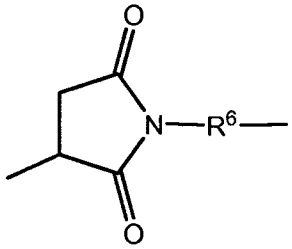
In another embodiment, the PML moiety of an AAC comprises a dendritic type linker for covalent attachment of more than one antibiotic moiety through a branching, multifunctional linker moiety to an antibody (Sun et al (2002) *Bioorganic & Medicinal Chemistry Letters* 12:2213-2215; Sun et al (2003) *Bioorganic & Medicinal Chemistry* 11:1761-1768). Dendritic linkers can increase the molar ratio of antibiotic to antibody, i.e. loading, which is related to the potency of the AAC. Thus, where a cysteine engineered antibody bears only one reactive cysteine thiol group, a multitude of antibiotic moieties may be attached through a dendritic linker.

In certain embodiments of Formula I AAC, the protease-cleavable, non-peptide linker PML has the formula:

$$-Str-PM-Y-$$

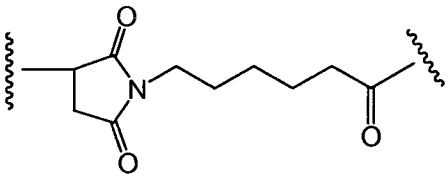
where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit;
abx is the rifamycin-type antibiotic; and
p is an integer from 1 to 8.

In one embodiment, a stretcher unit "Str" has the formula:

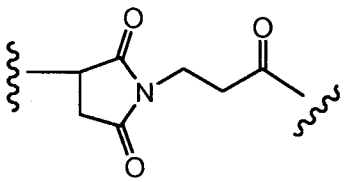


wherein R^6 is selected from the group consisting of C_1-C_{12} alkylene, C_1-C_{12} alkylene-C(=O), C_1-C_{12} alkylene-NH, $(CH_2CH_2O)_r$, $(CH_2CH_2O)_r$-C(=O), $(CH_2CH_2O)_r$-CH_2, and C_1-C_{12} alkylene-NHC(=O)CH_2CH(thiophen-3-yl), where r is an integer ranging from 1 to 10.

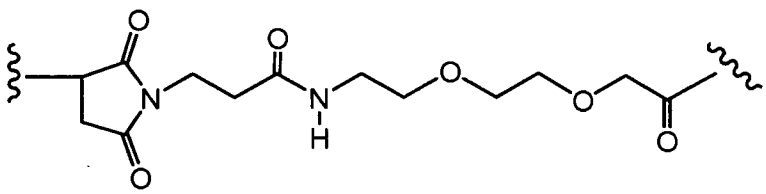
Exemplary stretcher units are shown below (wherein the wavy line indicates sites of covalent attachment to an antibody):

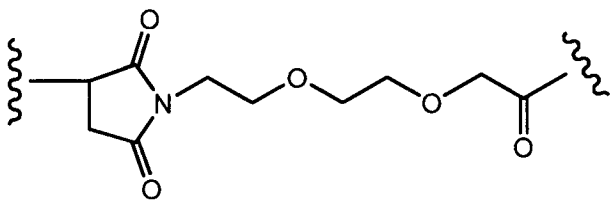


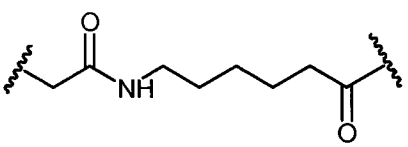
MC



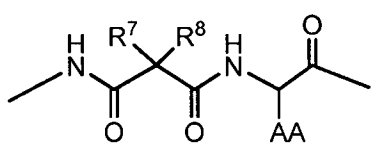
MP







In one embodiment, PM has the formula:



where R^7 and R^8 together form a C_3-C_7 cycloalkyl ring, and

AA is an amino acid side chain selected from H, $-CH_3$, $-CH_2(C_6H_5)$, $-CH_2CH_2CH_2CH_2NH_2$, $-CH_2CH_2CH_2NHC(NH)NH_2$, $-CHCH(CH_3)CH_3$, and $-CH_2CH_2CH_2NHC(O)NH_2$.

In one embodiment, spacer unit Y comprises para-aminobenzyl (PAB) or para-aminobenzyloxycarbonyl (PABC).

A spacer unit allows for release of the antibiotic moiety without a separate hydrolysis step. A spacer unit may be "self-immolative" or a "non-self-immolative." In certain embodiments, a spacer unit of a linker comprises a p-aminobenzyl unit (PAB). In one such embodiment, a p-aminobenzyl alcohol is attached to an amino acid unit via an amide bond, a carbamate, methylcarbamate, or carbonate between the p-aminobenzyl group and the antibiotic moiety (Hamann et al. (2005) *Expert Opin. Ther. Patents* (2005) 15:1087-1103). In one embodiment, the spacer unit is p-aminobenzyloxycarbonyl (PAB).

In one embodiment, the antibiotic comprises a quaternary amine, such as the dimethylaminopiperidyl group, when attached to the PAB spacer unit of the non-peptide linker. Examples of such quaternary amines are linker-antibiotic intermediates (PLA) are PLA-1 to 4 from Table 2. The quaternary amine group may modulate cleavage of the antibiotic moiety to optimize the antibacterial effects of the AAC. In another embodiment, the antibiotic is linked to the PABC spacer unit of the non-peptide linker, forming a carbamate functional group in the AAC. Such carbamate functional group may also optimize the antibacterial effects of the AAC. Examples of PABC carbamate linker-antibiotic intermediates (PLA) are PLA-5 and PLA-6 from Table 2.

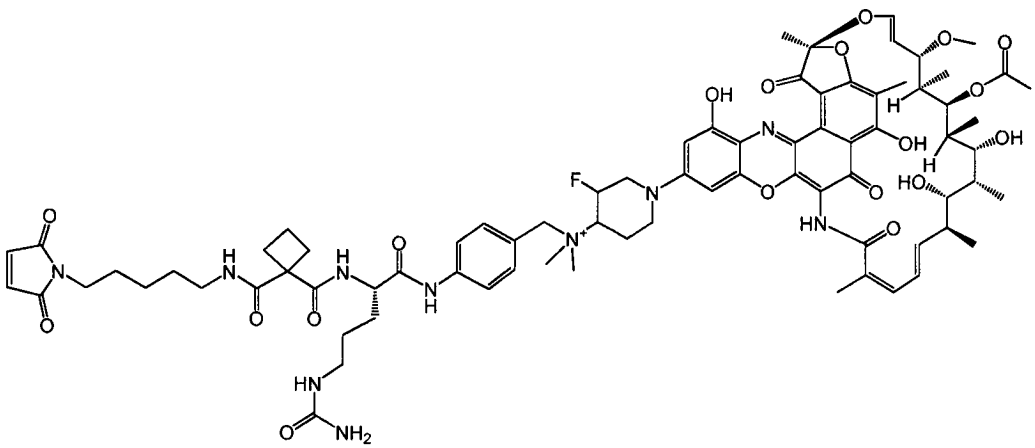
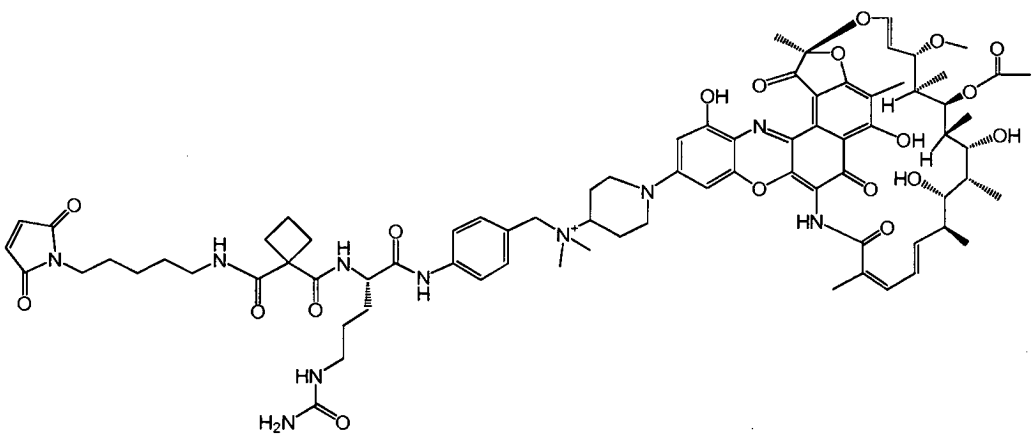
Other examples of self-immolative spacers include, but are not limited to, aromatic compounds that are electronically similar to the PAB group such as 2-aminoimidazol-5-methanol derivatives (US 7375078; Hay et al. (1999) *Bioorg. Med. Chem. Lett.* 9:2237) and ortho- or para-aminobenzylacetals. Spacers can be used that undergo cyclization upon amide bond hydrolysis, such as substituted and unsubstituted 4-aminobutyric acid amides (Rodrigues et al (1995) Chemistry Biology 2:223), appropriately substituted bicyclo[2.2.1] and bicyclo[2.2.2] ring systems (Storm et al (1972) *J. Amer. Chem. Soc.* 94:5815) and 2-aminophenylpropionic acid amides (Amsberry, et al (1990) *J. Org. Chem.* 55:5867). Elimination of amine-containing drugs that are substituted at glycine (Kingsbury et al (1984) *J. Med. Chem.* 27:1447) is also exemplary of self-immolative spacers useful in AAC.

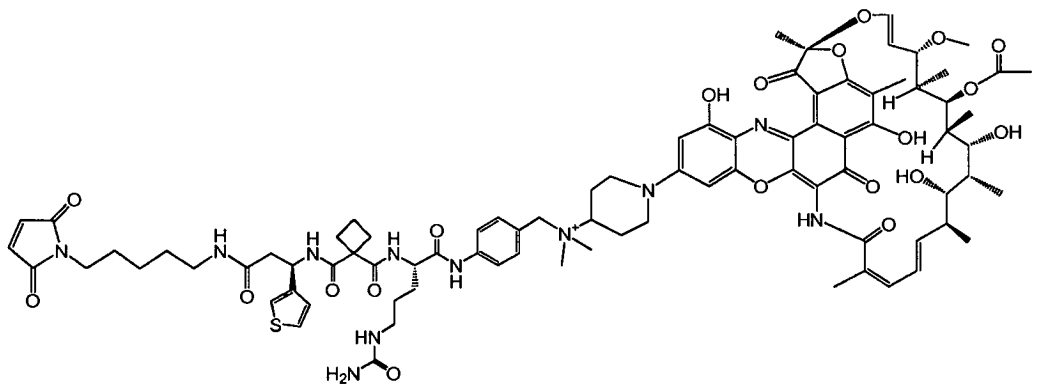
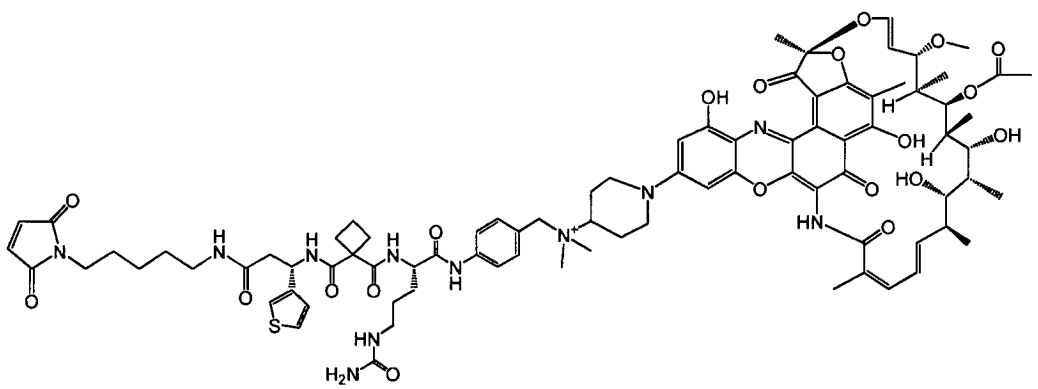
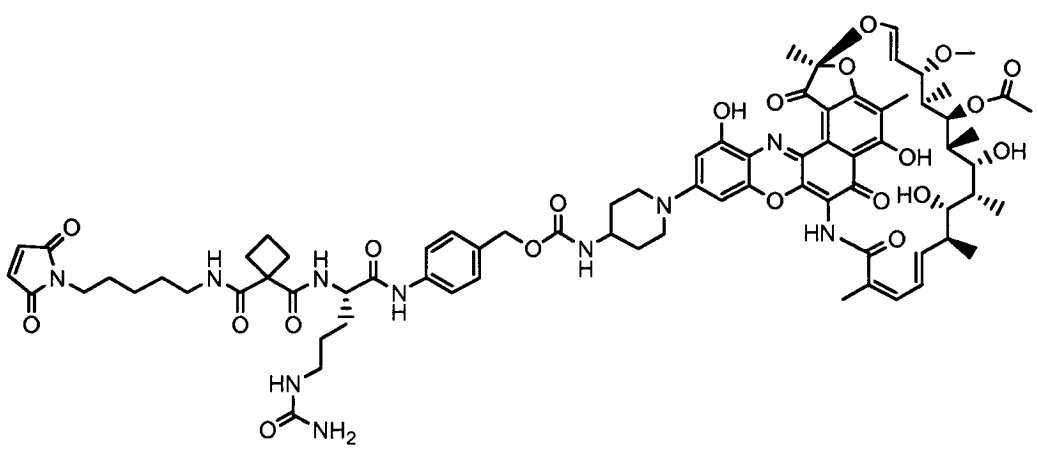
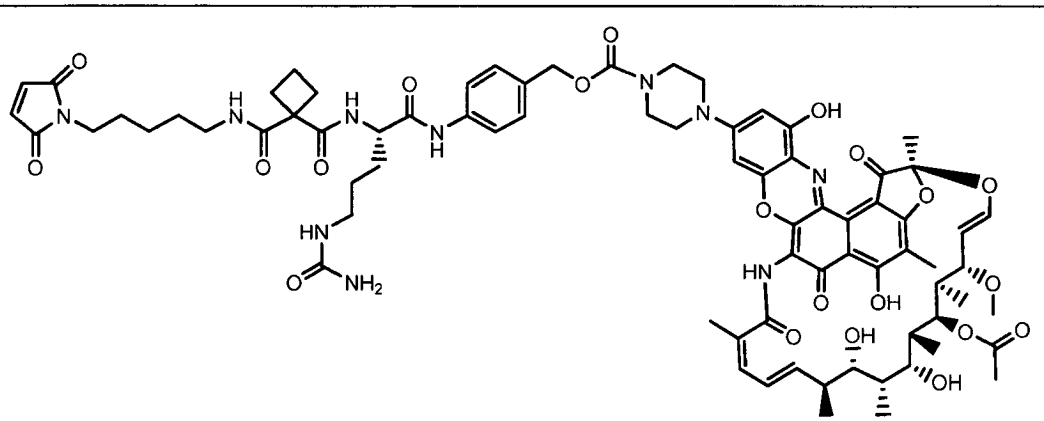
The amount of active antibiotic released from cleavage of AAC can be measured by a caspase release assay.

LINKER-ANTIBIOTIC INTERMEDIATES USEFUL FOR AAC

PML Linker-antibiotic intermediates (PLA) of Formula II and Table 2 were prepared by coupling a rifamycin-type antibiotic moiety with a linker reagent, Examples 7-17. Linker reagents were prepared by methods described in WO 2012/113847; US 7659241; US 7498298; US 20090111756; US 2009/0018086; US 6214345; Dubowchik et al (2002) Bioconjugate Chem. 13(4):855-869

Table 2 PML Linker-antibiotic intermediates

LA No.	Structure
PLA-1	
PLA-2	

PLA-3	
PLA-4	
PLA-5	
PLA-6	

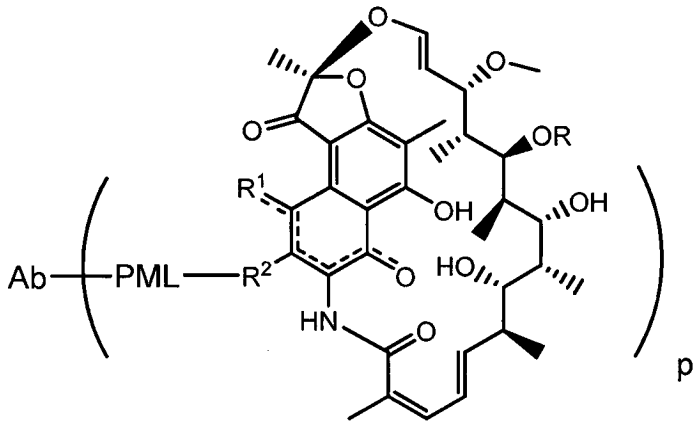
EMBODIMENTS OF ANTIBODY-ANTIBIOTIC CONJUGATES

Cysteine engineered, rF1 antibodies were linked via the free cysteine thiol group to derivatives of rifamycin, termed pipBOR and others, via a protease cleavable, non-peptide linker to form the antibody-antibiotic conjugate compounds (AAC) in Table 3. The linker is designed to be cleaved by lysosomal proteases including cathepsins B, D and others, Generation of the linker-antibiotic intermediate consisting of the antibiotic and the PML linker and others, is described in detail in Examples 7-17. The linker is designed such that cleavage of the amide bond at the PAB moiety separates the antibody from the antibiotic in an active state.

The AAC named "dimethylpipBOR" is identical to the "pipBOR" AAC except for the dimethylated amino on the antibiotic and the oxycarbonyl group on the linker.

Figure 3 shows a possible mechanism of drug activation for antibody-antibiotic conjugates (AAC). Active antibiotic (Ab) is only released after internalization of the AAC inside mammalian cells. The Fab portion of the antibody in AAC binds S. aureus whereas the Fc portion of the AAC enhances uptake of the bacteria by Fc-receptor mediated binding to phagocytic cells including neutrophils and macrophages. After internalization into the phagolysosome, the linker may be cleaved by lysosomal proteases releasing the active antibiotic inside the phagolysosome.

An embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes Formula I:



I

wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

R^1 is OH;

R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

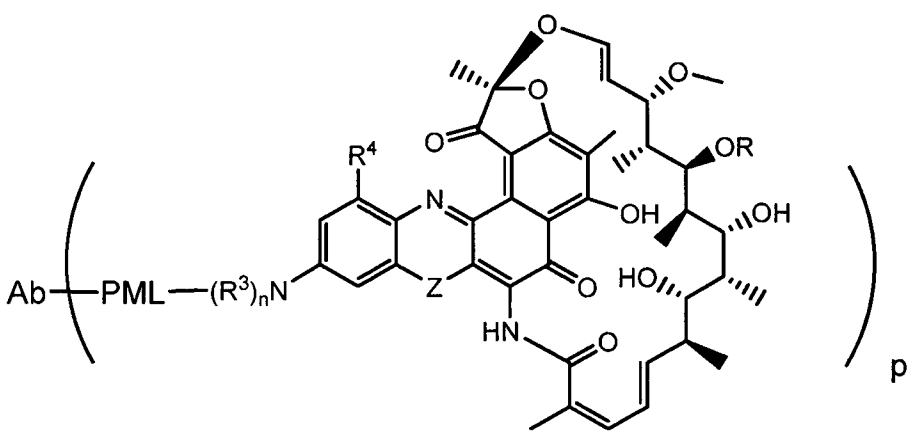
or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH;

PML is the protease-cleavable, non-peptide linker attached to R^2 or the fused heteroaryl or heterocyclyl formed by R^1 and R^2;

Ab is the rF1 antibody; and

p is an integer from 1 to 8.

Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



wherein

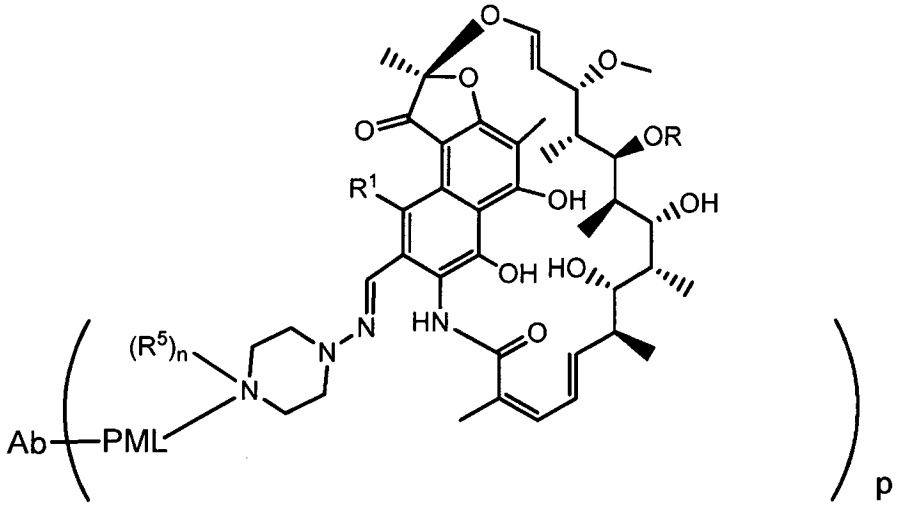
R^3 is independently selected from H and C_1–C_{12} alkyl;

n is 1 or 2;

R^4 is selected from H, F, Cl, Br, I, C_1–C_{12} alkyl, and OH; and

Z is selected from NH, N(C_1–C_{12} alkyl), O and S.

Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:

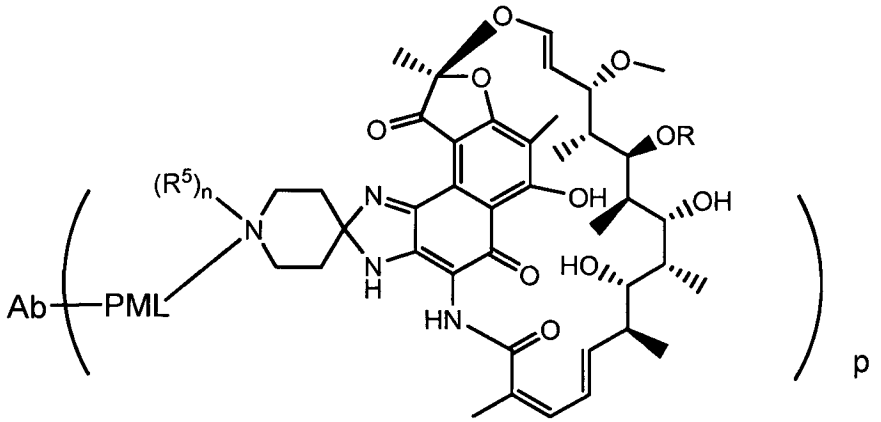


wherein

R^5 is selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:

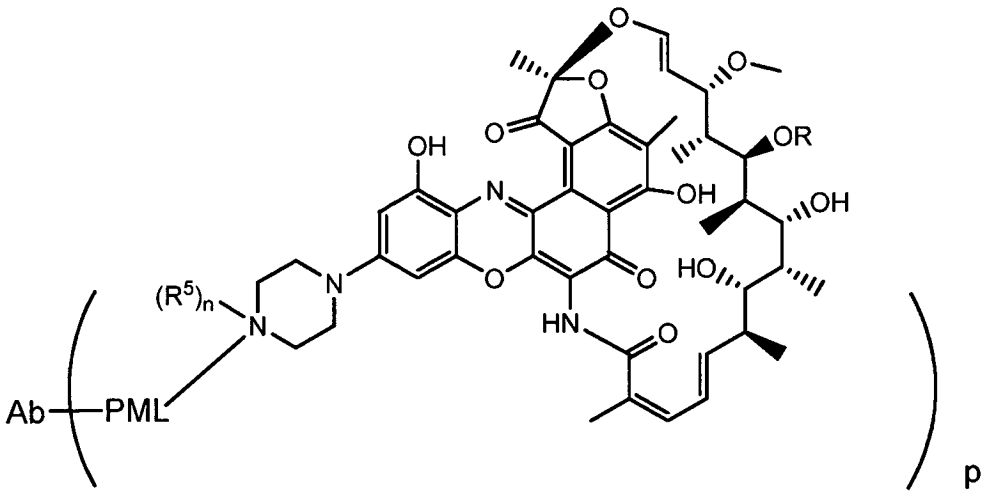


wherein

R^5 is selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:

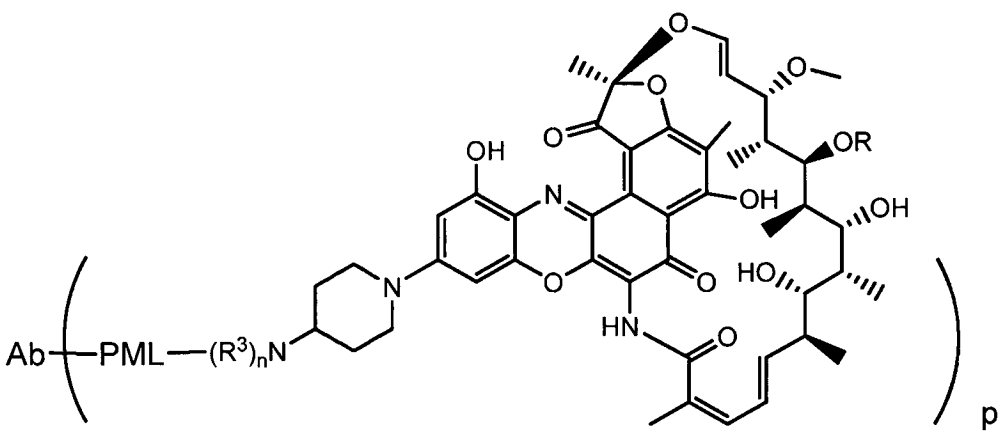


wherein

R^5 is independently selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:

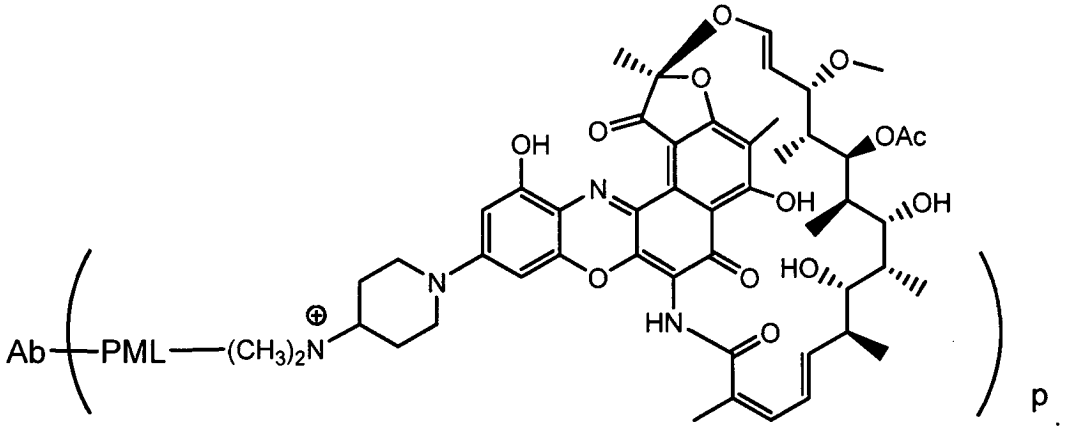


wherein

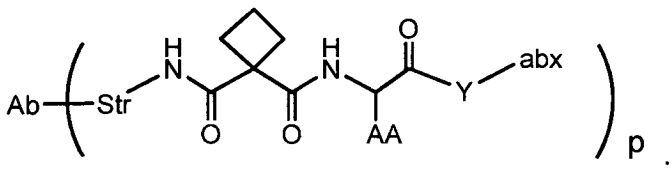
R^3 is independently selected from H and C_1–C_{12} alkyl; and

n is 1 or 2.

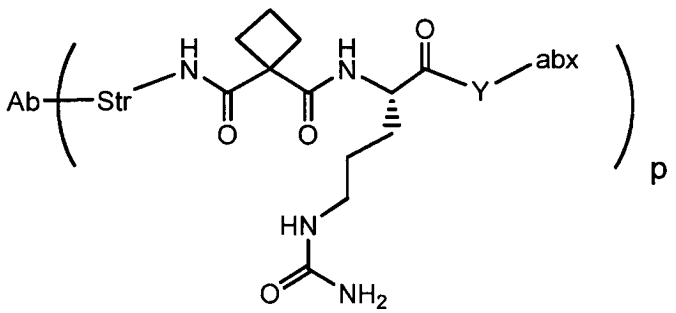
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



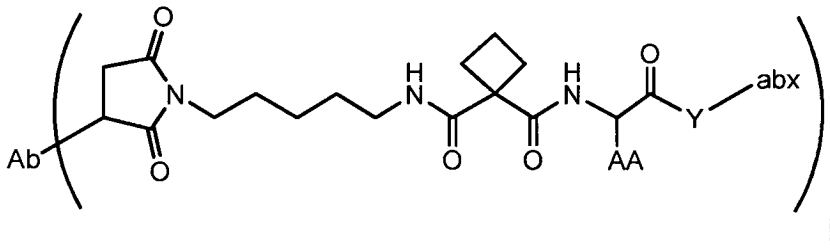
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



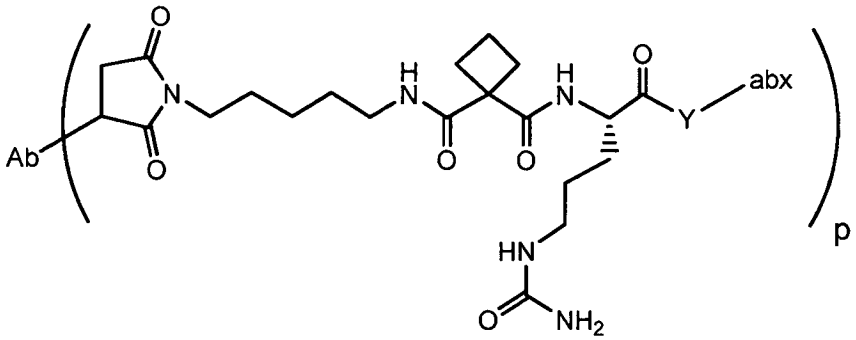
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



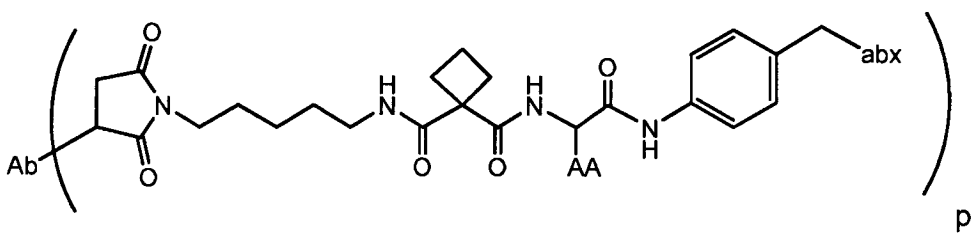
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



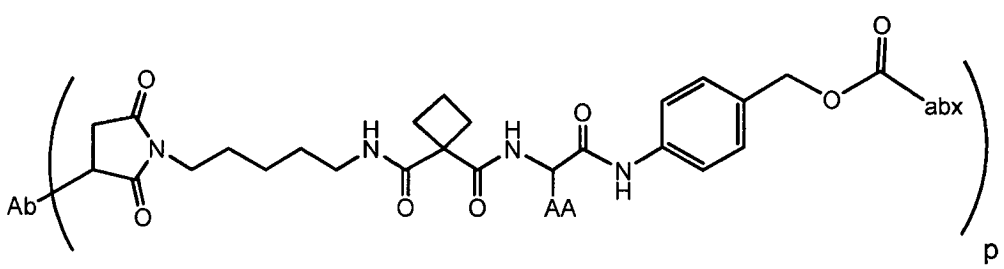
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



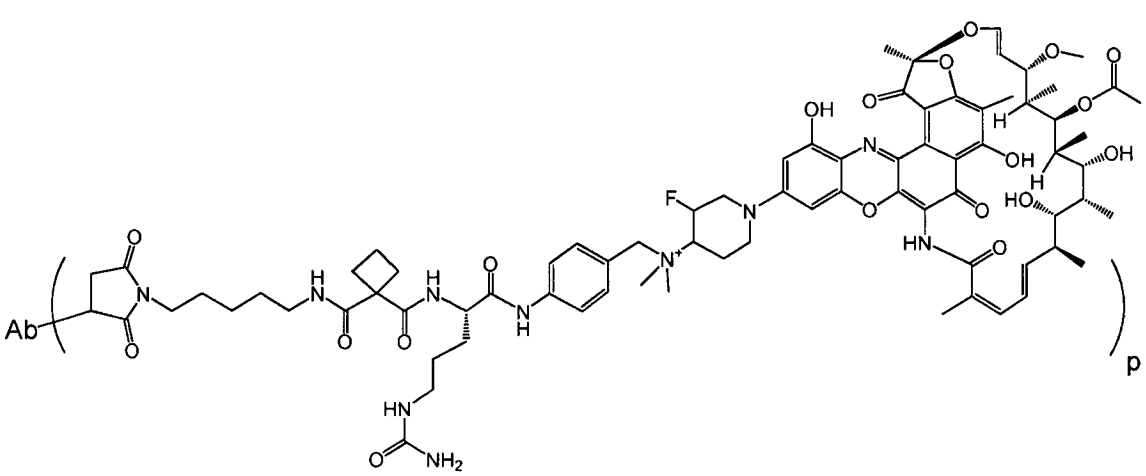
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formulas:



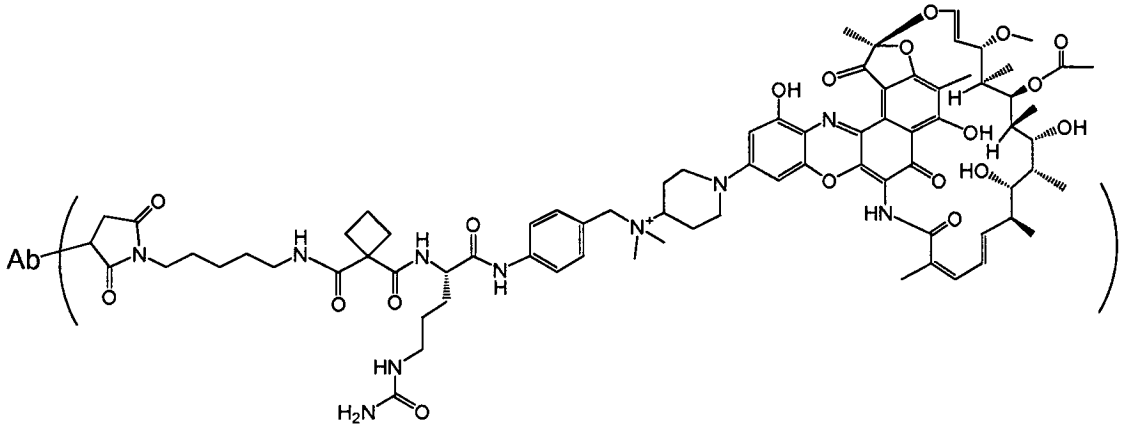
and



Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formulas:



;



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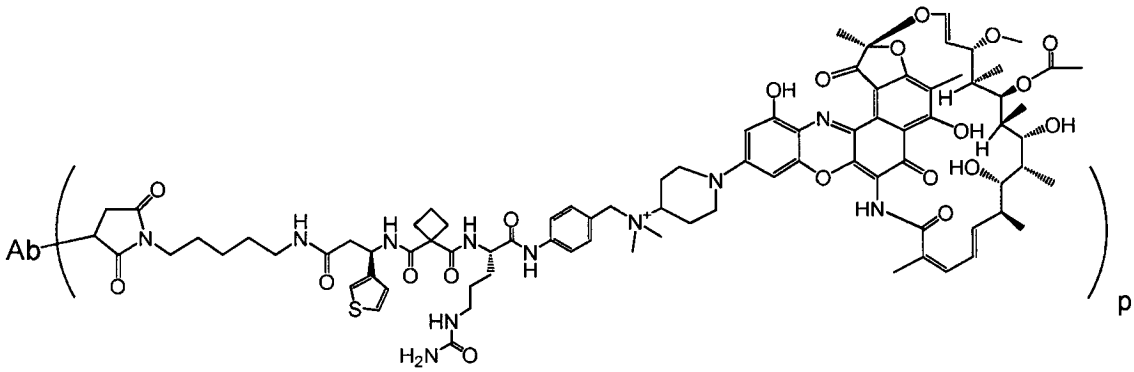

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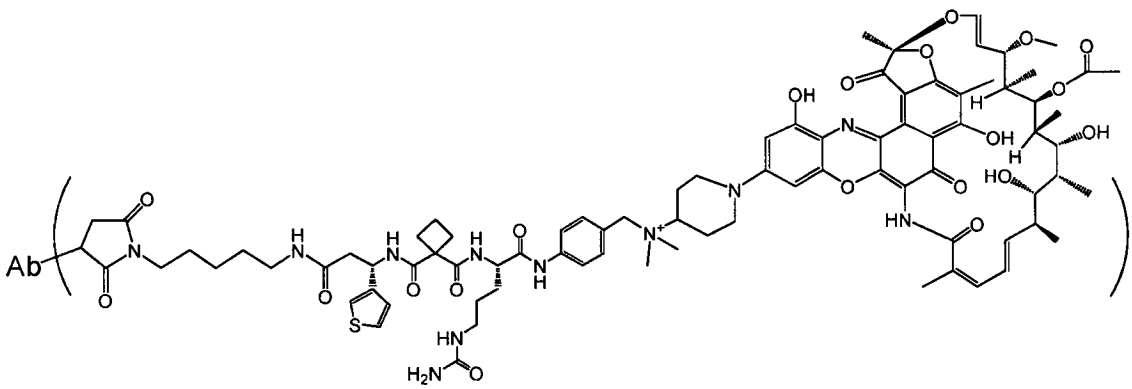

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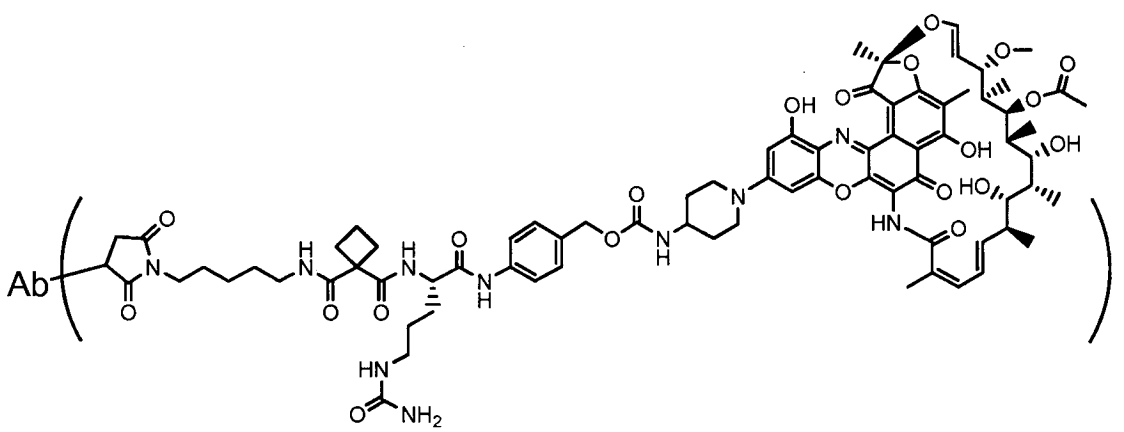
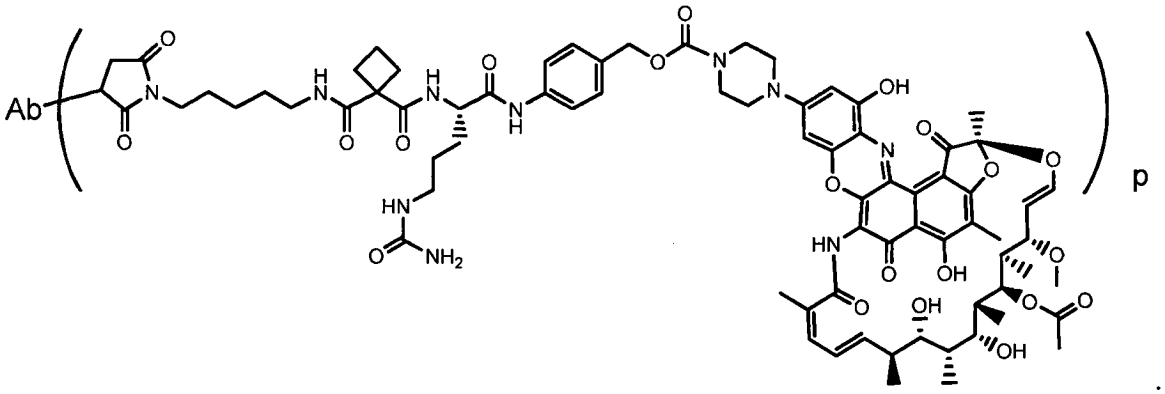


;

and



ANTIBIOTIC LOADING OF AAC

Antibiotic loading is represented by p, the average number of antibiotic (abx) moieties per antibody in a molecule of Formula I. Antibiotic loading may range from 1 to 20 antibiotic moieties (D) per antibody. The AAC of Formula I include collections or a pool of antibodies conjugated with a range of antibiotic moieties, from 1 to 20. The average number of antibiotic moieties per antibody in preparations of AAC from conjugation reactions may be characterized by conventional means such as mass spectroscopy, ELISA assay, and HPLC. The quantitative distribution of AAC in terms of p may also be determined. In some instances, separation, purification, and characterization of homogeneous AAC where p is a certain value from AAC with other antibiotic loadings may be achieved by means such as reverse phase HPLC or electrophoresis.

For some antibody-antibiotic conjugates, p may be limited by the number of attachment sites on the antibody. For example, where the attachment is a cysteine thiol, as in the exemplary embodiments above, an antibody may have only one or several cysteine thiol groups, or may have only one or several sufficiently reactive thiol groups through which a linker may be attached. In certain embodiments, higher antibiotic loading, e.g. p >5, may cause aggregation, insolubility, toxicity, or loss of cellular permeability of certain antibody-antibiotic conjugates. In certain embodiments, the antibiotic loading for an AAC of the invention ranges from 1 to about 8; from about 2 to about 6; from about 2 to about 4; or from about 3 to about 5; about 4; or about 2.

In certain embodiments, fewer than the theoretical maximum of antibiotic moieties are conjugated to an antibody during a conjugation reaction. An antibody may contain, for example, lysine residues that do not react with the antibiotic-linker intermediate or linker reagent, as discussed below. Generally, antibodies do not contain many free and reactive cysteine thiol

groups which may be linked to an antibiotic moiety; indeed most cysteine thiol residues in antibodies exist as disulfide bridges. In certain embodiments, an antibody may be reduced with a reducing agent such as dithiothreitol (DTT) or tricarbonylethylphosphine (TCEP), under partial or total reducing conditions, to generate reactive cysteine thiol groups. In certain embodiments, an antibody is subjected to denaturing conditions to reveal reactive nucleophilic groups such as lysine or cysteine.

The loading (antibiotic/antibody ratio, "AAR") of an AAC may be controlled in different ways, e.g., by: (i) limiting the molar excess of antibiotic-linker intermediate or linker reagent relative to antibody, (ii) limiting the conjugation reaction time or temperature, and (iii) partial or limiting reductive conditions for cysteine thiol modification. "DAR" if referred to herein or in the figures shall mean the same as "AAR".

It is to be understood that where more than one nucleophilic group reacts with an antibiotic-linker intermediate or linker reagent followed by antibiotic moiety reagent, then the resulting product is a mixture of AAC compounds with a distribution of one or more antibiotic moieties attached to an antibody. The average number of antibiotics per antibody may be calculated from the mixture by a dual ELISA antibody assay, which is specific for antibody and specific for the antibiotic. Individual AAC molecules may be identified in the mixture by mass spectroscopy and separated by HPLC, e.g. hydrophobic interaction chromatography (*see, e.g.*, McDonagh et al (2006) Prot. Engr. Design & Selection 19(7):299-307; Hamblett et al (2004) Clin. Cancer Res. 10:7063-7070; Hamblett, K.J., et al. "Effect of drug loading on the pharmacology, pharmacokinetics, and toxicity of an anti-CD30 antibody-drug conjugate," Abstract No. 624, American Association for Cancer Research, 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004; Alley, S.C., et al. "Controlling the location of drug attachment in antibody-drug conjugates," Abstract No. 627, American Association for Cancer Research, 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004). In certain embodiments, a homogeneous AAC with a single loading value may be isolated from the conjugation mixture by electrophoresis or chromatography. Cysteine-engineered antibodies of the invention enable more homogeneous preparations since the reactive site on the antibody is primarily limited to the engineered cysteine thiol. In one embodiment, the average number of antibiotic moieties per antibody is in the range of about 1 to about 20. In some embodiments the range is selected and controlled from about 1 to 4.

METHODS OF PREPARING ANTIBODY-ANTIBIOTIC CONJUGATES

An AAC of Formula I may be prepared by several routes employing organic chemistry reactions, conditions, and reagents known to those skilled in the art, including: (1) reaction of a nucleophilic group of an antibody with a bivalent linker reagent to form Ab-L via a covalent bond, followed by reaction with an antibiotic moiety (abx); and (2) reaction of a nucleophilic group of an antibiotic moiety with a bivalent linker reagent, to form L-abx, via a covalent bond, followed by reaction with a nucleophilic group of an antibody. Exemplary methods for preparing an AAC of Formula I via the latter route are described in US 7498298, which is expressly incorporated herein by reference.

Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, e.g. lysine, (iii) side chain thiol groups, e.g. cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, i.e. cysteine bridges. Antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol) or tricarbonylethylphosphine (TCEP), such that the antibody is fully or partially reduced. Each cysteine bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through modification of lysine residues, e.g., by reacting lysine residues with 2-iminothiolane (Traut's reagent), resulting in conversion of an amine into a thiol. Reactive thiol groups may be introduced into an antibody by introducing one, two, three, four, or more cysteine residues (e.g., by preparing variant antibodies comprising one or more non-native cysteine amino acid residues).

Antibody-antibiotic conjugates of the invention may also be produced by reaction between an electrophilic group on an antibody, such as an aldehyde or ketone carbonyl group, with a nucleophilic group on a linker reagent or antibiotic. Useful nucleophilic groups on a linker reagent include, but are not limited to, hydrazide, oxime, amino, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide. In one embodiment, an antibody is modified to introduce electrophilic moieties that are capable of reacting with nucleophilic substituents on the linker reagent or antibiotic. In another embodiment, the sugars of glycosylated antibodies may be oxidized, e.g. with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or antibiotic

moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, e.g. by borohydride reagents to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either galactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the antibody that can react with appropriate groups on the antibiotic (Hermanson, Bioconjugate Techniques). In another embodiment, antibodies containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid (Geoghegan & Stroh, (1992) *Bioconjugate Chem.* 3:138-146; US 5362852). Such an aldehyde can be reacted with an antibiotic moiety or linker nucleophile.

Nucleophilic groups on an antibiotic moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

The antibody-antibiotic conjugates (AAC) in Table 3 were prepared by conjugation of the described rF1 antibodies and linker-antibiotic intermediates of Table 2, and according to the described methods in Example 18. AAC were tested for efficacy by *in vitro* macrophage assay (Example 19) and *in vivo* mouse kidney model (Example 20).

Table 3 rF1 Antibody-PML-antibiotic conjugates (AAC)

AAC No.	AAC formula	linker-abx PLA No.	AAR *
101	thio-rF1-LC-V205C-MC-(CBDK-cit)-PAB-(dimethyl, fluoropipBOR)	PLA-1	2.0
102	thio-rF1-HC-121C, LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR)	PLA-2	3.9
103	thio-rF1-LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR)	PLA-2	1.9
104	thio-rF1-HC-A121C, LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR)	PLA-2	3.7

* AAR = antibiotic/antibody ratio average

Wild-type ("WT"), cysteine engineered mutant antibody ("thio"), light chain ("LC"), heavy chain ("HC"), 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), cyclobutyldiketo ("CBDK"), citrulline ("cit"), cysteine ("cys"), p-aminobenzyl ("PAB"), and p-aminobenzyloxycarbonyl ("PABC")

METHODS OF TREATING AND PREVENTING INFECTIONS WITH ANTIBODY-ANTIBIOTIC CONJUGATES

The rF1-AAC of the invention are useful as antimicrobial agents effective against human and veterinary Staphylococci, for example S. aureus, S. saprophyticus and S. simulans. In a specific aspect, the AAC of the invention are useful to treat *S. aureus* infections.

Following entry into the bloodstream, *S. aureus* can cause metastatic infection in almost any organ. Secondary infections occur in about one-third of cases before the start of therapy (Fowler et al., (2003) Arch. Intern. Med. 163:2066-2072), and even in 10% of patients after the start of therapy (Khatib et al., (2006) Scand. J. Infect. Dis., 38:7-14). Hallmarks of infections are large reservoirs of pus, tissue destruction, and the formation of abcesses (all of which contain large quantities of neutrophils). About 40% of patients develop complications if the bacteremia persists beyond three days.

The proposed mechanism of action of an AAC has been described above (under subheading Antibody-Antibiotic Conjugates). The rF1 antibody-antibiotic conjugates (AAC) of the invention have significant therapeutic advantages for treating intracellular pathogens. The AAC linker is cleaved by exposure to phagolysosomal enzymes, releasing an active antibiotic. Due to the confined space and relatively high local antibiotic concentration (about 10^4 per bacterium), the result is that the phagolysosome no longer supports the survival of the intracellular pathogen. Because the AAC is essentially an inactive prodrug, the therapeutic index of the antibiotic can be extended relative to the free (unconjugated) form. The antibody provides pathogen specific targeting, while the cleavable linker is cleaved under conditions specific to the intracellular location of the pathogen. The effect can be both directly on the opsonized pathogen as well as other pathogens that are co-localized in the phagolysosome. Antibiotic tolerance is the ability of a disease-causing pathogen to resist killing by antibiotics and other antimicrobials and is mechanistically distinct from multidrug resistance (Lewis K (2007). "Persister cells, dormancy and infectious disease". *Nature Reviews Microbiology* 5 (1): 48–56. doi:10.1038/nrmicro1557). Rather, this form of tolerance is caused by a small sub-population of microbial cells called persisters (Bigger JW (14 October 1944). "Treatment of staphylococcal infections with penicillin by intermittent sterilization". *Lancet* 244 (6320): 497–500). These cells are not multidrug resistant in the classical sense, but rather are dormant cells that are tolerant to antibiotic treatment that can kill their genetically identical siblings. This antibiotic tolerance is induced by a non-or extremely slow dividing physiological state. When antimicrobial treatment fails to eradicate these persister cells, they become a reservoir for

recurring chronic infections. The antibody-antibiotic conjugates of the invention possess a unique property to kill these persister cells and suppress the emergence of multidrug tolerant bacterial populations.

In another embodiment, the rF1-AAC of the invention may be used to treat infection regardless of the intracellular compartment in which the pathogen survives.

In another embodiment, rF1-AACs of the invention could also be used to target Staphylococci bacteria in planktonic or biofilm form. Bacterial infections treatable with antibody-antibiotic conjugates (AAC) of the invention include treating bacterial pulmonary infections, such as S. aureus pneumonia, osteomyelitis, recurrent rhinosinusitis, bacterial endocarditis, bacterial ocular infections, such as trachoma and conjunctivitis, heart, brain or skin infections, infections of the gastrointestinal tract, such as travellers' diarrhea, ulcerative colitis, irritable bowel syndrome (IBS), Crohn's disease, and IBD (inflammatory bowel disease) in general, bacterial meningitis, and abscesses in any organ, such as muscle, liver, meninges, or lung. The bacterial infections can be in other parts of the body like the urinary tract, the bloodstream, a wound or a catheter insertion site. The AACs of the invention are useful for difficult-to-treat infections that involve biofilms, implants or sanctuary sites (e.g., osteomyelitis and prosthetic joint infections), and high mortality infections such as hospital acquired pneumonia and bacteremia. Vulnerable patient groups that can be treated to prevent Staphylococcal aureus infection include hemodialysis patients, immune-compromised patients, patients in intensive care units, and certain surgical patients. In another aspect, the invention provides a method of killing, treating, or preventing a microbial infection in an animal, preferably a mammal, and most preferably a human, that includes administering to the animal an rF1 AAC or pharmaceutical formulation of an AAC of the invention. The invention further features treating or preventing diseases associated with or which opportunistically result from such microbial infections. Such methods of treatment or prevention may include the oral, topical, intravenous, intramuscular, or subcutaneous administration of a composition of the invention. For example, prior to surgery or insertion of an IV catheter, in ICU care, in transplant medicine, with or post cancer chemotherapy, or other activities that bear a high risk of infection, the AAC of the invention may be administered to prevent the onset or spread of infection.

The bacterial infection may be caused by bacteria with an active and inactive form, and the AAC is administered in an amount and for a duration sufficient to treat both the active and the inactive, latent form of the bacterial infection, which duration is longer than is needed to treat the active form of the bacterial infection.

An aspect of the invention is a method of treating a patient infected with S. aureus and/or Listeria monocytogenes by administering a therapeutically effective amount of an rF1-AAC of the invention. The invention also contemplates a method of preventing infections by one or more of S. aureus or S. Epidermidis, or S. saprophyticus or S. simulans by administering a therapeutically effective amount of an rF1-AAC of the invention in hospital settings such as surgery, burn patient, and organ transplantation.

The patient needing treatment for a bacterial infection as determined by a physician of skill in the art may have already been, but does not need to be diagnosed with the kind of bacteria that he/she is infected with. Since a patient with a bacterial infection can take a turn for the worse very quickly, in a matter of hours, the patient upon admission into the hospital can be administered the rF1-AACs of the invention along with one or more standard of care Abx such as vancomycin or ciprofloxacin. When the diagnostic results become available and indicate the presence of, e.g., S. aureus in the infection, the patient can continue with treatment with the rF1 AAC. Therefore, in one embodiment of the method of treating a bacterial infection or specifically a S. aureus infection, the patient is administered a therapeutically effective amount of an rF1 AAC. In the methods of treatment or prevention of the present invention, an AAC of the invention can be administered as the sole therapeutic agent or in conjunction with other agents such as those described below. The AACs of the invention show superiority to vancomycin in the treatment of MRSA in pre-clinical models. Comparison of AACs to SOC can be measured, e.g., by a reduction in mortality rate. The patient being treated would be assessed for responsiveness to the AAC treatment by a variety of measurable factors. Examples of signs and symptoms that clinicians might use to assess improvement in their patients includes the following: normalization of the white blood cell count if elevated at diagnosis, normalization of body temperature if elevated (fever) at the time of diagnosis, clearance of blood cultures, visual improvement in wound including less erythema and drainage of pus, reduction in ventilator requirements such as requiring less oxygen or reduced rate of ventilation in a patient who is ventilated, coming off of the ventilator entirely if the patient is ventilated at the time of diagnosis, use of less medications to support a stable blood pressure if these medications were required at the time of diagnosis, normalization of lab abnormalities that suggest end-organ failure such as elevated creatinine or liver function tests if they were abnormal at the time of diagnosis, and improvement in radiologic imaging (e.g. chest x-ray that previously suggested pneumonia showing resolution). In a patient in the ICU, these factors might be measured at least daily. Fever is monitored closely as is white blood cell count including absolute neutrophil

counts as well as evidence that a "left shift" (appearance of blasts indicating increased neutrophil production in response to an active infection) has resolved.

In the context of the present methods of treatment of the invention, a patient with a bacterial infection is considered to be treated if there is significant measurable improvement as assessed by the physician of skill in the art, in at least two or more of the preceding factors compared to the values, signs or symptoms before or at the start of treatment or at the time of diagnosis. In some embodiments, there is measurable improvement in 3, 4, 5, 6 or more of the aforementioned factors. If some embodiments, the improvement in the measured factors is by at least 50%, 60%, 70%, 80%, 90%, 95% or 100% compared to the values before treatment. Typically, a patient can be considered completely treated of the bacterial infection (e.g., S. aureus infection) if the patient's measurable improvements include the following: i) repeat blood or tissue cultures (typically several) that do not grow out the bacteria that was originally identified ; ii) fever is normalized; iii) WBC is normalized; and iv) evidence that end-organ failure (heart, lungs, liver, kidneys, vascular collapse) has resolved either fully or partially given the pre-existent co-morbidities that the patient had.

Dosing. In any of the foregoing aspects, in treating an infected patient, the dosage of an AAC is normally about 0.001 to 1000 mg/kg/day. In one embodiment the patient with a bacterial infection is treated at an AAC dose in the range of about 1 mg/kg to about 150mg/kg, typically about 5mg/kg to about 150mg/kg, more specifically, 25mg/kg to 125 mg/kg, 50mg/kg to 125mg/kg, even more specifically at about 50mg/kg to 100mg/kg. The AAC may be given daily (e.g., a single dose of 5 to 50 mg/kg/day) or less frequently (e.g., a single dose of 5, 10, 25 or 50 mg/kg/week). One dose may be split over 2 days, for example, 25mg/kg on one day and 25mg/kg the next day. The patient can be administered a dose once every 3 days (q3D), once a week to every other week (qOW), for a duration of 1-8 weeks. In one embodiment, the patient is administered an AAC of the invention via IV once a week for 2-6 weeks with standard of care (SOC) to treat the bacterial infection such as a staph A infection. Treatment length would be dictated by the condition of the patient or the extent of the infection, e.g. a duration of 2 weeks for uncomplicated bacteremia, or 6 weeks for bacteremia with endocarditis.

In one embodiment, an AAC administered at an initial dose of 2.5 to 100 mg/kg for one to seven consecutive days, followed by a maintenance dose of 0.005 to 10 mg/kg once every one to seven days for one month.

Route of administration. For treating the bacterial infections, the AACs of the invention can be administered at any of the preceding dosages intravenously (i.v.) or subcutaneously. In one embodiment, the rF1-AAC is administered intravenously. In a specific embodiment, the rF1-AAC is administered via i.v., wherein the rF1 antibody is one selected from the group of Abs with amino acid sequences as disclosed under SDR and rF1Abs and Tables 4A and 4B.

Combination therapy. An AAC may be administered in conjunction with one or more additional, e.g. second, therapeutic or prophylactic agents as appropriate as determined by the physician treating the patient.

In one embodiment, the second antibiotic administered in combination with the antibody-antibiotic conjugate compound of the invention is selected from the structural classes: : (i) aminoglycosides; (ii) beta-lactams; (iii) macrolides/cyclic peptides; (iv) tetracyclines; (v) fluoroquinolines/fluoroquinolones; (vi) and oxazolidinones. See: Shaw, K. and Barbachyn, M. (2011) Ann. N.Y. Acad. Sci. 1241:48-70; Sutcliffe, J. (2011) Ann. N.Y. Acad. Sci. 1241:122-152.

In one embodiment, the second antibiotic administered in combination with the antibody-antibiotic conjugate compound of the invention is selected from clindamycin, novobiocin, retapamulin, daptomycin, GSK-2140944, CG-400549, sitafloxacin, teicoplanin, triclosan, napthyridone, radezolid, doxorubicin, ampicillin, vancomycin, imipenem, doripenem, gemcitabine, dalbavancin, and azithromycin.

Additional examples of these additional therapeutic or prophylactic agents are anti-inflammatory agents (e.g., non-steroidal anti-inflammatory drugs (NSAIDs; e.g., detoprofen, diclofenac, diflunisal, etodolac, fenoprofen, flurbiprofen, ibuprofen, indomethacin, ketoprofen, meclofenameate, mefenamic acid, meloxicam, nabumeone, naproxen sodium, oxaprozin, piroxicam, sulindac, tolmetin, celecoxib, rofecoxib, aspirin, choline salicylate, salsalte, and sodium and magnesium salicylate) and steroids (e.g., cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone)), antibacterial agents (e.g., azithromycin, clarithromycin, erythromycin, gatifloxacin, levofloxacin, amoxicillin, metronidazole, penicillin G, penicillin V, methicillin, oxacillin, cloxacillin, dicloxacillin, nafcillin, ampicillin, carbenicillin, ticarcillin, mezlocillin, piperacillin, azlocillin, temocillin, cepalothin, cephapirin, cephradine, cephaloridine, cefazolin, cefamandole, cefuroxime, cephalexin, cefprozil, cefaclor, loracarbef, cefoxitin, cefmatozole, cefotaxime, ceftizoxime, ceftriaxone, cefoperazone, ceftazidime, cefixime, cefpodoxime, ceftibuten, cefdinir, cefpirome, cefepime, BAL5788, BAL9141, imipenem, ertapenem, meropenem, astreonam, clavulanate,

sulbactam, tazobactam, streptomycin, neomycin, kanamycin, paromycin, gentamicin, tobramycin, amikacin, netilmicin, spectinomycin, sisomicin, dibekalin, isepamicin, tetracycline, chlortetracycline, demeclocycline, minocycline, oxytetracycline, methacycline, doxycycline, telithromycin, ABT-773, lincomycin, clindamycin, vancomycin, oritavancin, dalbavancin, teicoplanin, quinupristin and dalfopristin, sulphanilamide, para-aminobenzoic acid, sulfadiazine, sulfisoxazole, sulfamethoxazole, sulfathalidine, linezolid, nalidixic acid, oxolinic acid, norfloxacin, perfloxacin, enoxacin, ofloxacin, ciprofloxacin, temafloxacin, lomefloxacin, fleroxacin, grepafloxacin, sparfloxacin, trovafloxacin, clinafloxacin, moxifloxacin, gemifloxacin, sitafloxacin, daptomycin, garenoxacin, ramoplanin, faropenem, polymyxin, tigecycline, AZD2563, or trimethoprim), antibacterial antibodies including antibodies to the same or different antigen from the AAC targeted Ag, platelet aggregation inhibitors (e.g., abciximab, aspirin, cilostazol, clopidogrel, dipyridamole, eptifibatide, ticlopidine, or tirofiban), anticoagulants (e.g., dalteparin, danaparoid, enoxaparin, heparin, tinzaparin, or warfarin), antipyretics (e.g., acetaminophen), or lipid lowering agents (e.g., cholestyramine, colestipol, nicotinic acid, gemfibrozil, probucol, ezetimibe, or statins such as atorvastatin, rosuvastatin, lovastatin simvastatin, pravastatin, cerivastatin, and fluvastatin). In one embodiment the AAC of the invention is administered in combination with standard of care (SOC) for S. aureus (including methicillin-resistant and methicillin-sensitive strains). MSSA is usually typically treated with nafcillin or oxacillin and MRSA is typically treated with vancomycin or cefazolin.

These additional agents may be administered within 14 days, 7 days, 1 day, 12 hours, or 1 hour of administration of an AAC, or simultaneously therewith. The additional therapeutic agents may be present in the same or different pharmaceutical compositions as an AAC. When present in different pharmaceutical compositions, different routes of administration may be used. For example, an AAC may be administered intravenous or subcutaneously, while a second agent may be administered orally.

PHARMACEUTICAL FORMULATIONS

The present invention also provides pharmaceutical compositions containing the rF1-AAC, and to methods of treating a bacterial infection using the pharmaceutical compositions containing AAC. Such compositions may further comprise suitable excipients, such as pharmaceutically acceptable excipients (carriers) including buffers, acids, bases, sugars, diluents, glidants, preservatives and the like, which are well known in the art and are described herein. The present methods and compositions may be used alone or in combinations with other conventions methods and/or agents for treating infectious diseases. In some embodiments, a

pharmaceutical formulation comprises 1) a rF1-AAC of the invention, and 2) a pharmaceutically acceptable carrier. In some embodiments, a pharmaceutical formulation comprises 1) an AAC of the invention and optionally, 2) at least one additional therapeutic agent.

Pharmaceutical formulations comprising an AAC of the invention are prepared for storage by mixing the AAC having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)) in the form of aqueous solutions or lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, histidine and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride); phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.,* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Pharmaceutical formulations to be used for *in vivo* administration are generally sterile, readily accomplished by filtration through sterile filtration membranes.

Active ingredients may also be entrapped in microcapsule prepared, for example, by co-acervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacrylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody or AAC of the invention, which matrices are in the form of shaped articles, *e.g.,* films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No.

3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies or AAC remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37 °C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

An AAC may be formulated in any suitable form for delivery to a target cell/tissue. For example, AACs may be formulated as liposomes, a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., (1985) *Proc. Natl. Acad. Sci. USA* 82:3688; Hwang et al., (1980) *Proc. Natl Acad. Sci. USA* 77:4030; US 4485045; US 4544545; WO 97/38731; US 5013556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter.

MATERIALS AND METHODS

Bacterial strains and culture:

All experiments were done with MRSA-USA300 NRS384 obtained from NARSA (http://www.narsa.net/control/member/repositories) unless noted otherwise.

Bacteria were grown on tryptic soy agar plates supplemented with 5% sheep blood (TSA plates) for 18 h at 37 °C. For liquid cultures, single colonies from TSA plates were inoculated into tryptic soy broth (TSB) and incubated at 37 °C while shaking at 200 rpm for 18 h; 100 fold dilutions of these cultures in fresh TSB were further subcultured for various times.

MIC determinations for extracellular bacteria

The MIC for extracellular bacteria was determined by preparing serial 2-fold dilutions of the antibiotic in Tryptic Soy Broth. Dilutions of the antibiotic were made in quadruplicate in 96 well culture dishes. MRSA (NRS384 strain of USA300) was taken from an exponentially growing culture and diluted to $1x10^4$ CFU/mL. The bacteria was cultured in the presence of antibiotic for 18-24 hours with shaking at 37°C and bacterial growth was determined by reading the Optical Density (OD) at 630 nM. The MIC was determined to be the dose of antibiotic that inhibited bacterial growth by >90%.

MIC determinations for intracellular bacteria

Intracellular MIC was determined on bacteria that were sequestered inside mouse peritoneal macrophages (see below for generation of murine peritoneal macrophages). Macrophages were plated in 24 well culture dishes at a density of $4x10^5$ cells/mL and infected with MRSA at a ratio of 10-20 bacteria per macrophage. Macrophage cultures were maintained in growth media supplemented with 50 ug/mL of gentamycin (an antibiotic that is active only on extracellular bacteria) to inhibit the growth of extracellular bacteria and test antibiotics were added to the growth media 1 day after infection. The survival of intracellular bacteria was assessed 24 hours after addition of the antibiotics. Macrophages were lysed with Hanks Buffered Saline Solution supplemented with .1% Bovine Serum Albumin and .1% Triton-X, and serial dilutions of the lysate were made in Phosphate Buffered Saline solution containing .05% Tween-20. The number of surviving intracellular bacteria was determined by plating on Tryptic Soy Agar plates with 5% defibrinated sheep blood.

Bacterial cell wall preparations (CWP), immunoblotting, and ELISA

CWP were generated by incubating 40 mg of pelleted *S. aureus* or *S. epidermidis* per mL of 10 mM Tris-HCl (pH 7.4) supplemented with 30% raffinose, 100 μg/ml of lysostaphin (Cell Sciences, Canton, MA), and EDTA-free protease inhibitor cocktail (Roche, Pleasanton, CA), for 30 min at 37°C. The lysates were centrifuged at 11,600 x g for 5 min, and the supernatants containing cell wall components were collected. For immunoprecipitation, CWP were diluted 4 times in NP-40 buffer (120 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40, complete protease inhibitor cocktail (Roche) and 2 mM dithiothreitol) containing 1 μg/mL of indicated primary antibodies and incubated for 2 h at 4°C, followed by a 1 h incubation with Protein A/G agarose (Thermo, Waltham, MA). Whole cell lysates (WCL) were generated by a 30 min incubation at 37°C in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 100 μg/ml of lysostaphin, 1% Triton-X100

(Thermo) and EDTA-free protease inhibitor cocktail. For immunoblot analysis, proteins were separated on a 4-12% Tris-glycine gel, and transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA), followed by blotting with indicated primary antibodies (1 μg/mL). Antibodies used are listed in Table 1. Lectin studies were performed by immunoprecipitating filtered (0.2 micron) overnight culture supernatants with concanavalin A (ConA)- or sWGA-agarose beads (Vector Labs, Burlingame, CA) supplemented with 0.1 mM $CaCl_2$ and 0.01 mM $MnCl_2$.

ELISA experiments were performed using standard protocols. Briefly, plates which were pre-coated with CWP were reacted with human IgG preparations, ie. purified human IgG (Sigma), intravenous immunoglobulin Gammagard Liquid (Baxter, Westlake Village, CA), pooled serum from healthy donors or from MRSA patients (both generated in-house). The concentrations of anti-staphylococcal IgG present in the serum or purified IgG were calculated by using a calibration curve that was generated with known concentrations of mAb 28.9.9 against peptidoglycan.

Treatment of bacteria with human neutrophil proteases or lysosomal extracts from human neutrophils and cultured cells

Lysosomal extracts were isolated from human neutrophils, THP-1 cells, and RAW cells, using a Lysosome Enrichment kit (Thermo). A total of $5x10^7$ cells was used to obtain 300 to 500 microgram of total proteins in the lysosomes. Protease inhibitors were omitted from all steps to maintain protease activity in the lysosomes. The plasma membranes of the cells were disrupted by 30 strokes using a dounce homogenizer (Wheaton, Millville, NJ). The homogenate was centrifuged at 500 x g for 5 min to obtain postnuclear supernatant, which was loaded onto the top of a gradient of 8%, 20%, 23%, 27% and 30% (from top to bottom) of iodixanol. After ultracentrifugation at 145,000 x g for 2 h at 4°C, we obtained the lysosomes layered between 8% and 20% iodixanol. This lysosomal fraction was diluted into PBS and pelleted by centrifugation at 18,000 x g for 30 min at 4 °C. The lysosomal pellets were washed with PBS and lysed in 2% CHAPS with Tris-buffered saline to obtain lysosomal extracts.

To analyze the cleavage of SDR proteins by host proteases, *S. aureus* bacteria were treated with 50 nM of purified human neutrophil serine proteases or 0.1 mg/ml of neutrophil lysosomal extracts in 50 mM Tris (pH 8.0) with 150 mM NaCl and 2mM $CaCl_2$; or with 0.1 mg/ml of RAW or THP-1 lysosomal extracts in 50 mM NaCitrate with 100 mM NaCl and 2 mM DTT (pH 5.5). Cathepsin G inhibitor (Calbiochem, Billerica, MA) was added at 100 g/ml. These mixtures were incubated at 37°C for 30 minutes when using purified proteases or for 1 h when using lysosomal lysates, and centrifuged to pellet bacteria. The supernatants were analyzed

by immunoblotting to detect cleavage products. In some experiments, cell wall preparations were obtained from the remaining bacterial pellets and also analyzed by immunoblotting.

EXAMPLES

Example 1 Intracellular MRSA are protected from conventional antibiotics

To confirm the hypothesis that mammalian cells provide a protective niche for *S. aureus* in the presence of antibiotic therapy, the efficacy was compared of three major antibiotics that are currently used as standard of care (SOC) for invasive MRSA infections (vancomycin, daptomycin and linezolid) against extracellular planktonic bacteria versus bacteria sequestered inside murine macrophages (Table 1).

For extracellular bacteria, MRSA was cultured overnight in Tryptic Soy Broth, and the MIC was determined to be the minimum antibiotic dose that prevented growth. For intracellular bacteria, murine peritoneal macrophages were infected with MRSA and cultured in the presence of gentamycin to kill extracellular bacteria. Test antibiotics were added to the culture medium one day post infection, and the total number of surviving intracellular bacteria was determined 24 hours later. The expected serum concentrations for clinically relevant antibiotics was reported in Antimicrobial Agents, Andre Bryskier. ASM Press, Washington DC (2005).

Table 1: Minimum inhibitory concentrations (MIC) for several antibiotics on extracellular bacteria grown in liquid culture *vs.* intracellular bacteria sequestered inside murine macrophages.

Antibiotics (Abx)	Extracellular MRSA MIC (μg/mL)	Intracellular MRSA MIC (μg/mL)	Serum Cmax (μg/mL)
Vancomycin	1	>100	50
Daptomycin	4	>100	60
Linezolid	0.3	>20	20
Rifampicin	0.004	50	20

This analysis with a highly virulent community-acquired MRSA strain USA300 revealed that although extracellular MRSA is highly susceptible to growth inhibition by low

concentrations of vancomycin, daptomycin, and linezolid in liquid culture, all three antibiotics failed to kill the same strain of MRSA sequestered inside macrophages exposed to clinically achievable concentrations of the antibiotics. Even rifampicin, thought to be relatively effective at eliminating intracellular pathogens(Vandenbroek, P.V. (1989) Antimicrobial Drugs, Microorganisms, and Phagocytes. *Reviews of Infectious Diseases* **11**, 213-245), required a 6,000-fold higher dose to eliminate intracellular MRSA compared to the dose required to inhibit growth (MIC) of planktonic bacteria (Table 1), consistent with other studies showing that the majority of existing antibiotics are inefficient at killing intracellular *S. aureus* both in vitro and in vivo(Sandberg, A., Hessler, J.H., Skov, R.L., Blom, J. & Frimodt-Moller, N. (2009) "Intracellular activity of antibiotics against Staphylococcus aureus in a mouse peritonitis model" *Antimicrob Agents Chemother* **53**, 1874-1883).

Example 2 Dissemination of infection with intracellular MRSA

These experiments compared the virulence of intracellular bacteria *versus* an equivalent dose of free-living planktonic bacteria, and determined whether the intracellular bacteria are able to establish infection in the presence of vancomycin in vivo. Four cohorts of mice were infected by intravenous injection with roughly equivalent doses of *S. aureus* viable free bacteria (2.9×10^6) taken directly from broth culture or intracellular bacteria (1.8×10^6) sequestered inside host macrophages and neutrophils that were generated by peritoneal infection of donor mice (Fig. 1A) and selected groups were treated with vancomycin immediately after infection and then once per day. Mice were examined 4 days after infection for bacterial colonization in the kidney, an organ that is consistently colonized by *S. aureus* in mice [23]. In three independent experiments, equivalent or higher bacterial burdens in the kidneys of mice infected with intracellular bacteria compared to those infected with an equivalent dose of planktonic bacteria was observed (Fig. 1B). Surprisingly, it was found that infection with intracellular bacteria resulted in more consistent colonization of the brain, an organ that is not efficiently colonized following infection with planktonic bacteria in this model (Fig. 1C). Furthermore, intracellular bacteria, but not planktonic bacteria, were able to establish infection in the face of vancomycin therapy in this model (Fig. 1B, Fig.1C)

Further analyses in vitro addressed more quantitatively the extent to which intracellular survival facilitates antibiotic evasion. To this end, MG63 osteoblasts were infected with either planktonic MRSA or intracellular MRSA, in the presence of vancomycin.

Infection of osteoblasts or HBMEC. MG63 cell line was obtained from ATCC (CRL-1427) and maintained in RPMI 1640 tissue culture media supplemented with 10 mM Hepes and

10 % Fetal Calf Serum (RPMI-10). HBMEC cells (Catalog #1000) and ECM media (catalog# 1001) were obtained from SciencCell Research Labs (Carlsbad, CA). Cells were plated in 24 well tissue culture plates and cultured to obtain a confluent layer. On the day of the experiment, the cells were washed once in RPMI (without supplements). MRSA or infected peritoneal cells were diluted in complete RPMI-10 and vancomycin was added at 5 ug/mL immediately prior to infection. Peritoneal cells were added to the osteoblasts at $1x10^6$ peritoneal cells/mL. A sample of the cells was lysed with .1% triton-x to determine the actual concentration of live intracellular bacteria at the time of infection. The actual titer for all infections was determined by plating serial dilutions of the bacteria on Tryptic Soy Agar with 5% defibrinated sheep blood.

MRSA (free bacteria) was seeded in media, media + vancomycin, or media + vancomycin and plated on a monolayer of MG63 osteoblasts (Fig.1E) or Human Brain Microvascular Endothelial Cells (HBMEC, Fig.1F). Plates were centrifuged to promote contact of the bacteria with the monolayer. At each time point, the culture supernatant was collected to recover extracellular bacteria or adherent cells were lysed to release intracellular bacteria.

Planktonic bacteria exposed to vancomycin alone were efficiently killed. Surviving bacteria were not recovered after one day in culture (Fig. 1D). When a similar number of planktonic bacteria were plated on MG63 osteoblasts, a small number of surviving bacteria (approximately 0.06% of input) associated with the MG63 cells one day after infection, which had been protected from vancomycin by invasion of the osteoblasts, was recovered.

MRSA that were sequestered inside peritoneal cells showed a dramatic increase in both survival and efficiency of infection in the presence of vancomycin. About 15% of intracellular MRSA in the leukocytes survived under identical conditions where vancomycin had sterilized the cultures of planktonic bacteria. Intracellular bacteria also were better able to infect the monolayer of MG63 osteoblasts in the presence of vancomycin, resulting in a doubling of the bacteria recovered one day after exposure to vancomycin (Fig. 1D). Moreover, intracellular *S. aureus* were able to increase by almost 10-fold over a 24 hour period in MG63 cells (Fig. 1E), primary human brain endothelial cells (Fig. 1F), and A549 bronchial epithelial cells (not shown) under constant exposure to a concentration of vancomycin that killed free living bacteria. Although protected from antibiotic killing, bacterial growth did not occur in cultures of infected peritoneal macrophages and neutrophils (not shown). Together these data support that intracellular reservoirs of MRSA in myeloid cells can promote dissemination of infection to new sites, even in the presence of active antibiotic treatment, and intracellular growth can occur in endothelial and epithelial cells, even under conditions of constant antibiotic therapy.

Example 3 Generation of anti-SDR and other antibodies

For generation of mAb rF1, $CD19^+CD3^-CD27^+IgD^-IgA^-$ memory B cells were isolated from peripheral blood of an MRSA-infected donor using a FACSAria cell sorter (BD, San Jose, CA). Before viral transduction with B-cell lymphoma (Bcl)-xL and Bcl-6 genes, the memory cells were activated on CD40L-expressing mouse L fibroblasts in the presence of interleukin-21, as described previously in Kwakkenbos MJ, et al. (2010) Nat Med 16: 123-128. Transduced B cells were maintained in the same culture system. The use of donor blood was approved by the institutional committee. Monoclonal antibody (mAb) rF1 was selected from culture supernatants by reactivity with lysates of MSSA strain Newman by ELISA; positive wells were subcloned and re-tested by ELISA twice. Recombinant rF1 was generated by cloning the heavy and light chain variable regions with human IgG1 kappa constant regions using pcDNA3.1 (Invitrogen) and transfection into 293T cells (ATCC). Purified IgG was obtained from culture supernatants using protein A-coupled SEPHAROSE® (Invitrogen). The generation of mAb rF1 and its variants are described in US 8,617,556 (Beaumont et al.) and Hazenbos et al. (2103) *PLOS Pathogens* 9(10): 1-18, incorporated by reference herein in their entirety.

The human IgG1 mAbs SD2, SD3 and SD4 (all against glycosylated SDR proteins) and 4675 (human IgG1 anti-ClfA),were cloned from peripheral B cells from patients post *S. aureus* infection using the Symplex™ technology which conserves the cognate pairing of antibody heavy and light chains [34]. Both plasma and memory B-cells were used as genetic source for the recombinant full length IgG repertoires (manuscript in preparation). Individual antibody clones were expressed by transfection of mammalian cells [35]. Supernatants containing full length IgG1 antibodies were harvested after seven days and used to screen for antigen binding by ELISA. Antibodies 4675, SD2, SD3 and SD4 were positive for binding to cell wall preparations from USA300 or Newman *S. aureus* strains. Antibodies were subsequently produced in 200-ml transient transfections and purified with Protein A chromatography (MabSelect SuRe, GE Life Sciences, Piscataway, NJ) for further testing. Isolation and usage of these antibodies were approved by the regional ethical review board. rF1 variants were generated.

Mouse mAb against ClfA (9E10), ClfB, (10D2), SdrD (17H4), IsdA (2D3) and non-modified SDR proteins (9G4) were generated by immunizing mice with the respective recombinant proteins, which were purified after expression in *E. coli*, using standard protocols; hybridoma supernatants were purified by protein A affinity chromatography. Rabbit mAb 28.9.9 was generated by immunizing rabbits with peptidoglycan (PGN)-derived peptide CKKGGG-(L-Ala)-(D-gamma-Glu)-(L-Lys)-(D-Ala)-D-Ala) followed by cloning of the IgG.

Example 4 Characterization of a highly opsonic monoclonal antibody (rF1) isolated from an MRSA infected donor

Several *S. aureus*-reactive monoclonal antibodies (mAb) from memory B cells from peripheral blood of MRSA-infected donors were isolated as described above. When characterizing these antibodies, one IgG1 mAb (hereafter referred to as rF1) was identified with broad reactivity to a panel of *S. aureus* strains that induced robust opsonophagocytic killing (OPK) by human polymorphonuclear leukocytes (PMN).

Maximum binding of mAb rF1 to bacteria from clinical MRSA strain USA300 was approximately 10 fold higher than that of an isotype-matched anti-ClfA mAb (Figure 5A). Consistent with increased binding, opsonization with rF1 resulted in increased uptake (Figure 5B) and killing (Figure 5C) of USA300 by PMN. In contrast, preopsonization with human anti-ClfA had no effect on bacterial viability (Figure 5C). The rF1 antibody did not affect viability of USA300 in the absence of PMN. Thus, rF1 is a mAb with the capacity to bind MRSA and induce potent killing of MRSA by PMN.

Example 5 Binding of rF1 to Staphylococcus strains

FACS analysis of rF1 binding to whole bacteria from culture or infected tissues

Whole bacteria were harvested from TSA plates or TSB cultures and washed with HBSS without phenol red supplemented with 0.1% IgG free BSA (Sigma) and 10 mM Hepes, pH 7.4 (HB buffer) Bacteria ($20x10^8$ CFU/mL) were incubated with 300 μg/mL of rabbit IgG (Sigma) in HB buffer for 1 h at room temperature (RT) to block nonspecific IgG binding. Bacteria were stained with 2 μg/mL of primary antibodies, including rF1 or isotype control IgG1 mAb gD:5237 (Nakamura GR, et al. (1993) J Virol 67: 6179-6191), and next with fluorescent anti-human IgG secondary antibodies (Jackson Immunoresearch, West Grove, PA). The bacteria were washed and analyzed by FACSCalibur® (BD).

For antibody staining of bacteria from infected mouse tissues, 6-8 weeks old female C57Bl/6 mice (Charles River, Wilmington, MA) were injected intravenously with 10^8 CFU of logphase-grown USA300 in PBS. Mouse organs were harvested two days after infection. Rabbit infective endocarditis (IE) was established as described in Tattevin P, et al. (2010) Antimicrobial agents and chemotherapy 54: 610-613. Rabbits were injected intravenously with $5x10^7$ CFU of stationary-phase grown MRSA strain COL, and heart vegetations were harvested eighteen hours later. Treatment with 30 mg/kg of vancomycin was given intravenously b.i.d. 18 h after infection with $7x10^7$ CFU stationary-phase COL.

To lyse mouse or rabbit cells, tissues were homogenized in M tubes (Miltenyi, Auburn, CA) using a gentleMACS® cell dissociator (Miltenyi), followed by incubation for 10 min at RT in PBS containing 0.1% Triton-X100 (Thermo), 10 μg/mL of DNAseI (Roche) and Complete Mini protease inhibitor cocktail (Roche). The suspensions were passed through a 40 micron filter (BD) and bacteria were stained with mAbs as described above. Bacteria were differentiated from mouse organ debris by double staining with 20 μg/mL mouse mAb 702 anti-*S. aureus* peptidoglycan (abcam, Cambridge, MA) and a fluorochrome-labeled anti-mouse IgG secondary antibody (Jackson Immunoresearch). During flow cytometry analysis, bacteria were gated for positive staining with mAb 702 from double fluorescence plots. All animal experiments were approved by the Institutional Review Boards of Genentech and the University of California, San Francisco.

Flow cytometry (FCM) analysis showed potent binding activity of rF1 to all 15 *S. aureus* strains tested (Figure 7). These strains were broadly distributed across the *S. aureus* phylogeny [8]. As expression levels of bacterial cell surface antigens might differ between *in vitro* and *in vivo* growth, we also tested the ability of rF1 to recognize USA300 isolated from various mouse tissues after systemic infection. The rF1 mAb strongly bound to USA300 derived from infected mouse kidneys, livers and lungs (Figure 6). The binding rF1 to USA300 from mouse kidneys was sustained until at least 8 days after infection (not shown), suggesting robust long-term expression of the rF1 epitope during infection. In addition, rF1 strongly bound to MRSA COL bacteria from heart vegetations in a rabbit model of infectious endocarditis. Treatment with vancomycin did not affect the reactivity of rF1 with MRSA (Figure 6). Thus, the antigen recognized by rF1 is conserved across various strains and stably expressed in various growth and infection conditions.

Given the ubiquitous nature of rF1-reactivity across all *S. aureus* strains, experiments were performed to see if such reactivity is extended to other gram-positive bacteria. Notably, rF1 binding was detectable only for the coagulase-negative human pathogen *S. epidermidis* (Figure 7). The rF1 mAb did not bind to any other staphylococcal species tested, including *S. saprophyticus*, *S. lugdunensis*, *S. simulans* and *S. carnosus*, or other Gram-positive species such as *Streptococcus pyogenes, Bacillus subtilis*, *Enterococcus faecalis*, and *Listeria monocytogenes* (Figure 7). Thus, rF1 is a human antibody that binds to stably-expressed surface antigen(s) on human-adapted staphylococcal pathogens and promotes bacterial killing by human PMNs.

Example 6 Amino acid modifications of rF1 antibodies

In summary, the VH region of each of the rF1 Abs were cloned out and linked to human H chain gamma1 constant region and the VL linked to kappa constant region to express the Abs as IgG1. Wild-type sequences were altered at certain positions to improve the antibody stability while maintaining antigen binding as described below. Cysteine engineered Abs (ThioMabs, also referred to as THIOMAB™) were then generated.

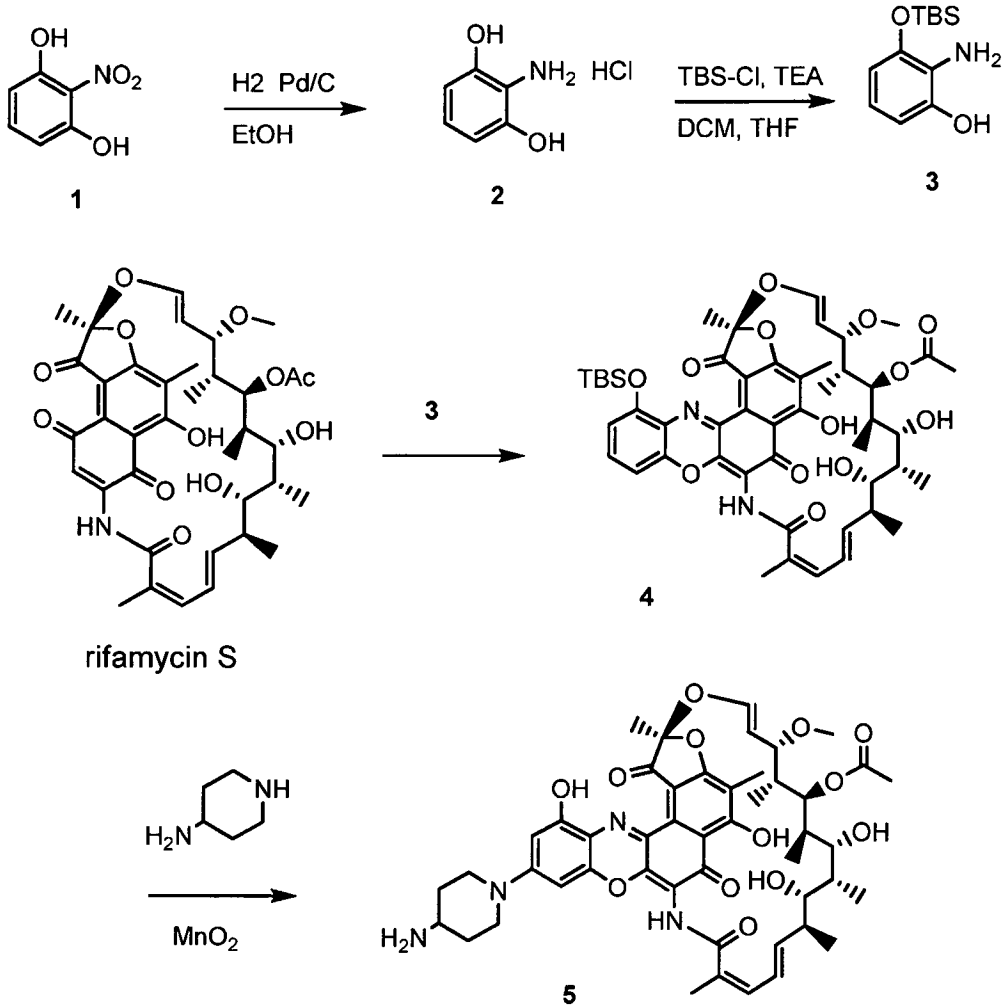
i. Generating stability variants

The rF1 Abs were engineered to improve certain properties (to avoid deamidation, aspartic acid isomerization, oxidation or N-linked glycosylation) and tested for retention of antigen binding as well as chemical stability after amino acid replacements . The amino acid alterations made were as described in US8,617,556.

iii. Generating Cys engineered mutants (ThioMabs)

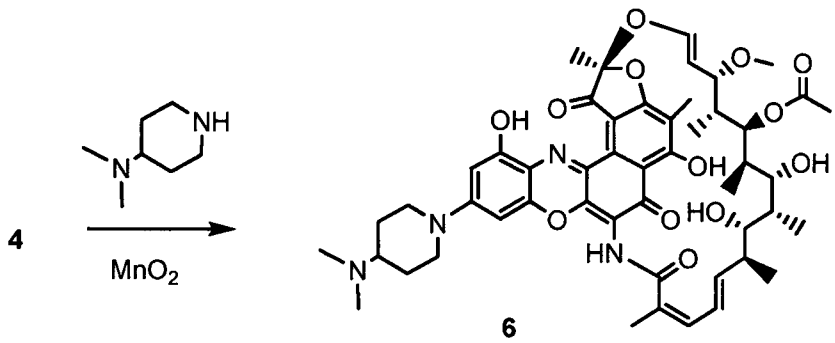
Full length ThioMabs were produced by introducing a Cysteine into the H chain (in CH1) or the L chain (Cκ) at a predetermined position as previously taught, e.g., at V205 in the kappa Constant region of the L chain and position A118 in the human Gamma 1 H chain (amino acid position numbers according to Eu convention) to allow conjugation of the antibody to a linker-antibiotic intermediate. H and L chains are then cloned into separate plasmids and the H and L encoding plasmids co-transfected into 293 cells where they are expressed and assembled into intact Abs. Both H and L chains can also be cloned into the same expression plasmid. IgG1 having 2 engineered Cys, one in each of H chains; or 2 engineered Cys, one in each of the L chains; or a combination of an engineered Cys in each of the H and L chains (HC LC Cys) leading to 4 engineered Cys per antibody tetramer, were generated by expressing the desired combination of cys mutant chains and wild type chains.

Example 7 Piperidyl benzoxazino rifamycin (pipBOR) **5**

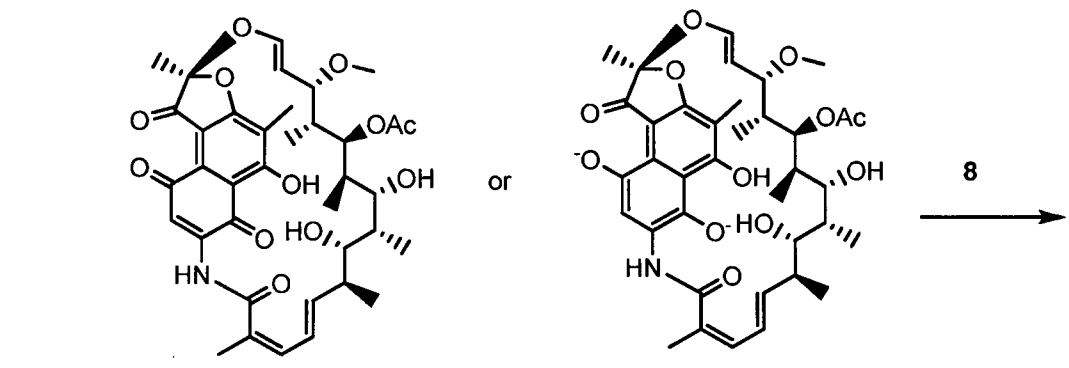


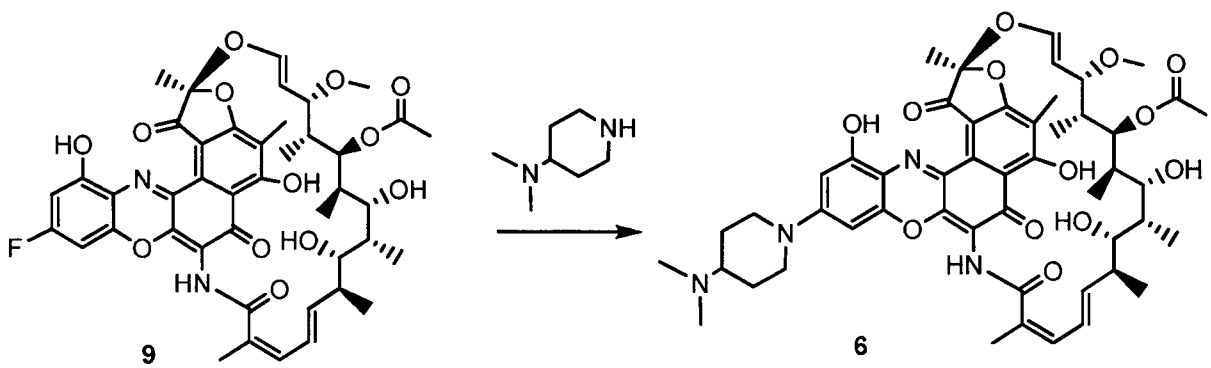
2-Nitrobenzene-1,3-diol **1** was hydrogenated under hydrogen gas with palladium/carbon catalyst in ethanol solvent to give 2-aminobenzene-1,3-diol **2**, isolated as the hydrochloride salt. Mono-protection of **2** with tert-butyldimethylsilyl chloride and triethylamine in dichloromethane/tetrahydrofuran gave 2-amino-3-(tert-butyldimethylsilyloxy)phenol **3**. Rifamycin S (ChemShuttle Inc., Fremont, CA, US 7342011; US 7271165; US 7547692) was reacted with **3** by oxidative condensation with manganese oxide or oxygen gas in toluene at room temperature to give TBS-protected benzoxazino rifamycin **4**. LCMS (ESI): $M+H^+$ = 915.41. Reaction of **4** with piperidin-4-amine and manganese oxide gave piperidyl benzoxazino rifamycin (pipBOR) **5**. LCMS (ESI): $M+H^+$ = 899.40

Example 8 DimethylpipBOR **6**



Reaction of N,N-dimethylpiperidin-4-amine with TBS-protected benzoxazino rifamycin **4** gave dimethylpiperidyl benzoxazino rifamycin (dimethylpipBOR) **6**



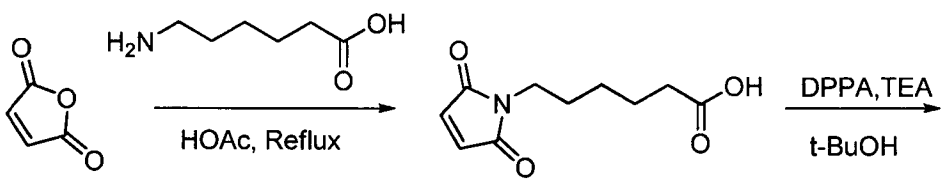


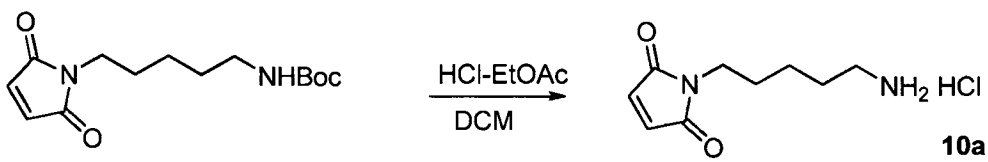
Alternatively, (5-fluoro-2-nitro-1,3-phenylene)bis(oxy)bis(methylene)dibenzene **7** was hydrogenated under hydrogen gas with palladium/carbon catalyst in tetrahydrofuran/methanol solvent to remove the benzyl groups to give 2-amino-5-fluorobenzene-1,3-diol **8**. LCMS (ESI): $M+H^+ = 144.04$. Commercially available Rifamycin S or Rifamycin SV sodium salt

(ChemShuttle Inc., Fremont, CA) was reacted with 2-amino-5-fluorobenzene-1,3-diol **8** by oxidative condensation in air or potassium ferric cyanide in ethyl acetate at 60 °C to give fluorobenzoxazino rifamycin **9**. Displacement of fluoride with N,N-dimethylpiperidin-4-amine gave dimethylpipBOR **6**. LCMS (ESI): $M+H^+ = 927.43$

Example 9 (S)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-N-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)cyclobutane-1,1-dicarboxamide **10**

Step 1: Preparation of 1-(5-aminopentyl)-1H-pyrrole-2,5-dione hydrochloride **10a**

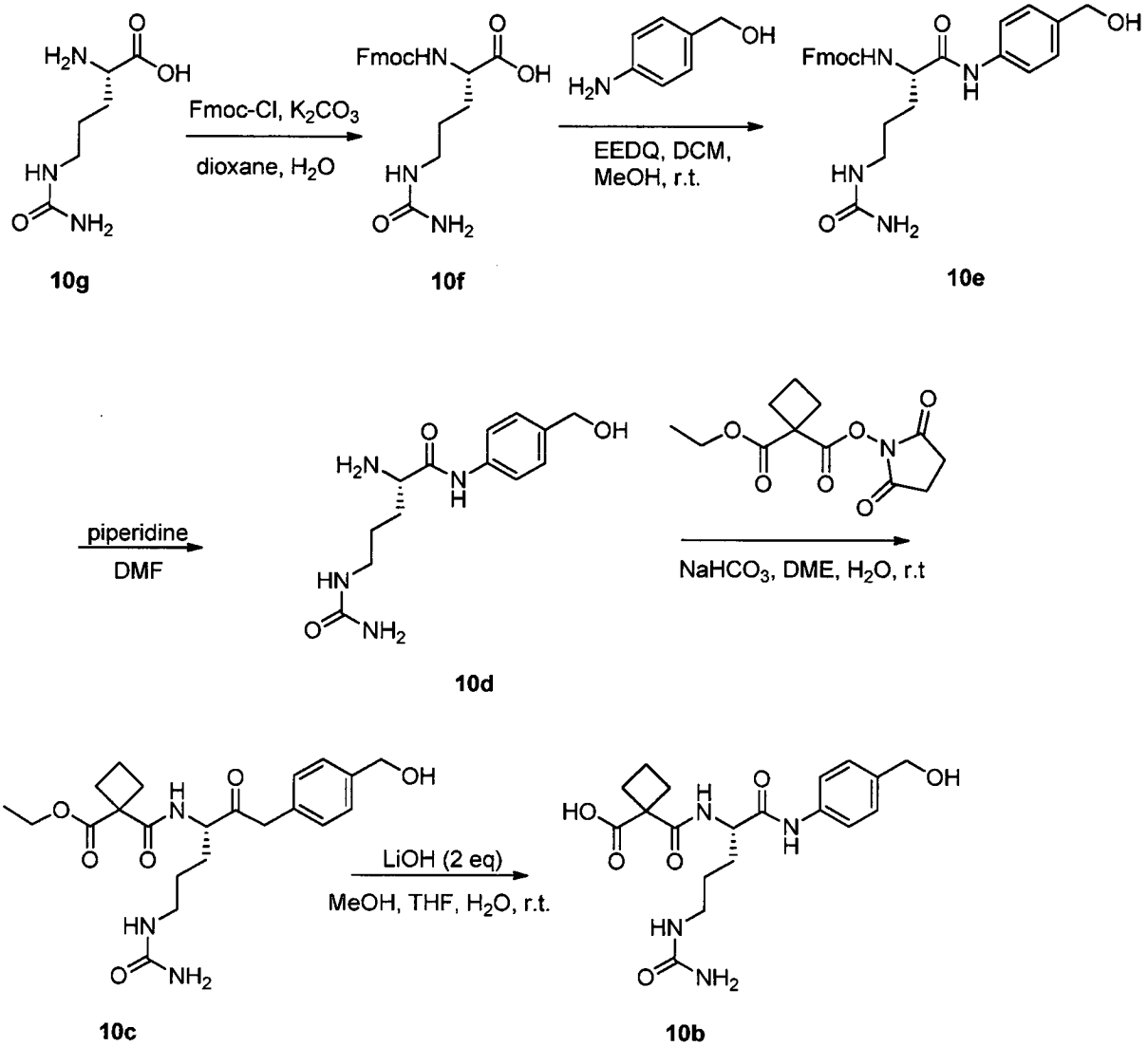




10a

Maleic anhydride, furan-2,5-dione (150 g, 1.53 mol) was added to a stirred solution of 6-aminohexanoic acid (201 g, 1.53 mol) in HOAc (1000 mL). After the mixture was stirred at r.t. for 2 h, it was heated at reflux for 8 h. The organic solvents were removed under reduced pressure and the residue was extracted with EtOAc (500 mL × 3), washed with H_2O. The combined organic layers was dried over Na_2SO_4 and concentrated to give the crude product. It was washed with petroleum ether to give 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoic acid as white solid (250 g, 77.4 %). DPPA (130 g, 473 mmol) and TEA (47.9 g, 473 mmol) was added to a solution of 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoic acid (100 g, 473 mmol) in t-BuOH (200 mL). The mixture was heated at reflux for 8 h under N_2. The mixture was concentrated, and the residue was purified by column chromatography on silica gel (PE:EtOAc= 3:1) to give tert-butyl 5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylcarbamate (13 g, 10 %). To a solution of tert-butyl 5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylcarbamate (28 g, 992 mmol) in anhydrous EtOAc (30 mL) was added HCl/EtOAc (50 mL) dropwise. After the mixture was stirred at r.t. for 5 h, it was filtered and the solid was dried to give 1-(5-aminopentyl)-1H-pyrrole-2,5-dione hydrochloride **10a** (16 g, 73.7 %). ^1H NMR (400 MHz, DMSO-d_6): δ 8.02 (s, 2H), 6.99 (s, 2H), 3.37-3.34 (m, 2H), 2.71-2.64 (m, 2H), 1.56-1.43 (m, 4H), 1.23-1.20 (m, 2H).

Step 2: Preparation of (S)-1-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-ylcarbamoyl)cyclobutanecarboxylic acid **10b**



10g **10f** **10e** **10d** **10c** **10b**

To a mixture of (S)-2-amino-5-ureidopentanoic acid **10g** (17.50 g, 0.10 mol) in a mixture of dioxane and H_2O (50 mL / 75 mL) was added K_2CO_3 (34.55 g, 0.25 mol). Fmoc-Cl (30.96 g, 0.12 mol) was added slowly at 0 °C. The reaction mixture was warmed to r.t. over 2 h. Organic solvent was removed under reduced pressure, and the water slurry was adjusted to pH = 3 with 6 M HCl solution, and extracted with EtOAc (100 mL × 3). The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-ureidopentanoic acid **10f** (38.0 g, 95.6 %). **10f** is commercially available.

To a solution of **10f** (4 g, 10 mmol) in a mixture of DCM and MeOH (100 mL / 50 mL) were added (4-aminophenyl)methanol (1.6 g, 13 mmol, 1.3 eq) and 2-Ethoxy-1-ethoxycarbonyl-

1,2-dihydroquinoline, EEDQ, Sigma-Aldrich CAS Reg. No. 16357-59-8 (3.2 g, 13 mmol, 1.3 eq). After the mixture was stirred at r.t. for 16 h under N_2, it was concentrated to give a brown solid. MTBE (200 mL) was added and it was stirred at 15°C for 2 h. The solid was collected by filtration, washed with MTBE (50 mL × 2) to give (S)-(9H-fluoren-9-yl)methyl (1-((4-(hydroxymethyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)carbamate **10e** as an orange solid (4.2 g, 84%). LCMS (ESI): m/z 503.0 [M+1].

To a stirred solution of **10e** (4.2 g, 8.3 mmol) in dry DMF (20 ml) was added piperidine (1.65 mL, 17 mmol, 2 eq) dropwise at r.t. The mixture was stirred at r.t. for 30 min, and solid precipitate formed. Dry DCM (50 mL) was added, and the mixture became transparent immediately. The mixture was stirred at r.t. for another 30 min, and LCMS showed **10e** was consumed. It was concentrated to dryness under reduced pressure (make sure no piperidine remained), and the residue was partitioned between EtOAc and H_2O (50 mL / 20 mL). Aqueous phase was washed with EtOAc (50 mL × 2) and concentrated to give (S)-2-amino-N-(4-(hydroxymethyl)phenyl)-5-ureidopentanamide **10d** as an oily residual (2.2 g, 94%) (contained small amount of DMF).

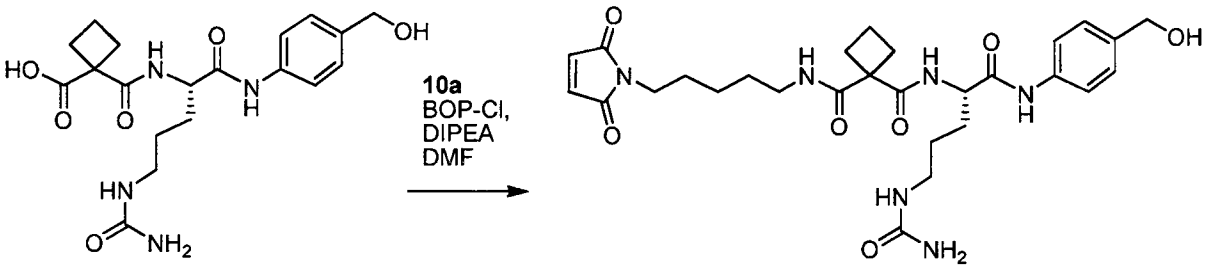
Commercially available 1,1-cyclobutanedicarboxylic acid, 1,1-diethyl ester (CAS Reg. No. 3779-29-1) was converted by limited saponification with aqueous base to the half acid/ester 1,1-cyclobutanedicarboxylic acid, 1-ethyl ester (CAS Reg No. 54450-84-9) and activation with a coupling reagent such as TBTU (*O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate, also called: *N,N,N',N'*-Tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate, CAS No. 125700-67-6, Sigma-Aldrich B-2903), and N-hydroxysuccinimide to the NHS ester, 1-(2,5-dioxopyrrolidin-1-yl) 1-ethyl cyclobutane-1,1-dicarboxylate.

To a solution of 1-(2,5-dioxopyrrolidin-1-yl) 1-ethyl cyclobutane-1,1-dicarboxylate (8 g, 29.7 mmol) in DME (50 mL) was added a solution of **10d** (6.0 g, 21.4 mmol) and $NaHCO_3$ (7.48 g, 89.0 mmol) in water (30 mL). After the mixture was stirred at r.t. for 16 h, it was concentrated to dryness under reduced pressure and the residue was purified by column chromatography (DCM:MeOH = 10:1) to give (S)-ethyl 1-((1-(4-(hydroxymethyl)phenyl)-2-oxo-6-ureidohexan-3-yl)carbamoyl)cyclobutanecarboxylate **10c** as white solid (6.4 g, 68.7%). LCMS (ESI): m/z 435.0 [M+1]

To a stirred solution of **10c** (6.4 g, 14.7 mmol) in a mixture of THF and MeOH (20 mL / 10 mL) was added a solution of LiOH • H_2O (1.2 g, 28.6 mmol) in H_2O (20 mL) at r.t. After the reaction mixture was stirred at r.t. for 16 h, solvent was removed under reduced pressure, the residue obtained was purified by prep-HPLC to give (S)-1-(1-(4-(hydroxymethyl)phenylamino)-

1-oxo-5-ureidopentan-2-ylcarbamoyl)cyclobutanecarboxylic acid **10b** (3.5 g, yield: 58.5%). LCMS (ESI): m/z 406.9 [M+1]. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.86 (d, J = 8.4 Hz, 2 H), 8.51 (d, J = 8.4 Hz, 2 H), 5.88 - 5.85 (m, 1 H), 5.78 (s, 2 H), 4.54 - 4.49 (m, 3 H), 4.38 - 4.32 (m, 1 H), 3.86 - 3.75 (m, 1 H), 3.84 - 3.80 (m, 2 H), 3.28 - 3.21 (m, 1 H), 3.30 - 3.24 (m, 1 H), 3.00 - 2.80 (m, 1 H), 2.37 - 2.28 (m, 2 H).

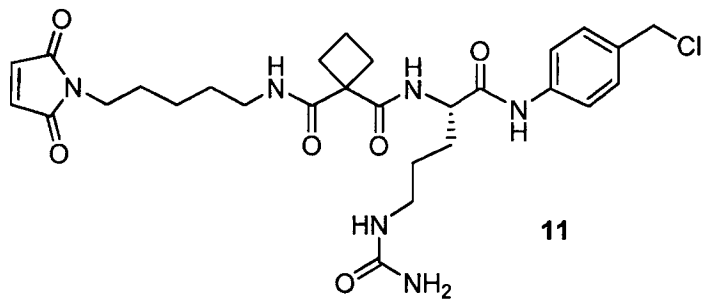
Step 3: Preparation of S)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-N-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)cyclobutane-1,1-dicarboxamide **10**



10b **10**

Diisopropylethylamine, DIPEA (1.59 g, 12.3 mmol) and bis(2-oxo-3-oxazolidinyl)phosphinic chloride, BOP-Cl (CAS Reg. No. 68641-49-6, Sigma-Aldrich, 692 mg, 2.71 mmol) was added to a solution of (S)-1-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-ylcarbamoyl)cyclobutanecarboxylic acid **10b** (1 g, 2.46 mmol) in DMF (10 mL) at 0 °C, followed by 1-(5-aminopentyl)-1H-pyrrole-2,5-dione hydrochloride 10a (592 mg, 2.71 mmol). The mixture was stirred at 0 °C for 0.5h. The reaction mixture was quenched with citric acid solution (10 mL), extracted with DCM/MeOH (10:1). The organic layer was dried and concentrated, and the residue was purified by column chromatography on silica gel (DCM:MeOH = 10:1) to give to give S)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-N-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)cyclobutane-1,1-dicarboxamide 10 (1.0 g, 71 %), also referred to as MC-CBDK-cit-PAB-OH. LCMS (ESI): M+H$^+$ = 571.28. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.00 (s, 1H), 7.82-7.77 (m, 2H), 7.53 (d, J = 8.4 Hz, 2 H), 7.19 (d, J = 8.4 Hz, 2 H), 6.96 (s, 2H), 5.95 (t, J = 6.4 Hz, 1H), 5.39 (s, 2H), 5.08 (t, J = 5.6 Hz, 1H), 4.40-4.35 (m, 3H), 4.09 (d, J = 4.8 Hz, 1 H), 3.01 (d, J = 3.2 Hz, 2 H), 3.05-2.72 (m, 4H), 2.68-2.58 (m, 3H), 2.40-2.36 (m, 4H), 1.72-1.70 (m, 3H), 1.44-1.42 (m, 1H), 1.40-1.23 (m, 6H), 1.21-1.16 (m, 4H).

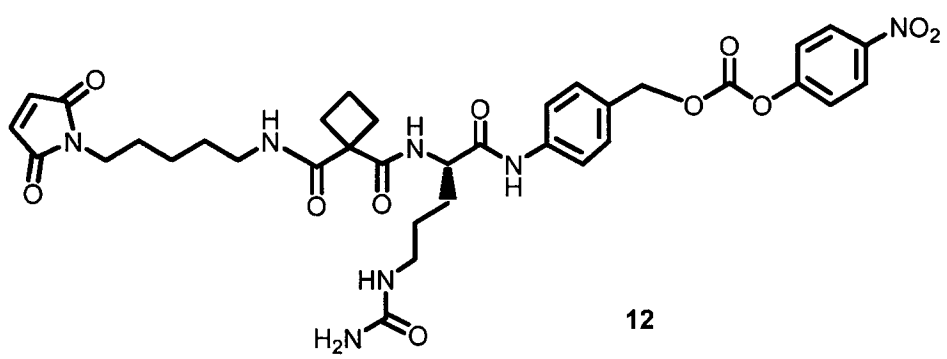
Example 10 (S)-N-(1-(4-(chloromethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)cyclobutane-1,1-dicarboxamide **11**



11

A solution of (S)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-N-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)cyclobutane-1,1-dicarboxamide **10** (2.0 g, 3.5 mmol) in N,N-dimethylformamide, DMF or N-methylpyrrolidone, NMP (50 mL) was treated with thionyl chloride, $SOCl_2$ (1.25 g, 10.5 mmol) in portions dropwise at 0 °C. The reaction remained yellow. The reaction was monitored by LC/MS indicating >90% conversion. After the reaction mixture was stirred at 20 °C for 30 min or several hours, it was diluted with water (50 mL) and extracted with EtOAc (50 mL x 3). The organic layer was dried, concentrated and purified by flash column (DCM : MeOH = 20 : 1) to form **11**, also referred to as MC-CBDK-cit-PAB-Cl as a gray solid. LCMS: (5-95, AB, 1.5 min), 0.696 min, *m/z* = 589.0 $[M+1]^+$.

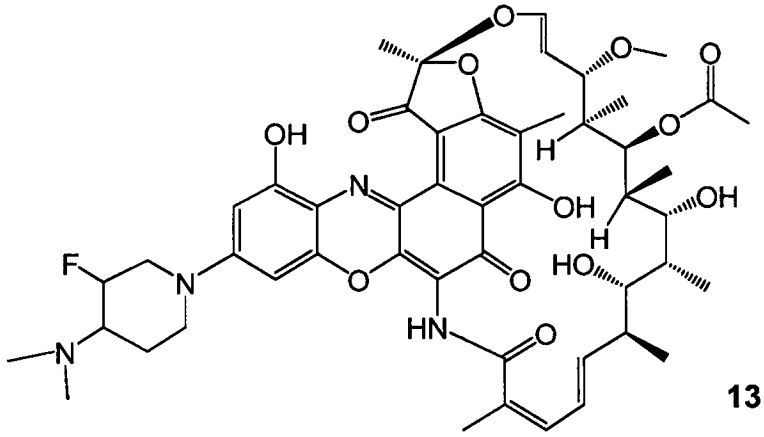
Example 11 (S)-4-(2-(1-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylcarbamoyl)cyclobutanecarboxamido)-5-ureidopentanamido)benzyl 4-nitrophenyl carbonate **12**



12

To a solution of (S)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-N-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)cyclobutane-1,1-dicarboxamide **10** in anhydrous DMF was added diisopropylethylamine (DIEA), followed by PNP carbonate (bis(4-nitrophenyl) carbonate). The reaction solution was stirred at room temperature (r.t.) for 4 hours and the mixture was purified by prep-HPLC to afford **12**. LCMS (ESI): $M+H^+$ = 736.29.

Example 12 Preparation of MC-(CBDK-cit)-PAB-(dimethyl, fluoropipBOR) - PLA-1



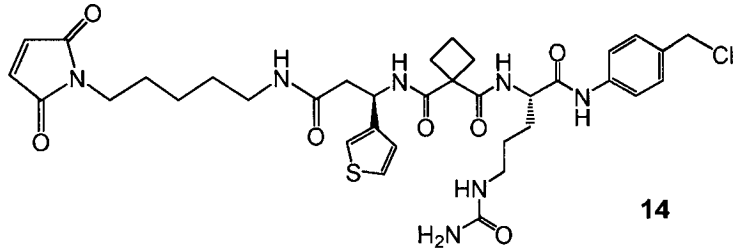
13

Following the procedure for PLA-2, (S)-N-(1-(4-(chloromethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)cyclobutane-1,1-dicarboxamide **11** and the fluorinated rifamycin-derivative, dimethylfluoropipBOR **13** (LCMS (ESI): $M+H^+$ = 945.43) were reacted to form MC-(CBDK-cit)-PAB-(dimethyl, fluoropipBOR) - PLA-1, Table 2. LCMS (ESI): $M+H^+$ = 1499.7

Example 13 Preparation of MC-(CBDK-cit)-PAB-(dimethylpipBOR) - PLA-2

(S)-N-(1-(4-(chloromethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)cyclobutane-1,1-dicarboxamide **11** (0.035 mmol) in DMF was cooled to 0 °C and dimethylpipBOR **6**, (10 mg, 0.011 mmol) was added. The mixture was diluted with another 0.5 mL of DMF. Stirred open to air for 30 minutes. N,N-diisopropylethylamine (DIEA, 10 µL, 0.05 mmol) was added and the reaction stirred overnight open to air. By LC/MS, 50% of desired product was observed. An additional 0.2 eq N,N-diisopropylethylamine base was added while the reaction stirred open to air for another 6 hours until the reaction appeared to stop progressing. The reaction mixture was diluted with DMF and purified on HPLC (20-60% ACN/HCOOH in H_2O) to give MC-(CBDK-cit)-PAB-(dimethylpipBOR) - PLA-2, Table 2. LCMS (ESI): $M+H^+$ = 1481.8, yield 31%.

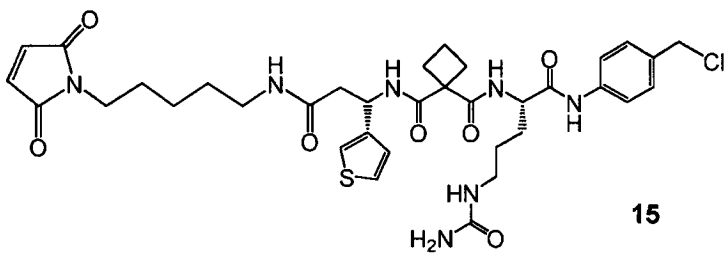
Example 14 Preparation of MC-((*R*)-thiophen-3-yl-CBDK-cit)-PAB-(dimethylpipBOR) (PLA-3)



14

Following the procedure for PLA-2, (N-((S)-1-(4-(chloromethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)-N-((R)-3-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylamino)-3-oxo-1-(thiophen-3-yl)propyl)cyclobutane-1,1-dicarboxamide **14** (LCMS (ESI): $M+H^+$ = 742.3) and dimethylpipBOR **6** were reacted to give MC-((*R*)-thiophen-3-yl-CBDK-cit)-PAB-(dimethylpipBOR) (PLA-3, Table 2). LCMS (ESI): $M+H^+$ = 1633.9

Example 15 Preparation of MC-((*S*)-thiophen-3-yl-CBDK-cit)-PAB-(dimethylpipBOR) (PLA-4)



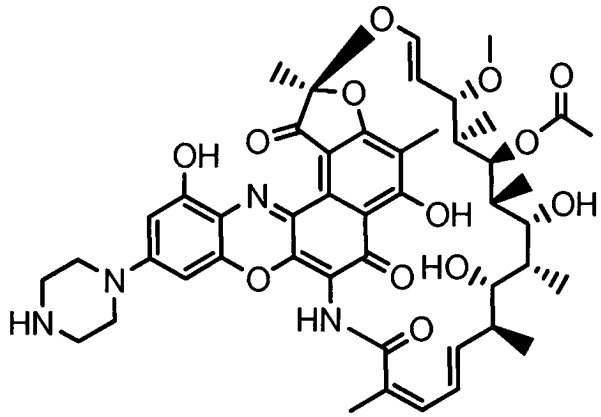
15

Following the procedure for PLA-2, (N-((R)-1-(4-(chloromethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)-N-((R)-3-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylamino)-3-oxo-1-(thiophen-3-yl)propyl)cyclobutane-1,1-dicarboxamide **15** (LCMS (ESI): $M+H^+$ = 742.3) and dimethylpipBOR **6** were reacted to give MC-((*R*)-thiophen-3-yl-CBDK-cit)-PAB-(dimethylpipBOR) (PLA-4, Table 2). LCMS (ESI): $M+H^+$ = 1633.9

Example 16 Preparation of MC-(CBDK-cit)-PABC-(pipBOR) (PLA-5)

Piperidyl benzoxazino rifamycin (pipBOR) **5** (15 mg, 0.0167 mmol), and then (S)-4-(2-(1-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylcarbamoyl)cyclobutanecarboxamido)-5-ureidopentanamido)benzyl 4-nitrophenyl carbonate **12** (12 mg, 0.0167 mmol) were weighed into a vial. Dimethylformamide, DMF (0.3 mL) was added, followed by diisopropylethylamine, DIEA (0.006 mL, 0.0334 mmol), and the reaction was allowed to stir at room temperature for 2 h. The reaction solution was directly purified by HPLC (30 to 70% MeCN/water + 1% formic acid) to give MC-(CBDK-cit)-PABC-(pipBOR) (PLA-5, Table 2). LCMS (ESI): $M+H^+$ = 1496.5

Example 17 Preparation of MC-(CBDK-cit)-PABC-(piperazBTR) (PLA-6)



16

Following the procedures for PLA-5,the piperidine rifamycin derivative, piperazBOR **16** (LCMS (ESI): M+H$^+$ = 885.4) and (S)-4-(2-(1-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylcarbamoyl)cyclobutanecarboxamido)-5-ureidopentanamido)benzyl 4-nitrophenyl carbonate **12** were reacted to give MC-(CBDK-cit)-PABC-(piperazBTR) (PLA-6. Table 2). LCMS (ESI): M+H$^+$ = 1482.5

Example 18 Preparation of rF1 Antibody-Antibiotic Conjugates

Antibody-antibiotic conjugates (AAC) Table 3 were prepared by conjugating an rF1 antibody to a PML Linker-Antibiotic intermediate, including those from Table 2. Prior to conjugation, the rF1 antibodies were partially reduced with TCEP using standard methods in accordance with the methodology described in WO 2004/010957, the teachings of which are incorporated by reference for this purpose. The partially reduced antibodies were conjugated to the linker-antibiotic intermediate using standard methods in accordance with the methodology described, e.g., in Doronina et al. (2003) *Nat. Biotechnol.* 21:778-784 and US 2005/0238649 A1. Briefly, the partially reduced antibodies were combined with the linker-antibiotic intermediate to allow conjugation of the linker-antibiotic intermediate to reduced cysteine residues of the antibody. The conjugation reactions were quenched, and the AAC were purified. The antibiotic load (average number of antibiotic moieties per antibody) for each AAC was determined and was between about 1 to about 2 for the rF1 antibodies engineered with a single cysteine mutant site.

Reduction/Oxidation of ThioMabs for Conjugation: Full length, cysteine engineered monoclonal antibodies (ThioMabs - Junutula, et al., 2008b Nature Biotech., 26(8):925-932; Dornan et al (2009) Blood 114(13):2721-2729; US 7521541; US 7723485; WO2009/052249, Shen et al (2012) Nature Biotech., 30(2):184-191; Junutula et al (2008) Jour of Immun. Methods 332:41-52) expressed in CHO cells were reduced with about a 20-40 fold excess of TCEP

(tris(2-carboxyethyl)phosphine hydrochloride or DTT (dithiothreitol) in 50 mM Tris pH 7.5 with 2 mM EDTA for 3 hrs at 37 °C or overnight at room temperature.(Getz *et al* (1999) *Anal. Biochem.* Vol 273:73-80; Soltec Ventures, Beverly, MA). The reduced ThioMab was diluted and loaded onto a HiTrap S column in 10 mM sodium acetate, pH 5, and eluted with PBS containing 0.3M sodium chloride. Alternatively, the antibody was acidified by addition of $1/20^{th}$ volume of 10 % acetic acid, diluted with 10 mM succinate pH 5, loaded onto the column and then washed with 10 column volumes of succinate buffer. The column was eluted with 50 mM Tris pH7.5, 2 mM EDTA.

The eluted reduced ThioMab was treated with 15 fold molar excess of DHAA (dehydroascorbic acid) or 200 nM aqueous copper sulfate ($CuSO_4$). Oxidation of the interchain disulfide bonds was complete in about three hours or more. Ambient air oxidation was also effective. The re-oxidized antibody was dialyzed into 20 mM sodium succinate pH 5, 150 mM NaCl, 2 mM EDTA and stored frozen at -20 °C.

Conjugation of ThioMabs with linker-antibiotic intermediates: The deblocked, reoxidized, thio-antibodies (ThioMab) were reacted with 6-8 fold molar excess of the linker-antibiotic intermediate of Table 2 (from a DMSO stock at a concentration of 20 mM) in 50 mM Tris, pH 8, until the reaction was complete (16-24 hours) as determined by LC-MS analysis of the reaction mixture.

The crude antibody-antibiotic conjugates (AAC) were then applied to a cation exchange column after dilution with 20 mM sodium succinate, pH 5. The column was washed with at least 10 column volumes of 20 mM sodium succinate, pH 5, and the antibody was eluted with PBS. The AAC were formulated into 20 mM His/acetate, pH 5, with 240 mM sucrose using gel filtration columns. AAC were characterized by UV spectroscopy to determine protein concentration, analytical SEC (size-exclusion chromatography) for aggregation analysis and LC-MS before and after treatment with Lysine C endopeptidase.

Size exclusion chromatography was performed using a Shodex KW802.5 column in 0.2M potassium phosphate pH 6.2 with 0.25 mM potassium chloride and 15% IPA at a flow rate of 0.75 ml/min. Aggregation state of AAC was determined by integration of eluted peak area absorbance at 280 nm.

LC-MS analysis was performed using an Agilent QTOF 6520 ESI instrument. As an example, an AAC generated using this chemistry was treated with 1:500 w/w Endoproteinase Lys C (Promega) in Tris, pH 7.5, for 30 min at 37 °C. The resulting cleavage fragments were loaded onto a 1000A, 8 um PLRP-S column heated to 80°C and eluted with a gradient of 30% B to 40% B in 5 minutes. Mobile phase A: H_2O with 0.05% TFA. Mobile phase B: acetonitrile

with 0.04% TFA. Flow rate: 0.5ml/min. Protein elution was monitored by UV absorbance detection at 280 nm prior to electrospray ionization and MS analysis. Chromatographic resolution of the unconjugated Fc fragment, residual unconjugated Fab and antibiotic-Fab was usually achieved. The obtained m/z spectra were deconvoluted using Mass Hunter™ software (Agilent Technologies) to calculate the mass of the antibody fragments.

The AAC, **103** (AAR = 1.9) thio-rF1-HC-121C, LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR) was made using the rF1 L chain of SEQ ID NO. 9 containing the engineered Cys 205, and the rF1 H chain comprising SEQ ID NO. 10. The AAC **102** (AAR = 3.9) thio-rF1-HC-121C, LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR) was made using the rF1 L chain of SEQ ID NO. 9 in the preceding containing the engineered Cys 205, and the rF1 H chain comprising SEQ ID NO. 12 which contains the engineered Cys 114 (114 Kabat numbering is the same as 118 Eu numbering and 121 sequential numbering). The Cys engineered L and/or H chain was conjugated to the PML linker and rifamycin-type antibiotic as shown in Table 2.

Example 19 *In vitro* efficacy of rF1-AACs

S. aureus (USA300 NRS384 strain) was incubated with various doses (100 ug/mL, 10 ug/mL, 1 ug/mL or 0.1 ug/mL) of an anti-S. aureus unconjugated antibody, **103** AAC loaded with 1.9 average antibiotic molecules per antibody (AAR2) or with **102** AAC loaded with 3.9 average antibiotic molecules per antibody (AAR4) for 1 hour to permit binding of the antibody to the bacteria. The resulting opsonized bacteria were fed to murine macrophages and incubated at 37°C to permit phagocytosis *(in vitro* macrophage assay). After 2 hours, the infection mix was removed and replaced with normal growth media supplemented with 50 ug/mL of gentamycin to kill any remaining extracellular bacteria. The total number of surviving intracellular bacteria was determined 2 days later by plating serial dilutions of the macrophage lysates on Tryptic Soy Agar plates.

The results are shown in Figure 10. Both of the AACs tested (AAR2 vs. AAR4) showed a similar dose response and yielded maximal killing at a dose of 10 ug/mL or above with partial to no killing at 1 ug/mL and below, suggesting that the dose response for the AAC is limited by the number of antibody binding sites on the bacterium. By loading 4 antibiotic molecules per antibody, bacterial killing by AACs and overall killing of bacteria was superior with the AAR4 AAC at all doses tested. At the highest dose tested, the 2DAR AAC reduced bacterial loads by 350-fold, whereas the 4AAR AAC reduced bacterial loads by more than 4,000-fold. (dashed line indicates the limit of detection for the assays shown).

This example demonstrates that rF1-AAC, **102** (AAR = 3.9) and **103** (AAR = 1.9) thio-rF1-HC-121C, LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR) from Table 3 killed intracellular MRSA in a macrophage assay in vitro. The results are shown in Figure 10.

Example 20 *In vivo* efficacy of rF1-AACs

This example demonstrates that the rF1-AACs were effective in greatly reducing or eradicating intracellular S. aureus infections, in a murine intravenous infection model.

Peritonitis Model. 7 week old female A/J mice (Jackson Laboratories) are infected by peritoneal injection with $5x10^7$ CFU of USA300. Mice are sacrificed 2 days post infection and the peritoneum is flushed with 5 mL of cold phosphate buffered saline solution (PBS). Kidneys are homogenized in 5 mL of PBS as described below for the intravenous infection model. Peritoneal washes are centrifuged for 5 minutes at 1,000 rpm at 4°C in a table top centrifuge. The supernatant is collected as the extracellular bacteria and the cell pellet containing peritoneal cells is collected as the intracellular fraction. The cells are treated with 50 μg/mL of lysostaphin for 20 minutes at 37°C to kill contaminating extracellular bacteria. Peritoneal cells are washed 3x in ice cold PBS to remove the lysostaphin prior to analysis. To count the number of intracellular CFUs, peritoneal cells are lysed in HB (Hanks Balanced Salt Solution supplemented with 10 mM HEPES and .1% Bovine Serum Albumin) with 0.1% Triton-X, and serial dilutions of the lysate are made in PBS with 0.05% tween-20.

Murine intravenous infection model. For studies involving competing human IgG (SCID IVIG model), CB17.SCID mice (Charles River Laboratories, Hollister, CA) were reconstituted with GammaGard S/D IGIV Immune Globulin (ASD Healthcare, Brooks KY) using a dosing regimen optimized to achieve constant serum levels of at least 10 mg/mL of human IgG in serum. IGIV was administered with an initial intravenous dose of 30 mg per mouse followed by a second dose of 15 mg/mouse by intraperitoneal injection after 6 hours, and subsequent daily dosing of 15 mg per mouse by intraperitoneal injection for 3 consecutive days.

Mice (n=8 for each of antibody or AAC) were infected 4 hours after the first dose of IGIV with $1x10^7$ CFU of MRSA (USA300 NRS384 strain) diluted in phosphate buffered saline by intravenous injection. Infected mice were treated with 50 mg/kg of rF1 naked antibody, **103** AAC DAR2 or **102** AAC DAR4. Mice were given a single dose of AAC 26h post infection by intravenous injection, sacrificed on day 4 post infection, and kidneys and hearts were harvested in 5 mL of phosphate buffered saline. The tissue samples were homogenized using a GentleMACS Dissociator™ (Miltenyi Biotec, Auburn, CA). The total number of bacteria

recovered per organ was determined by plating serial dilutions of the tissue homogenate in PBS .05% Tween on Tryptic Soy Agar with 5% defibrinated sheep blood.

Figure 11A shows the results of in vivo treatment with AACs on the bacterial load in the kidneys of the infected mice. Treatment with AAC containing 2 antibiotic molecules per antibody (DAR2) reduced bacterial load by approximately 30-fold and treatment with the AAC containing 4 antibiotic molecules per antibody (AAR4) reduced bacterial burdens by more than 30,000-fold.

Figure 11B shows the results of in vivo treatment with AACs on the bacterial count in the heart. Treatment with AAC AAR2 reduced bacterial burdens by approximately 70-fold with 6 out of 8 mice having undetectable level of bacteria in hearts; treatment with the AAC DAR4 completely eradicated infection in hearts resulting in 8 out of 8 mice having undetectable levels of bacteria.

Although the foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. All patents, patent applications, and references cited throughout the specification are expressly incorporated by reference.

CLAIMS

We claim:

1. An antibody-antibiotic conjugate compound comprising an anti-serine-aspartate repeat (SDR) antibody, covalently attached by a protease-cleavable, non-peptide linker to a rifamycin-type antibiotic.

2. The antibody-antibiotic conjugate compound of claim 1 having the formula:

$$Ab–(PML–abx)_p$$

wherein:

Ab is the rF1 antibody;

PML is the protease-cleavable, non-peptide linker having the formula:

$$–Str–PM–Y–$$

where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit;

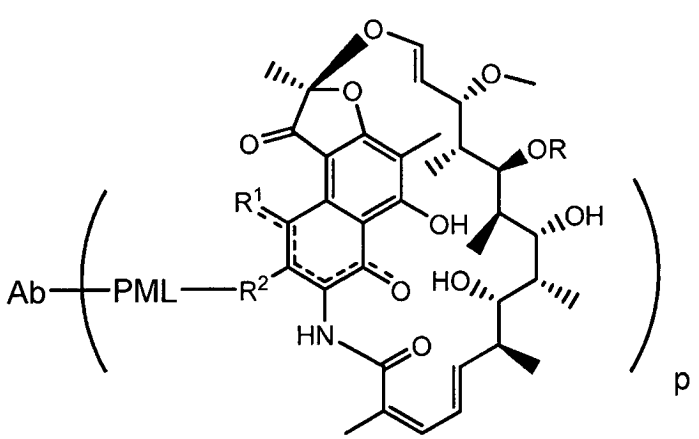
abx is the rifamycin-type antibiotic; and

p is an integer from 1 to 8.

3. The antibody-antibiotic conjugate compound of claim 2 wherein the rifamycin-type antibiotic is a rifalazil-type antibiotic.

4. The antibody-antibiotic conjugate compound of claim 2 wherein the rifamycin-type antibiotic comprises a quaternary amine attached to the protease-cleavable, non-peptide linker.

5. The antibody-antibiotic conjugate compound of claim 2 having Formula I:



I

wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

R^1 is OH;

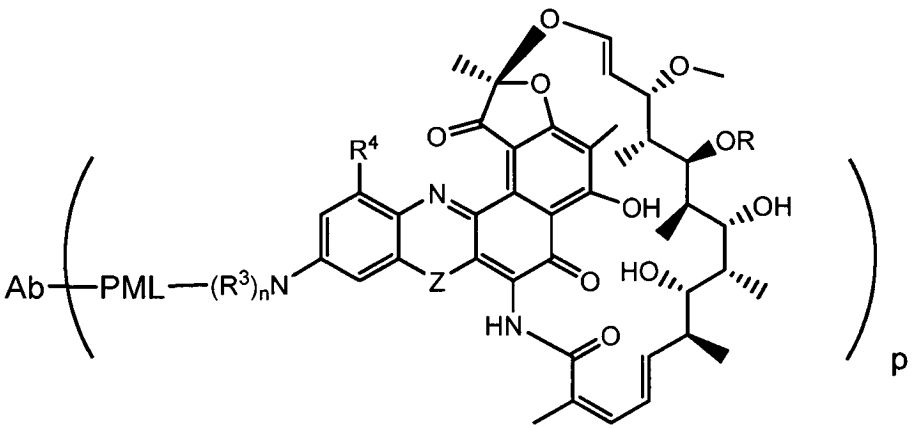
R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH;

PML is the protease-cleavable, non-peptide linker attached to R^2 or the fused heteroaryl or heterocyclyl formed by R^1 and R^2; and

Ab is the rF1 antibody.

6. The antibody-antibiotic conjugate compound of claim 5 having the formula:



wherein

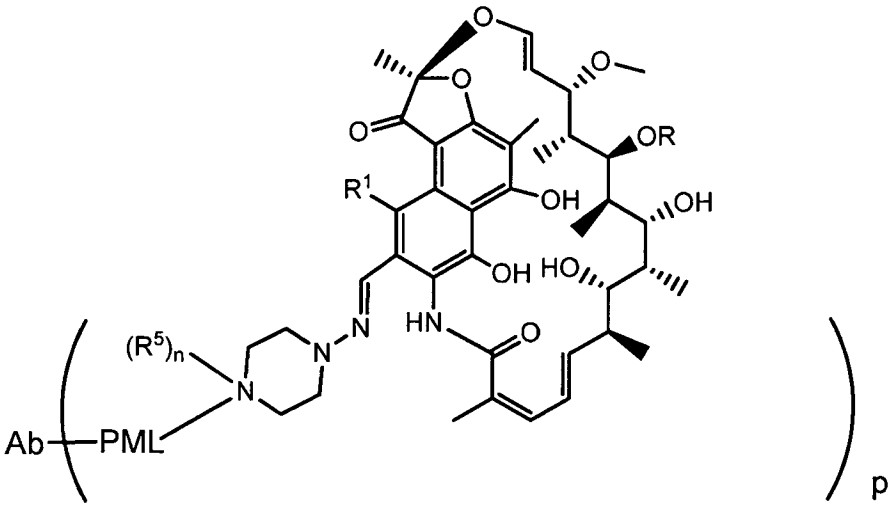
R^3 is independently selected from H and C_1–C_{12} alkyl;

n is 1 or 2;

R^4 is selected from H, F, Cl, Br, I, C_1–C_{12} alkyl, and OH; and

Z is selected from NH, N(C_1–C_{12} alkyl), O and S.

7. The antibody-antibiotic conjugate compound of claim 2 having the formula:

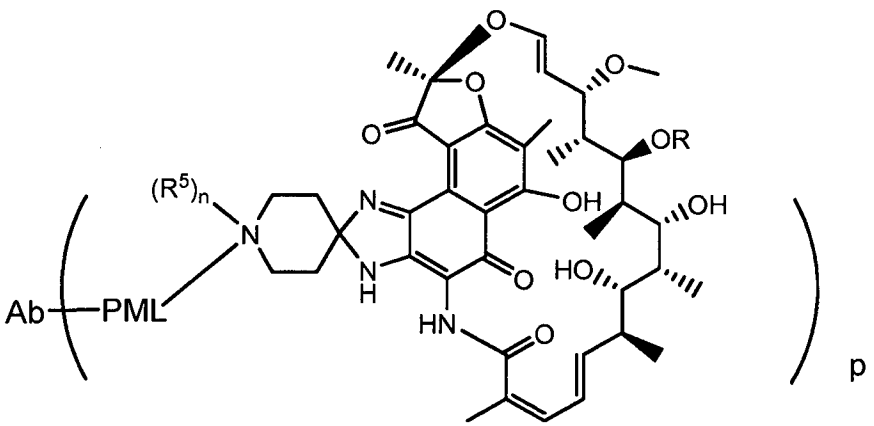


wherein

R^5 is selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

8. The antibody-antibiotic conjugate compound of claim 2 having the formula:

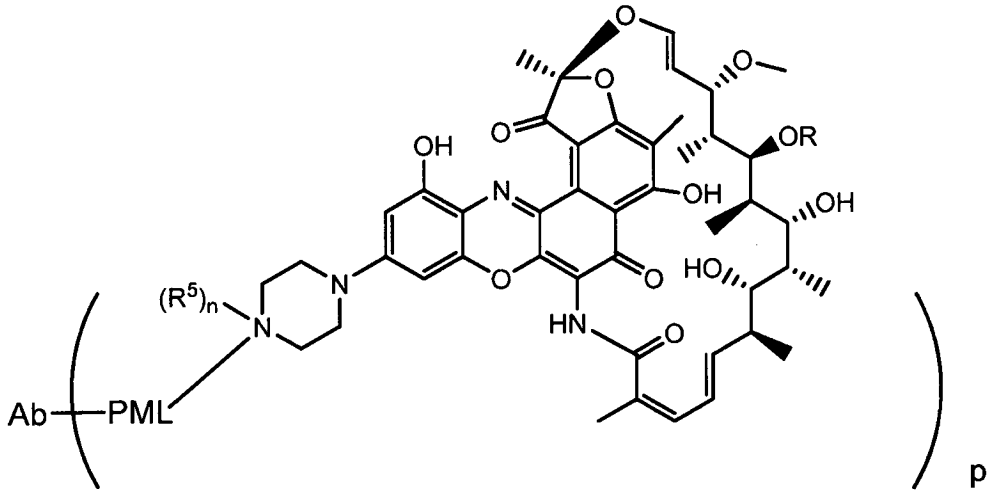


wherein

R^5 is selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

9. The antibody-antibiotic conjugate compound of claim 2 having the formula:

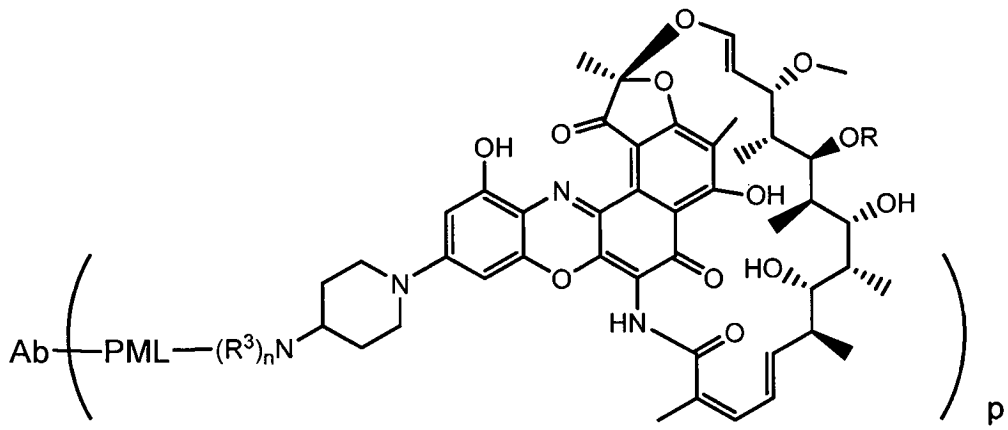


wherein

R^5 is independently selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

10. The antibody-antibiotic conjugate compound of claim 2 having the formula:

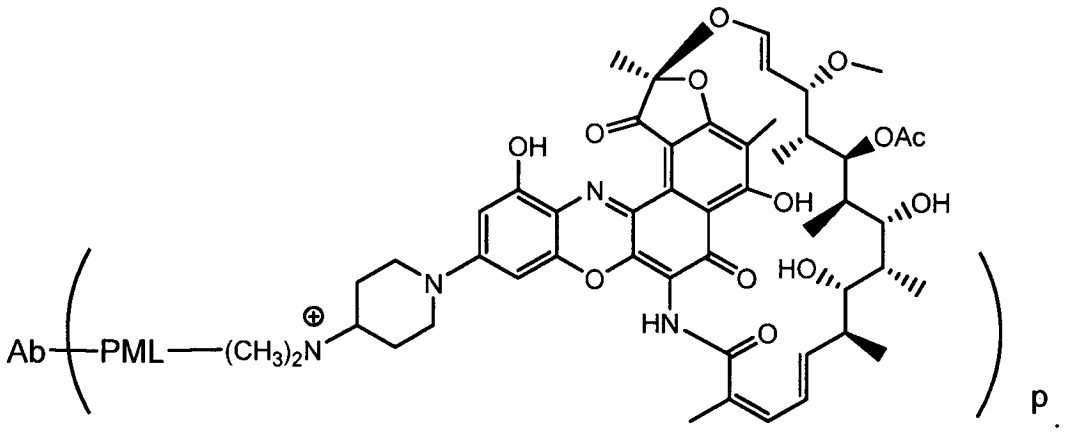


wherein

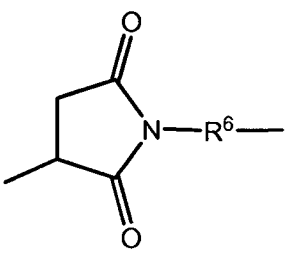
R^3 is independently selected from H and C_1–C_{12} alkyl; and

n is 1 or 2.

11. The antibody-antibiotic conjugate compound of claim 10 having the formula:



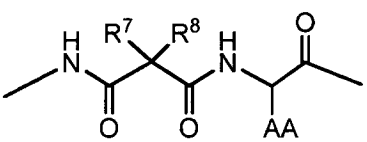
12. The antibody-antibiotic conjugate compound of claim 2 wherein Str has the formula:



wherein R^6 is selected from the group consisting of C_1-C_{12} alkylene, C_1-C_{12} alkylene-C(=O), C_1-C_{12} alkylene-NH, $(CH_2CH_2O)_r$, $(CH_2CH_2O)_r$-C(=O), $(CH_2CH_2O)_r$-CH_2, and C_1-C_{12} alkylene-NHC(=O)CH_2CH(thiophen-3-yl), where r is an integer ranging from 1 to 10.

13. The antibody-antibiotic conjugate compound of claim 12 wherein R^6 is $(CH_2)_5$.

14. The antibody-antibiotic conjugate compound of claim 2 wherein PM has the formula:

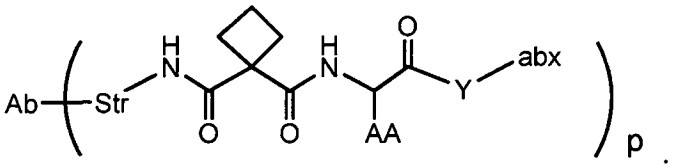


where R^7 and R^8 together form a C_3-C_7 cycloalkyl ring, and

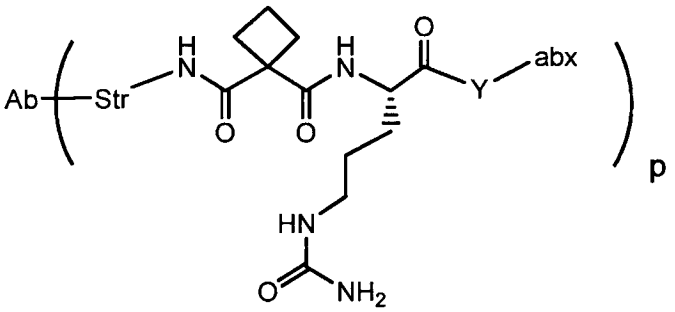
AA is an amino acid side chain selected from H, $-CH_3$, $-CH_2(C_6H_5)$, $-CH_2CH_2CH_2CH_2NH_2$, $-CH_2CH_2CH_2NHC(NH)NH_2$, $-CHCH(CH_3)CH_3$, and $-CH_2CH_2CH_2NHC(O)NH_2$.

15. The antibody-antibiotic conjugate compound of claim 2 wherein Y comprises para-aminobenzyl or para-aminobenzyloxycarbonyl.

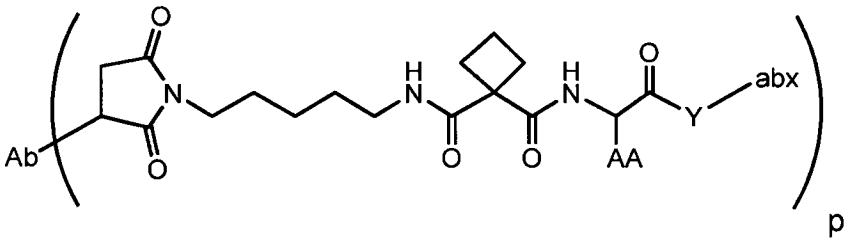
16. The antibody-antibiotic conjugate compound of claim 2 having the formula:



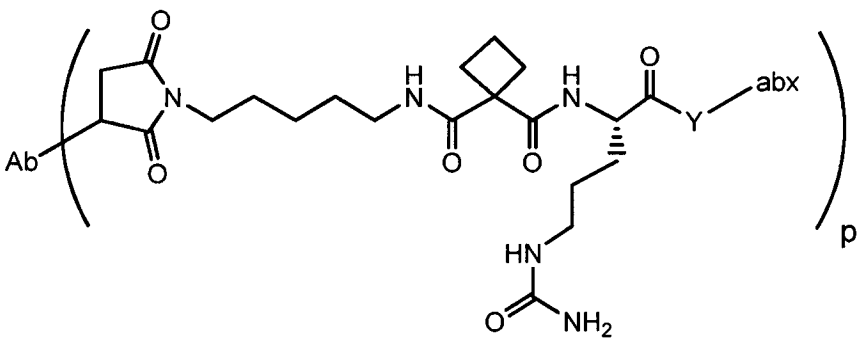
17. The antibody-antibiotic conjugate compound of claim 16 having the formula:



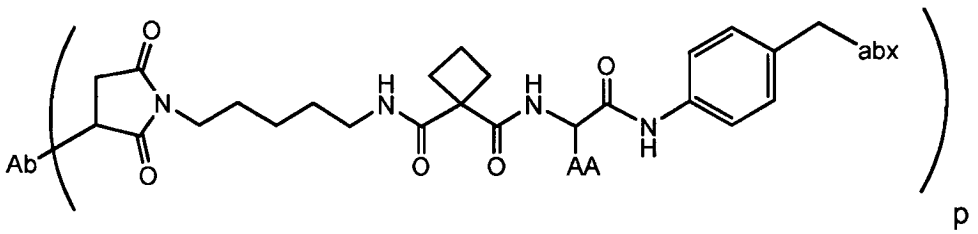
18. The antibody-antibiotic conjugate compound of claim 15 having the formula:



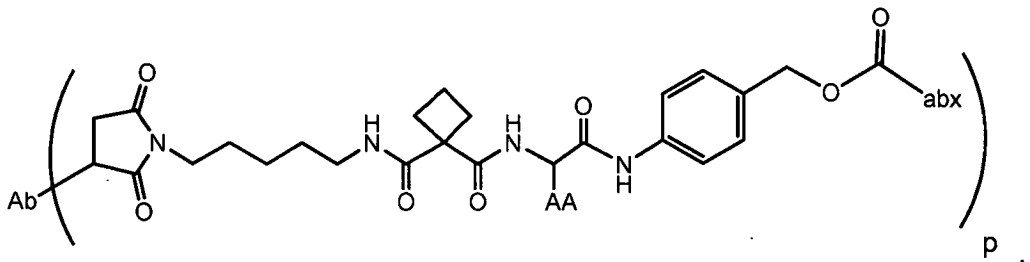
19. The antibody-antibiotic conjugate compound of claim 18 having the formula:



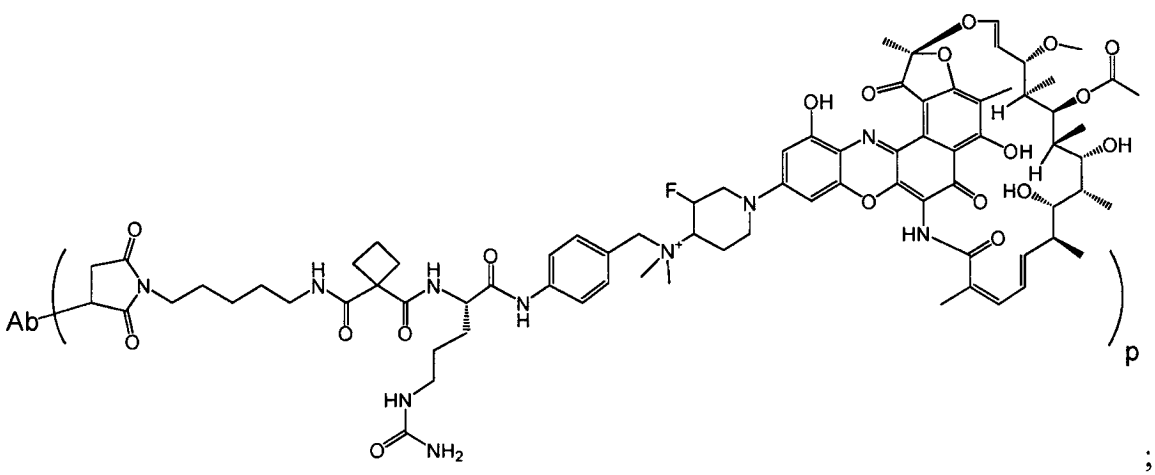
20. The antibody-antibiotic conjugate compound of claim 15 selected from the formulas:



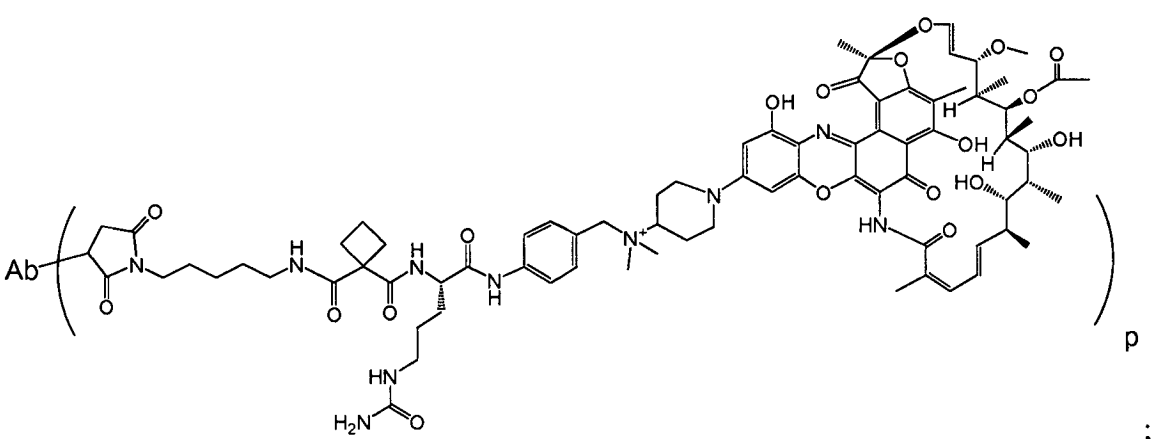
and



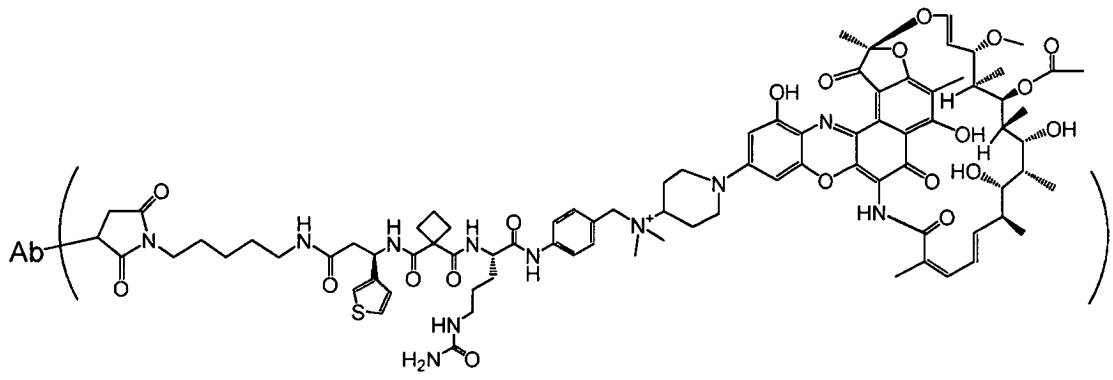
21. The antibody-antibiotic conjugate compound of claim 16 selected from the formulas:



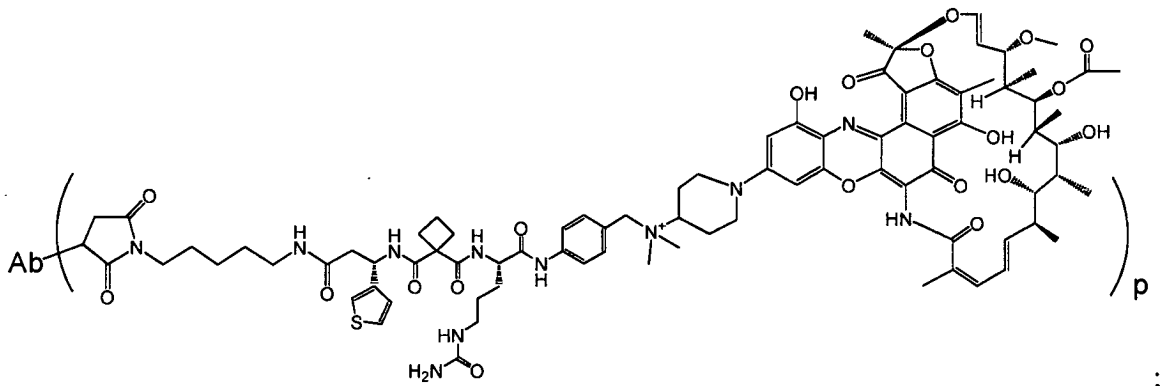
;



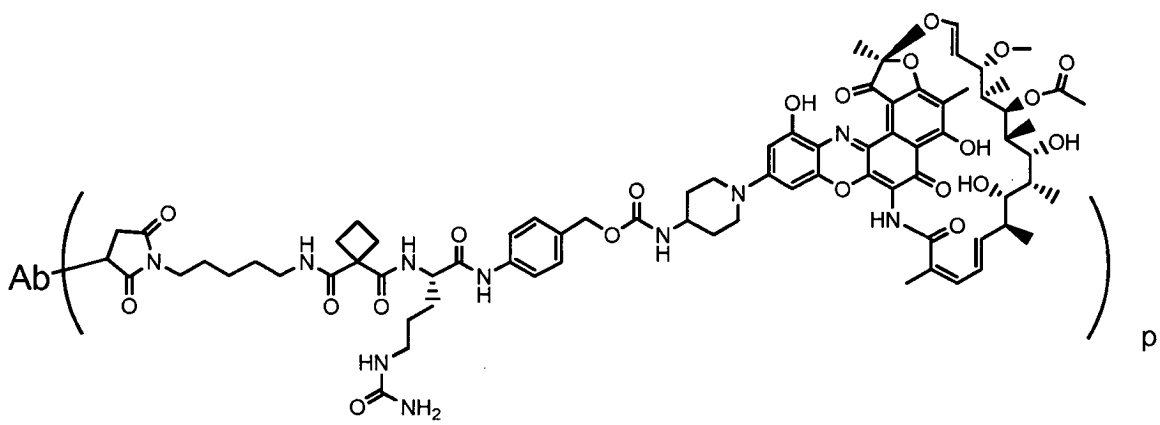
;



;

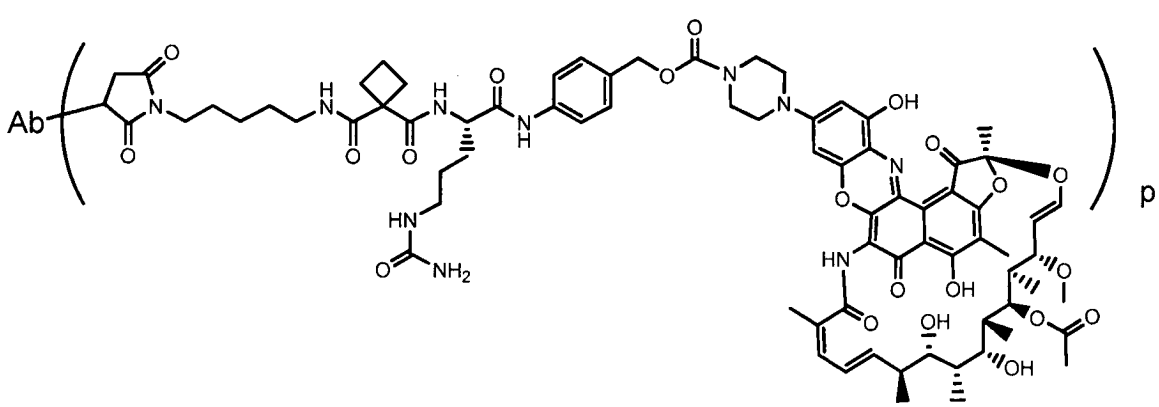


;



;

and



.

22. The antibody-antibiotic conjugate compound of claim 1, wherein the anti-SDR antibody is a rF1 antibody.

23. The antibody-antibiotic conjugate of claim 22, wherein the rF1 antibody comprises a light (L) chain and a heavy (H) chain, the L chain comprising CDR L1, CDR L2, and CDR L3 and the H chain comprising CDR H1, CDR H2 and CDR H3, wherein the CDR L1, CDR L2, and CDR L3 and CDR H1, CDR H2 and CDR H3 comprise the amino acid sequences of the CDRs of each of Abs F1, rF1, rF1.v1 and rF1.v6 (SEQ ID NO.1-8), respectively, as shown in Table 4A and Table 4B .

24. The antibody-antibiotic conjugate of claim 22 wherein the rF1 antibody comprises a heavy chain variable region (VH), wherein the VH comprises at least 95% sequence identity over the length of the VH region of SEQ ID NO.13.

25. The antibody-antibiotic conjugate compound of claim 24, wherein the VL comprises at least 95% sequence identity over the length of the VL region of SEQ ID NO. 14 or SEQ ID NO.15.

26. The antibody-antibiotic conjugate compound of any one of claim 1 or claim 22 wherein the anti-SDR antibody binds to *Staphylococcus aureus* and/or *Staphylococcus epidermidis* in vivo.

27. The antibody-antibiotic conjugate compound of any of the preceding claims, wherein the antibody is a F(ab) or a $F(ab')_2$.

28. A pharmaceutical composition comprising the antibody-antibiotic conjugate compound of claim 1, and a pharmaceutically acceptable carrier, glidant, diluent, or excipient.

29. A method of treating a Staphyloccocal bacterial infection in a patient comprising administering to the patient a therapeutically-effective amount of the antibody-antibiotic conjugate compound of claim 1.

30. The method of claim 29 wherein the patient is infected with Staphylococcus aureus.

31. The method of claim 30 wherein the patient is infected with Staphylococcus epidermidis.

32. The method of claim 29 wherein the antibody-antibiotic conjugate compound is administered to the patient at a dose in the range of about 50mg/kg to 100mg/kg.

33. The method of claim 29 wherein the patient is administered the antibody-antibiotic conjugate compound in conjunction with treatment with a second antibiotic.

34. A method of killing intracellular Staph aureus in the cells of a staph aureus infected patient without killing the host cells by administering an antibody-antibiotic conjugate compound of claim 1.

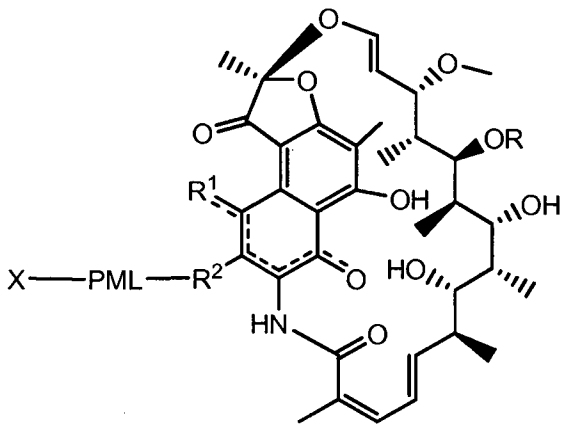
35. A process for making the antibody-antibiotic conjugate compound of claim 1 comprising conjugating a rifamycin-type antibiotic to an rF1 antibody.

36. A kit for treating a bacterial infection, comprising:

a) the pharmaceutical composition of claim 23; and

b) instructions for use.

37. An antibiotic-linker intermediate having Formula II:



II

wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

R^1 is OH;

R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH;

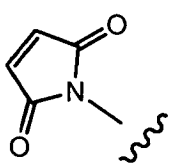
PML is a protease-cleavable, non-peptide linker attached to R^2 or the fused heteroaryl or heterocyclyl formed by R^1 and R^2, and having the formula:

–Str–PM–Y–

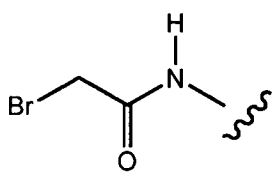
where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit; and

X is a reactive functional group selected from maleimide, thiol, amino, bromide, bromoacetamido, iodoacetamido, p-toluenesulfonate, iodide, hydroxyl, carboxyl, pyridyl disulfide, and N-hydroxysuccinimide.

38. The antibiotic-linker intermediate of claim 37 wherein X is

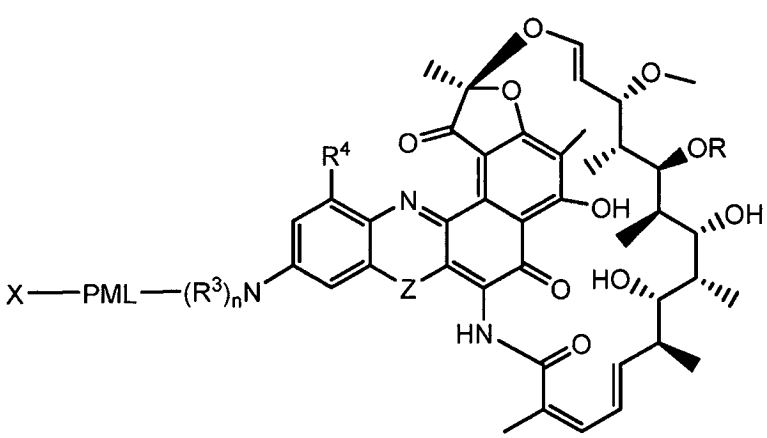


or



.

39. The antibiotic-linker intermediate of claim 37 having the formula:



wherein

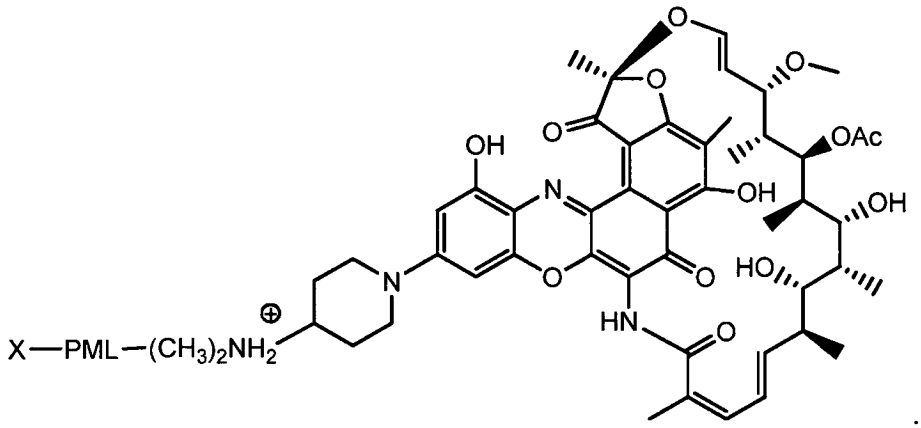
R^3 is independently selected from H and C_1–C_{12} alkyl;

n is 1 or 2;

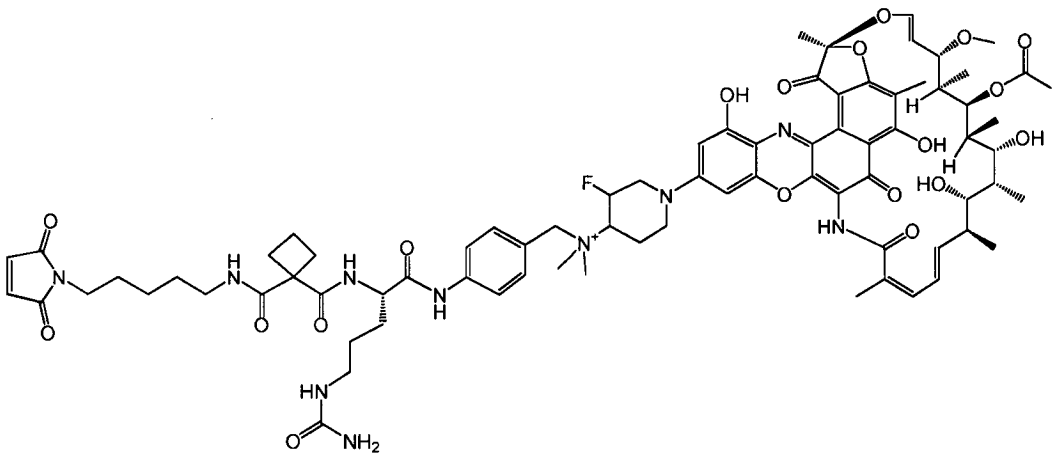
R^4 is selected from H, F, Cl, Br, I, C_1–C_{12} alkyl, and OH; and

Z is selected from NH, N(C_1–C_{12} alkyl), O and S.

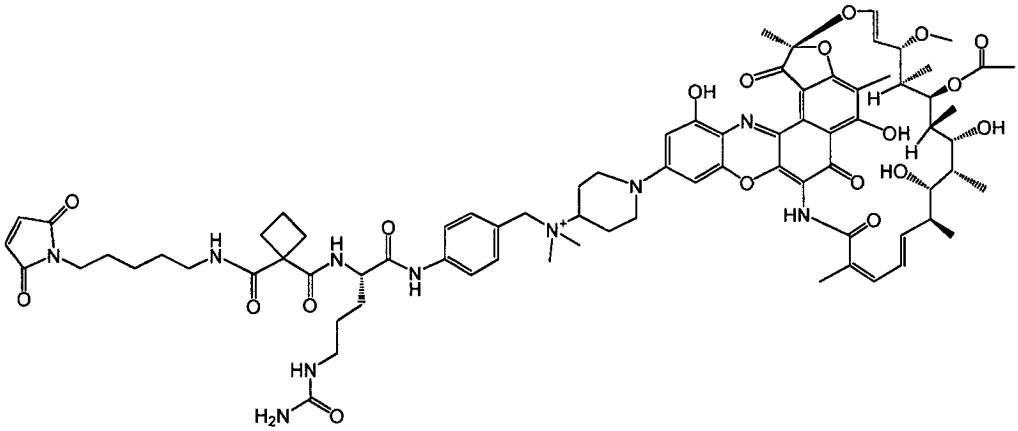
40. The antibiotic-linker intermediate of claim 37 having the formula:



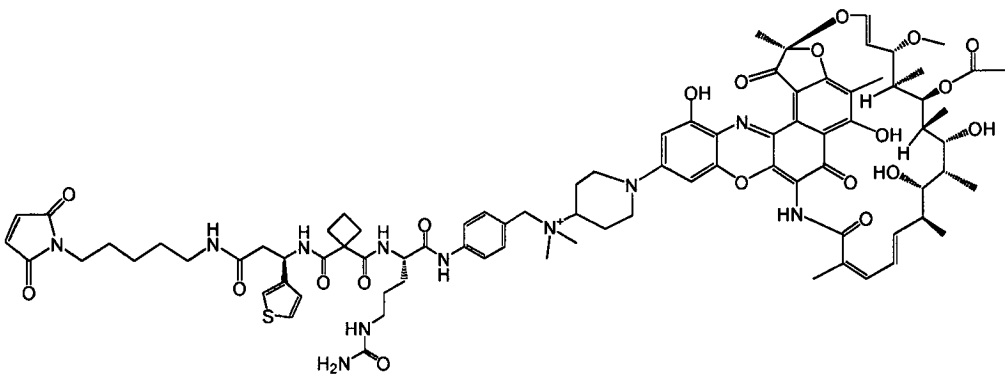
41. The antibiotic-linker intermediate of claim 37 selected from the formulas:



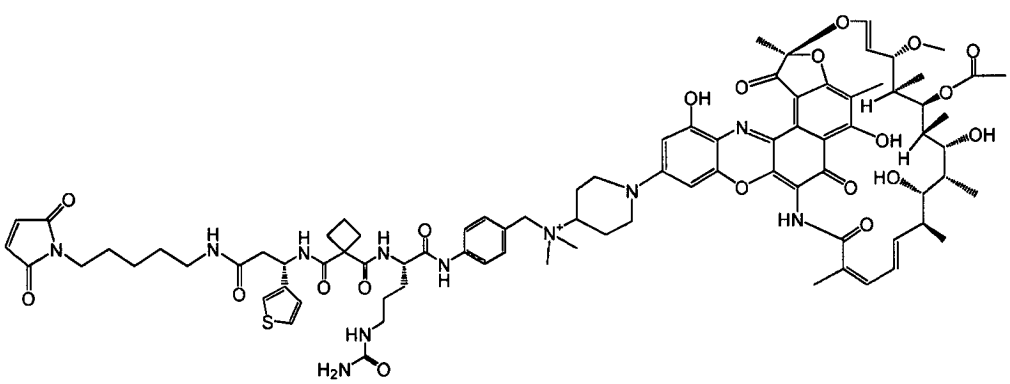
;



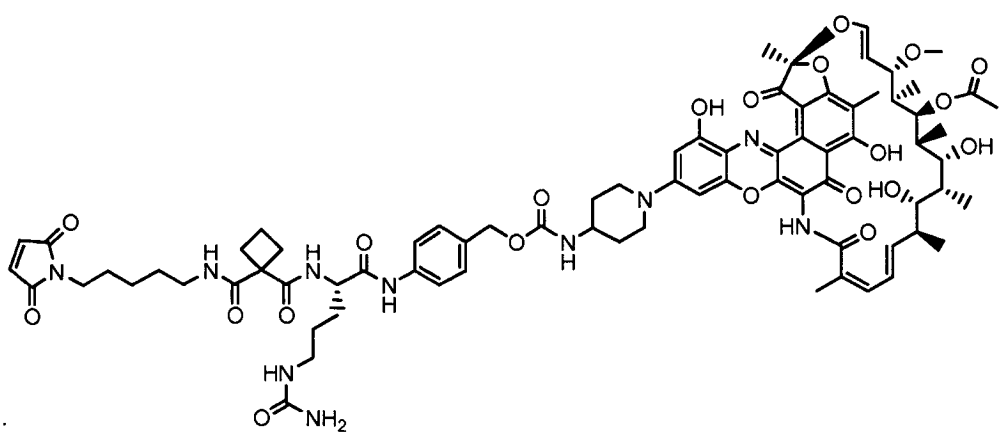
;



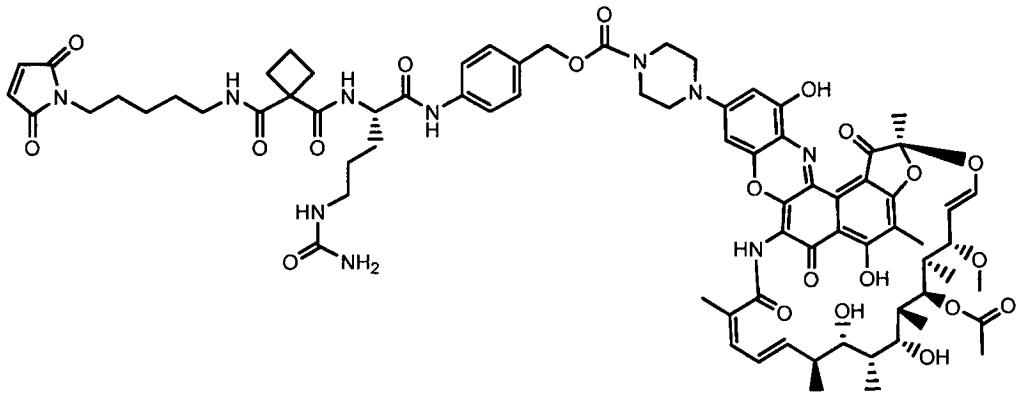
;



;



; and



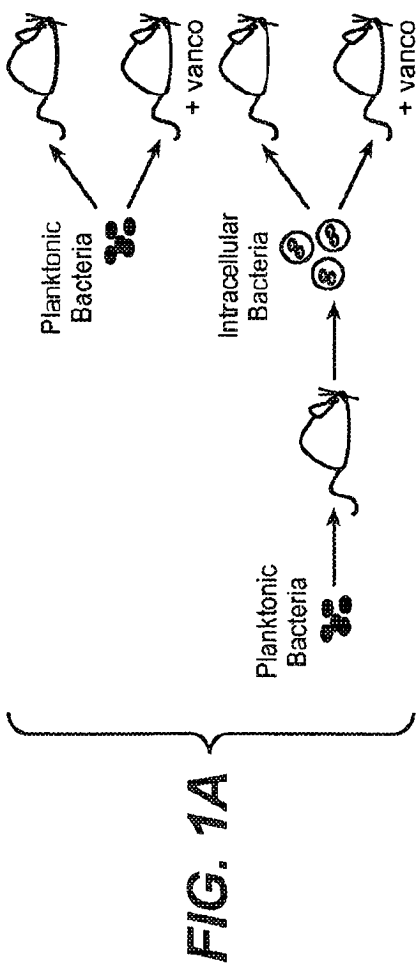


FIG. 1A

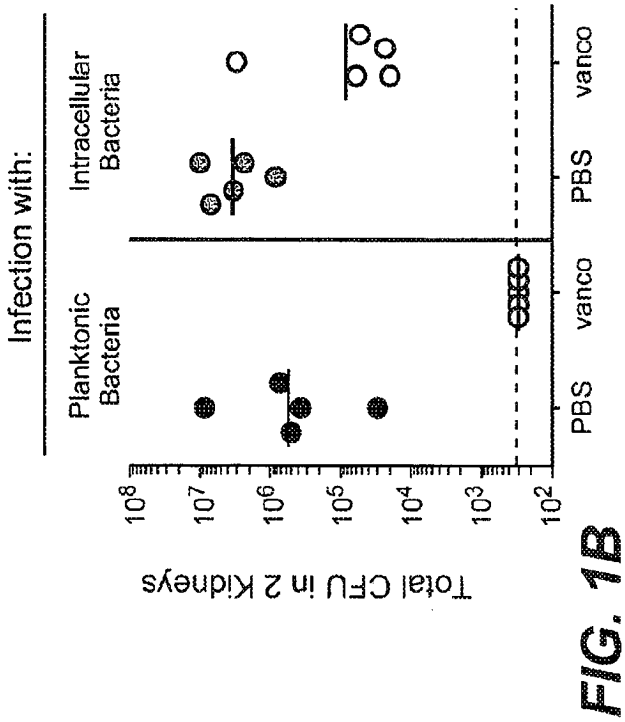


FIG. 1B

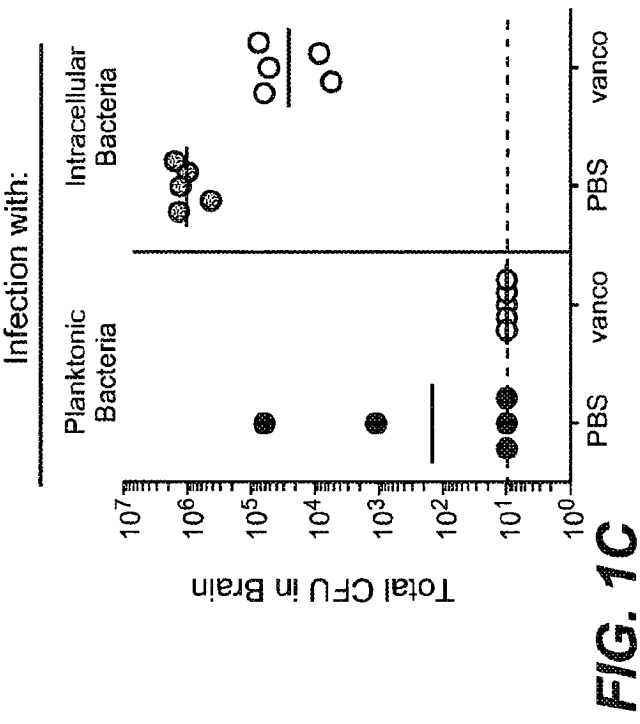


FIG. 1C

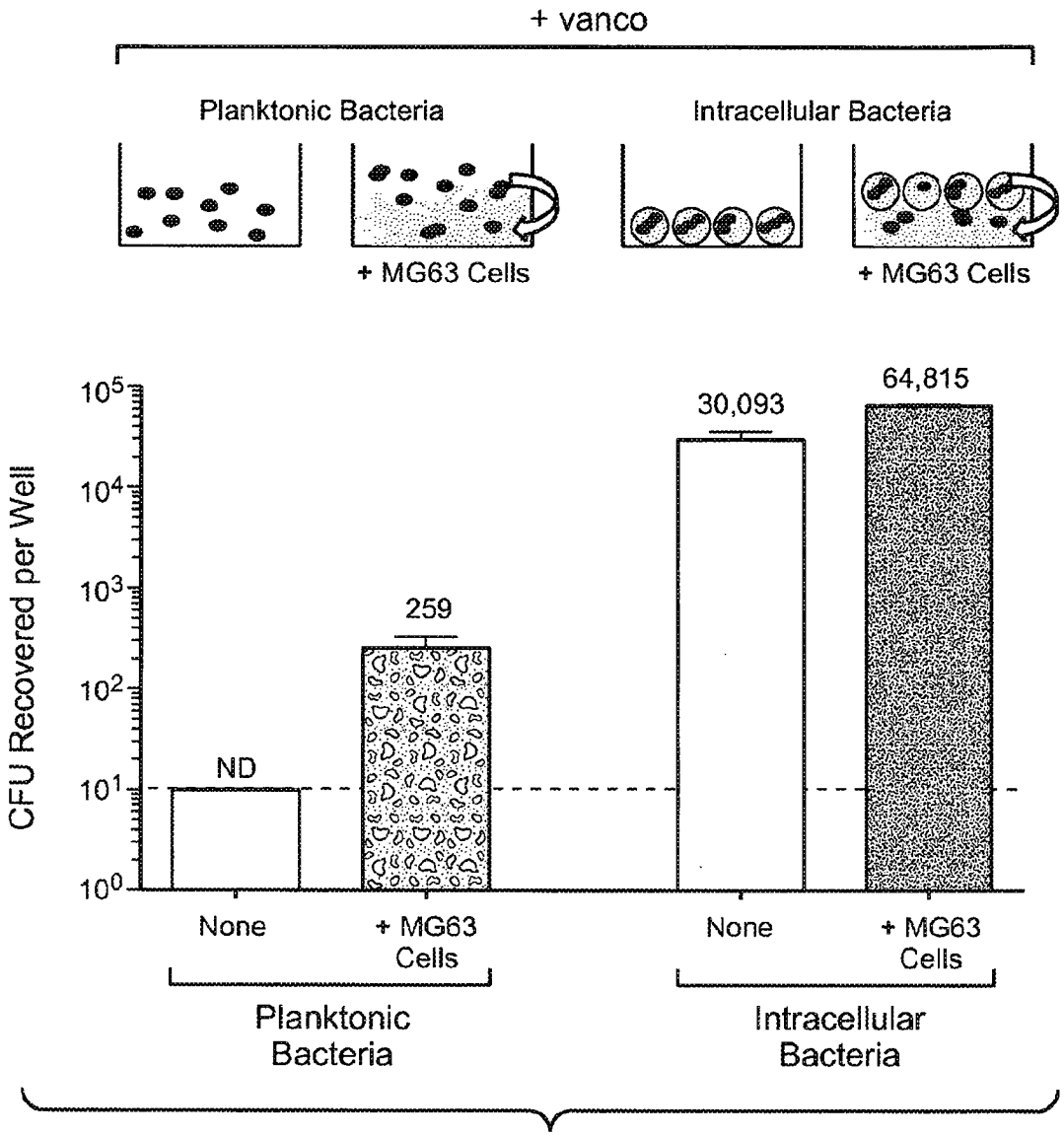

+ vanco
Planktonic Bacteria
Intracellular Bacteria
+ MG63 Cells
+ MG63 Cells
CFU Recovered per Well
10^5
10^4
10^3
10^2
10^1
10^0
ND
259
30,093
64,815
None
+ MG63 Cells
None
+ MG63 Cells
Planktonic Bacteria
Intracellular Bacteria

FIG. 1D

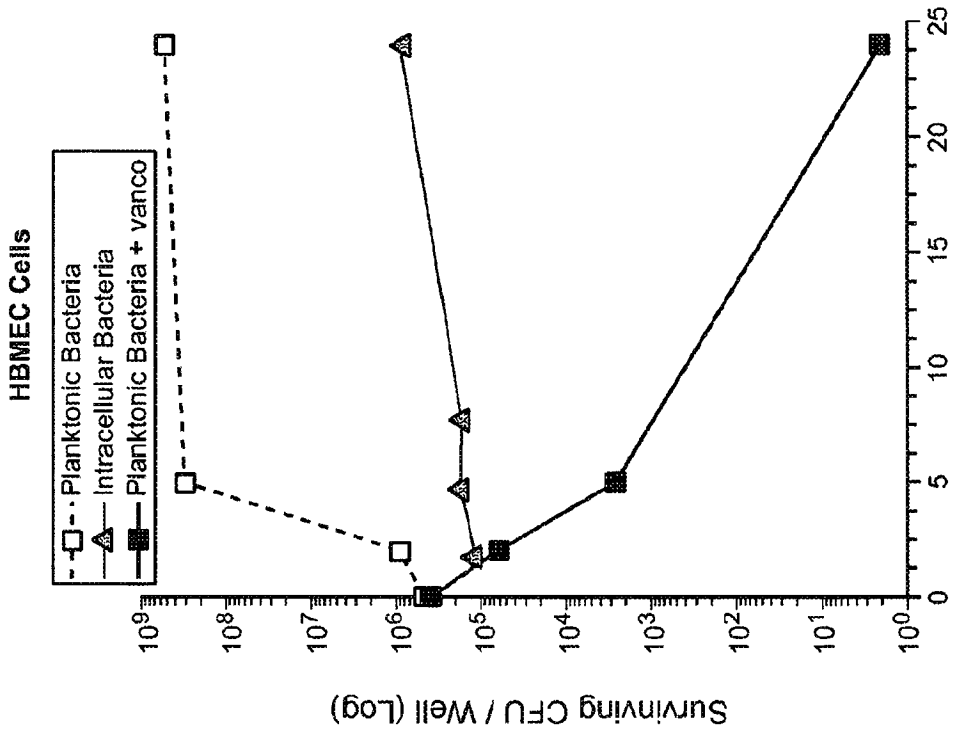

HBMEC Cells
Planktonic Bacteria
Intracellular Bacteria
Planktonic Bacteria + vanco
Surviving CFU / Well (Log)
Time (Hrs)

FIG. 1F

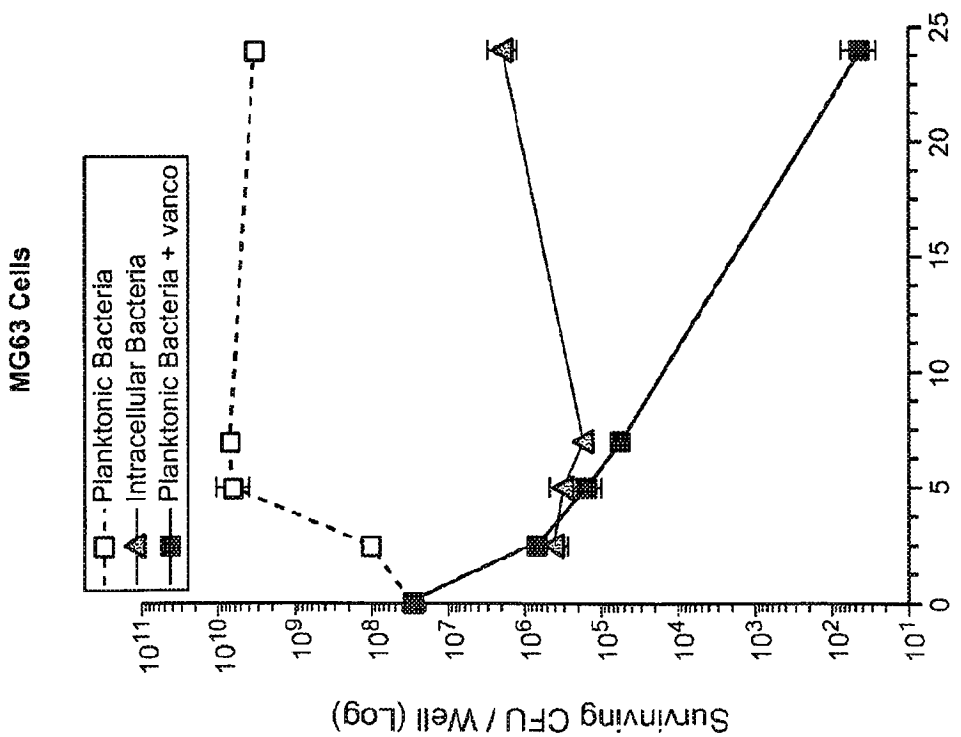
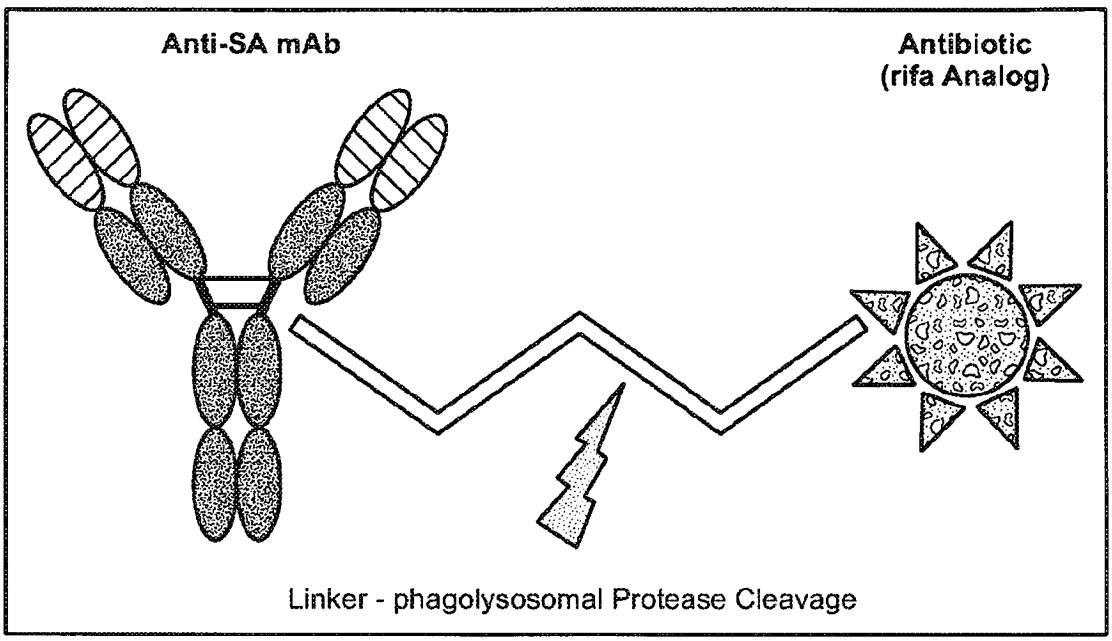

MG63 Cells
Planktonic Bacteria
Intracellular Bacteria
Planktonic Bacteria + vanco
Surviving CFU / Well (Log)
Time (Hrs)

FIG. 1E



• **Concept of TAC:**
Antibiotic is released from TAC by
Phagolysosomal Proteases

FIG. 2

FIG. 4A

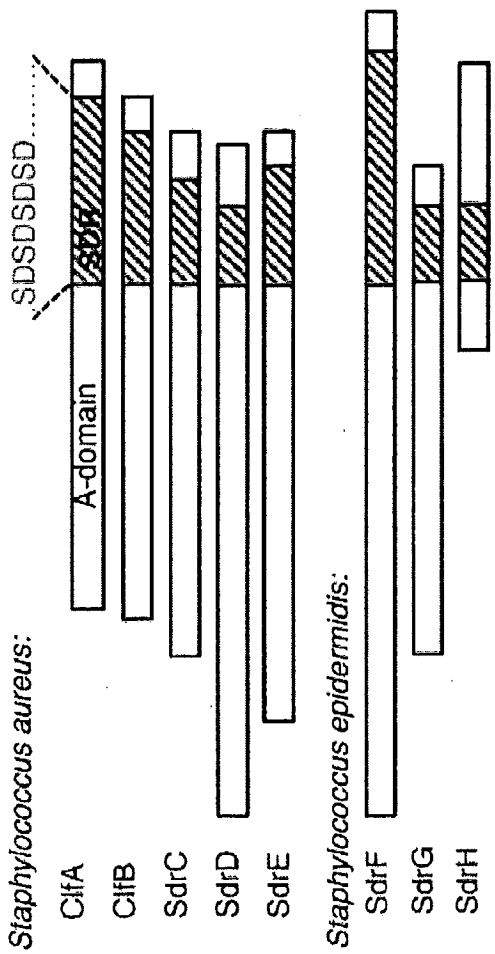
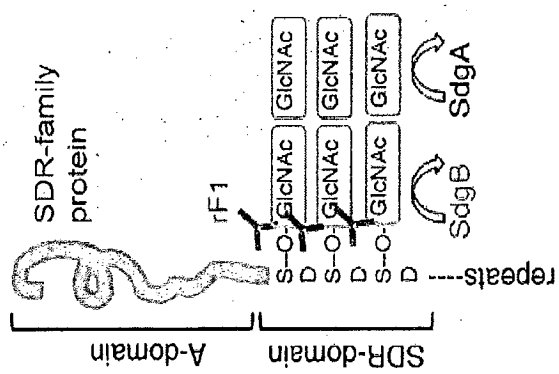

Staphylococcus aureus:
ClfA
ClfB
SdrC
SdrD
SdrE
A-domain
SDSDSDSD.....
Staphylococcus epidermidis:
SdrF
SdrG
SdrH

FIG. 4B


SDR-family protein
rF1
GlcNAc
SdgB
SdgA
repeats
A-domain
SDR-domain

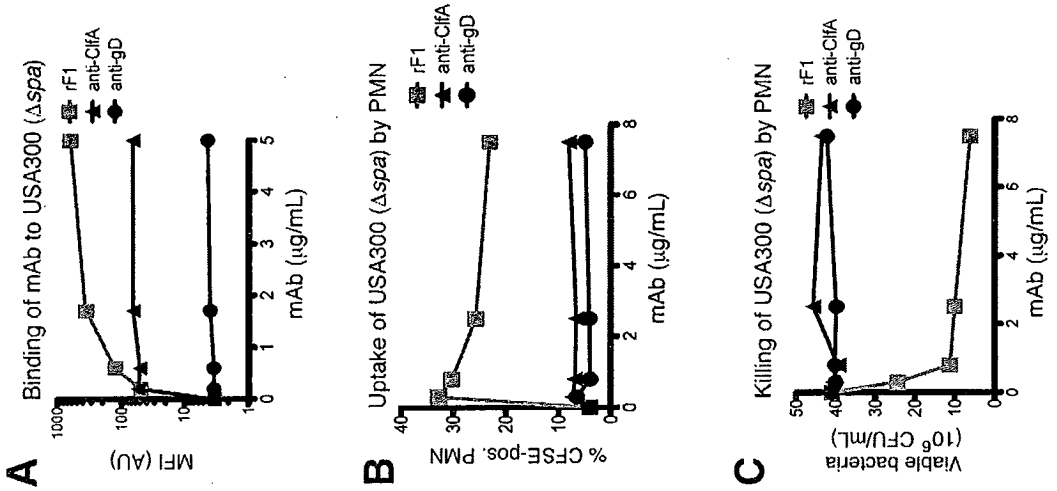

A
Binding of mAb to USA300 (Δspa)
rF1
anti-ClfA
anti-gD
MFI (AU)
1000
100
10
1
0
1
2
3
4
5
mAb (μg/mL)
B
Uptake of USA300 (Δspa) by PMN
rF1
anti-ClfA
anti-gD
% CFSE-pos. PMN
40
30
20
10
0
0
2
4
6
8
mAb (μg/mL)
C
Killing of USA300 (Δspa) by PMN
rF1
anti-ClfA
anti-gD
Viable bacteria
(10^6 CFU/mL)
50
40
30
20
10
0
0
2
4
6
8
mAb (μg/mL)

FIG. 5

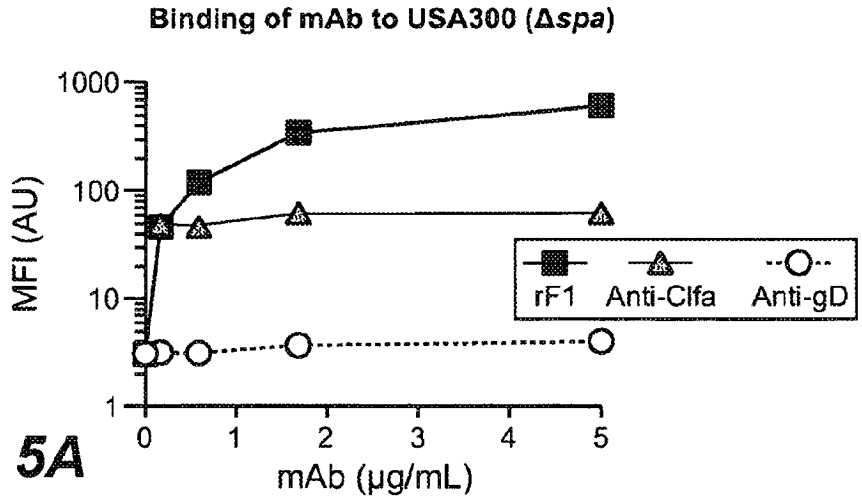


FIG. 5A

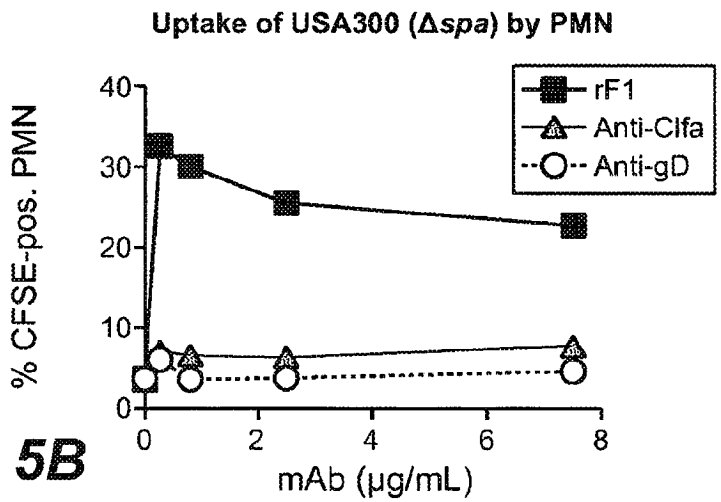


FIG. 5B

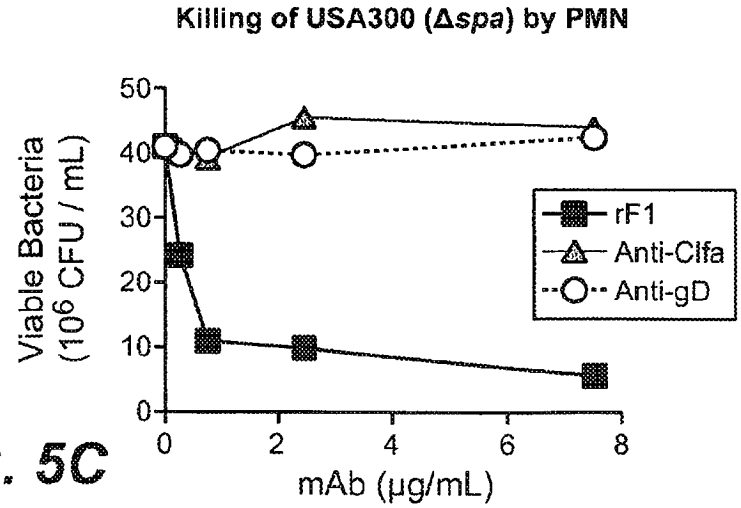


FIG. 5C

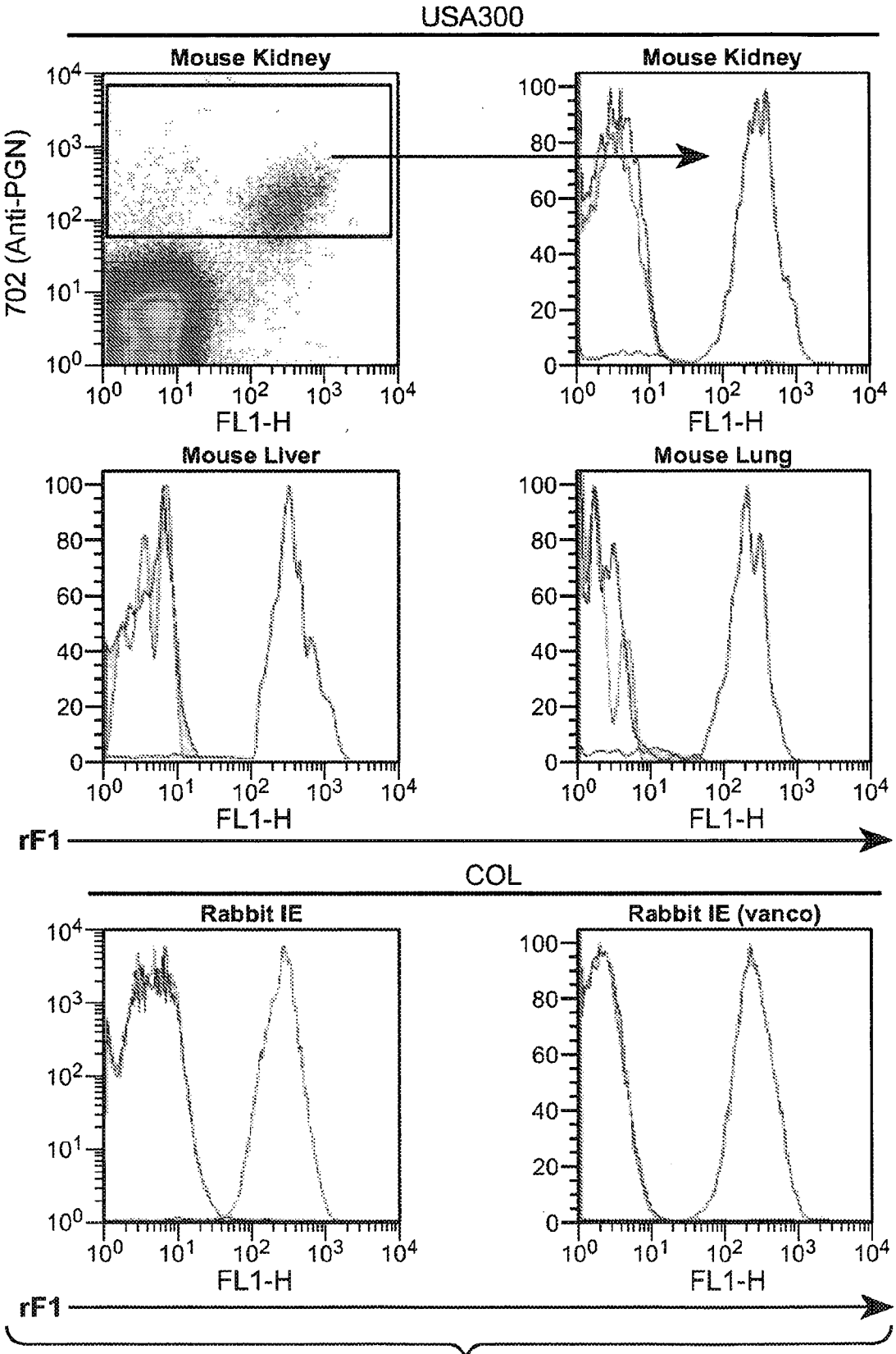

USA300
Mouse Kidney
Mouse Kidney
702 (Anti-PGN)
FL1-H
FL1-H
Mouse Liver
Mouse Lung
FL1-H
FL1-H
rF1
COL
Rabbit IE
Rabbit IE (vanco)
FL1-H
FL1-H
rF1

FIG. 6

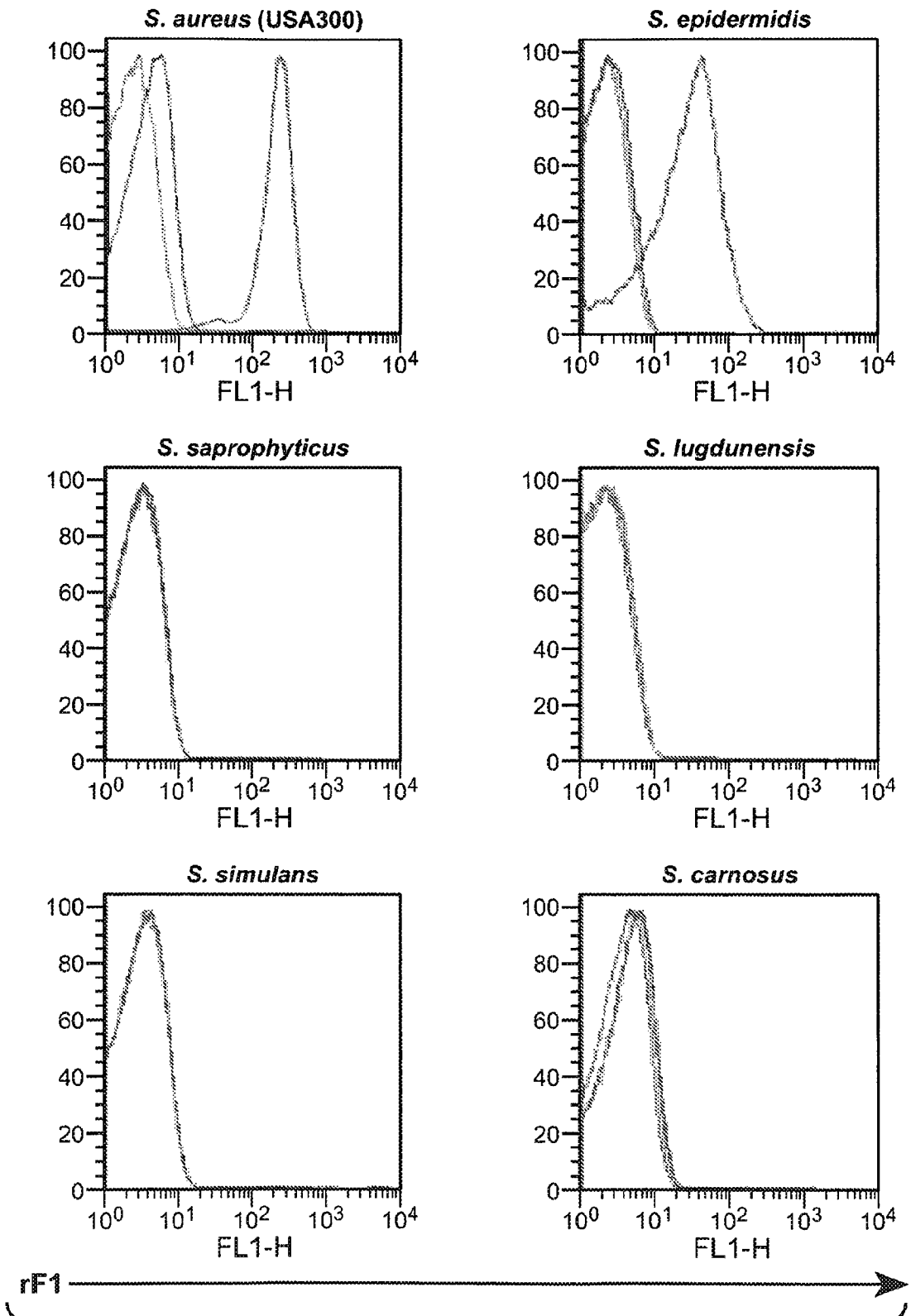

S. aureus (USA300)
S. epidermidis
S. saprophyticus
S. lugdunensis
S. simulans
S. carnosus
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4
FL1-H
rF1

FIG. 7A

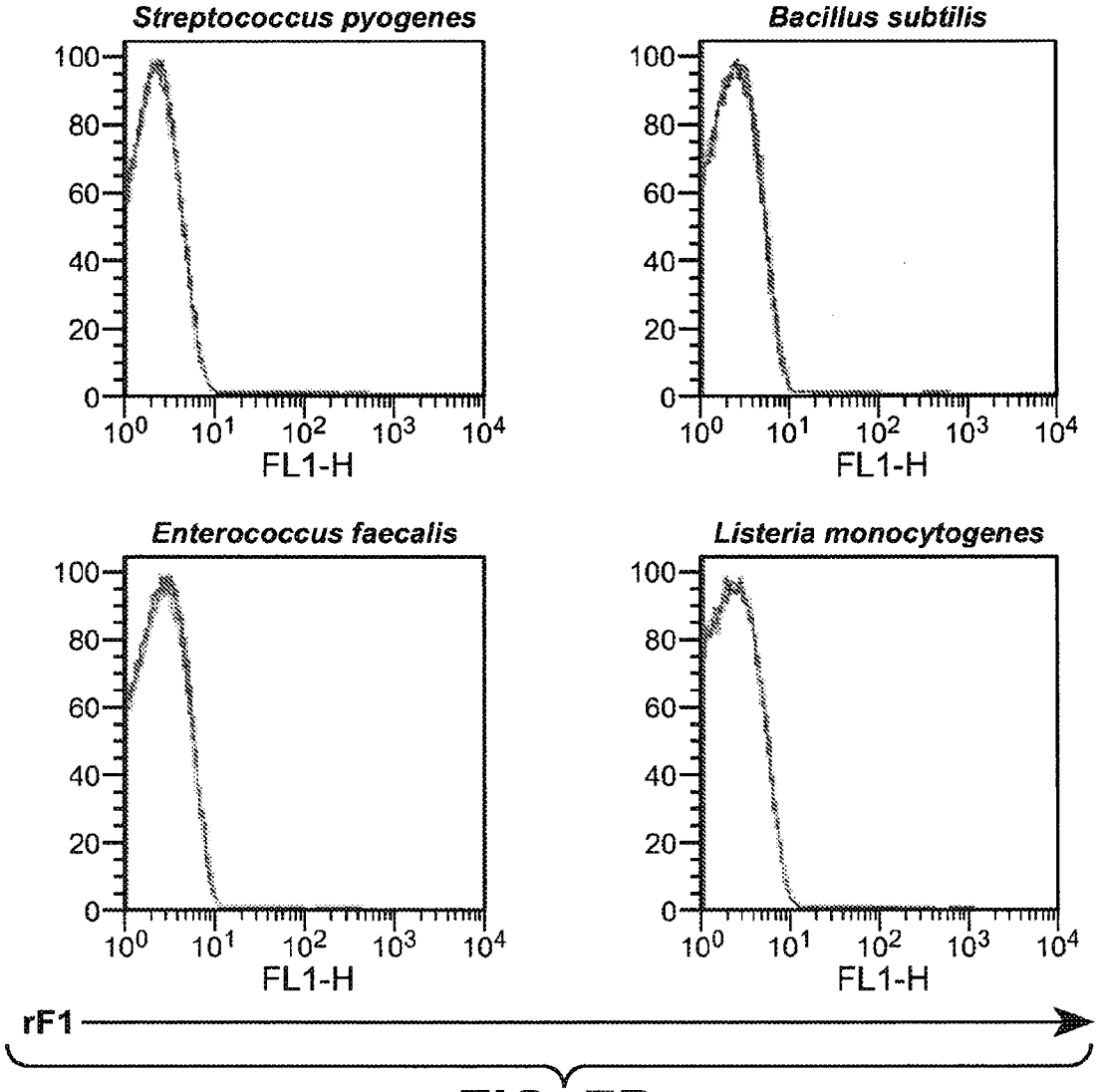

Streptococcus pyogenes
Bacillus subtilis
Enterococcus faecalis
Listeria monocytogenes
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4
FL1-H
rF1

FIG. 7B

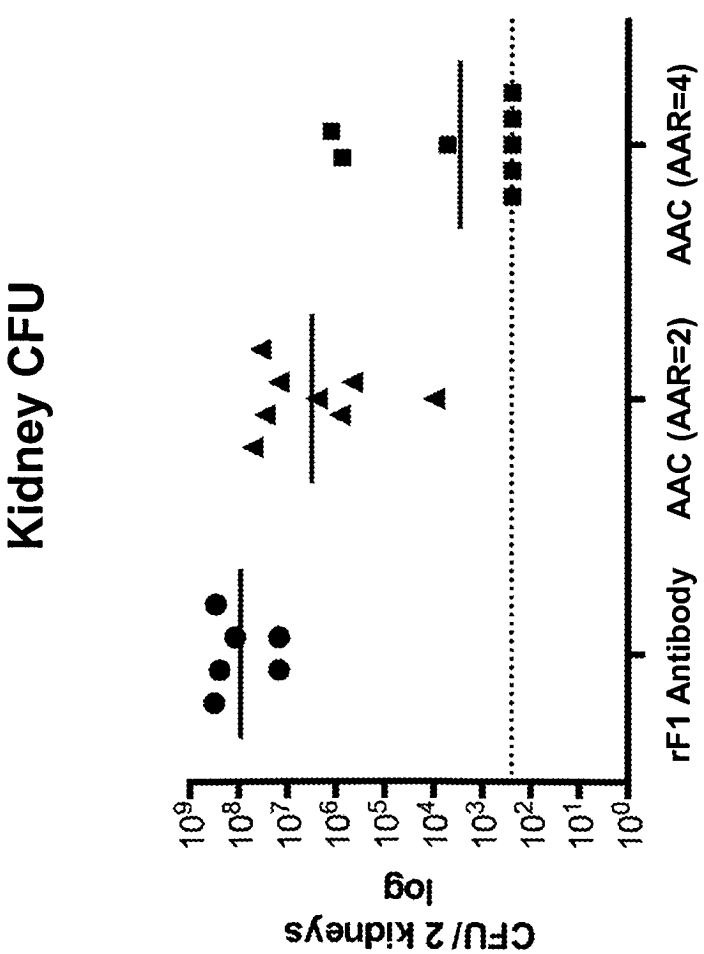


Figure 11A

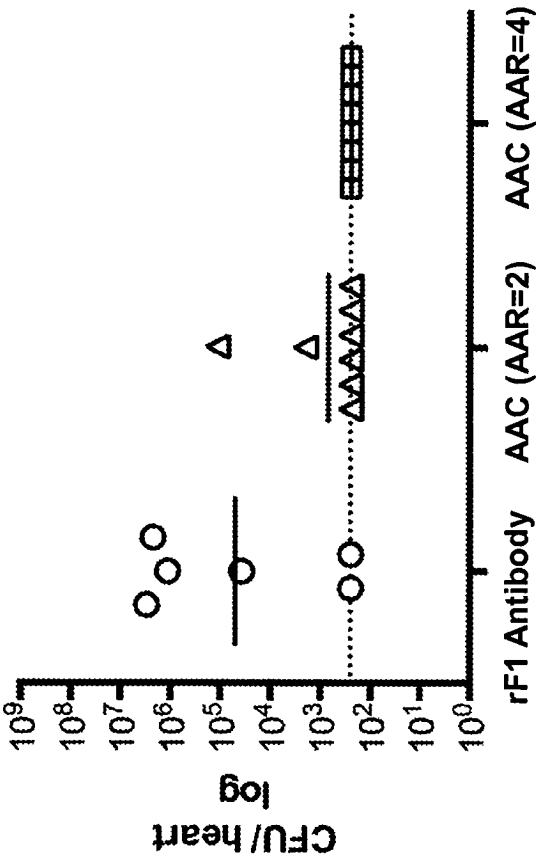


Figure 11B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/063515

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2011/008092 A2 (AIMM THERAPEUTICS BV; GENENTECH INC) 20 January 2011 (2011-01-20) pages 19-20; claims 1-7,14-16,25; example 5; sequences 1-10;27-30 pages 2-11	1,22-36 1-13, 15-21, 37-41

X A	WO 2014/080251 A1 (HANGZHOU DAC BIOTECH CO LTD) 30 May 2014 (2014-05-30) pages 35-36; claims 14; 11,12; figures 18,19 pages 43-44	12,13, 15,37,38 1-3,14, 16-20, 22, 26-30, 32,34,35

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 February 2016	12/02/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Kanbier, Titia

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/063515

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/020885 A2 (UNIV CALIFORNIA) 13 March 2003 (2003-03-13)	1,3, 28-30, 34,35
A	pages 44,47-48, paragraphs 10,25,33-35,86,90-94; claims 33-35,37-39,41	6-11, 16-22, 26,27, 32,33, 37-41

Y	WO 2005/081711 A2 (SEATTLE GENETICS INC) 9 September 2005 (2005-09-09) cited in the application claims 4,12-13,19-21,52,54,5829-30	1-13, 15-21, 37-41

Y	WO 2005/082023 A2 (GENENTECH INC) 9 September 2005 (2005-09-09) pages 14-15; claims 5,6,12,19	1-13, 15-21, 37-41

Y	WO 2008/141044 A2 (GENENTECH INC) 20 November 2008 (2008-11-20) page 68, paragraph 269 pages 59-67,90	1-13, 15-21, 37-41

X	WO 2007/096703 A2 (TARGANTA THERAPEUTICS INC) 30 August 2007 (2007-08-30) page 66, lines 9-11; example 2 page 1, line 34 - page 2, line 9; claims 1-20,22,29-36 page 41, lines 11-14 page 42, line 28 - page 47, line 13 page 66, lines 15-34 page 69, lines 13-18	5-11,21, 37-41

A	WO 03/045319 A2 (ACTIVBIOTICS INC) 5 June 2003 (2003-06-05) pages 2-13; figures 1,3; examples 2-4 pages 16-19 pages 35-42; claims 1-10,14-19	5-11,21, 37-41

X,P	WO 2014/194247 A1 (GENENTECH INC) 4 December 2014 (2014-12-04) pages 18-26 pages 69-81; claims	1-41

X,P	WO 2014/193722 A1 (GENENTECH INC) 4 December 2014 (2014-12-04) pages 58-80; claims; examples 4,14,17	1-41

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/063515

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011008092 A2	20-01-2011	AR 077756 A1	21-09-2011
		AU 2010271582 A1	01-03-2012
		CA 2768204 A1	20-01-2011
		CN 102666583 A	12-09-2012
		EA 201390967 A1	30-01-2014
		EP 2454284 A2	23-05-2012
		IL 217497 A	30-11-2015
		JP 2012533293 A	27-12-2012
		KR 20120065326 A	20-06-2012
		NZ 598063 A	25-07-2014
		NZ 616921 A	26-06-2015
		SG 177653 A1	28-02-2012
		TW 201107470 A	01-03-2011
		US 2011020323 A1	27-01-2011
		US 2012157665 A1	21-06-2012
		US 2013253175 A1	26-09-2013
		US 2013261293 A1	03-10-2013
		US 2014235828 A1	21-08-2014
		US 2015376266 A1	31-12-2015
		WO 2011008092 A2	20-01-2011
WO 2014080251 A1	30-05-2014	AU 2012395148 A1	21-05-2015
		CA 2891280 A1	30-05-2014
		EP 2922818 A1	30-09-2015
		US 2015250896 A1	10-09-2015
		WO 2014080251 A1	30-05-2014
WO 03020885 A2	13-03-2003	AU 2002326691 A1	18-03-2003
		CA 2455435 A1	13-03-2003
		EP 1416955 A2	12-05-2004
		US 2003153020 A1	14-08-2003
		WO 03020885 A2	13-03-2003
WO 2005081711 A2	09-09-2005	AU 2004316290 A1	09-09-2005
		BR PI0416028 A	02-01-2007
		CA 2543888 A1	09-09-2005
		CA 2841741 A1	09-09-2005
		CN 1938046 A	28-03-2007
		CN 103394083 A	20-11-2013
		CN 104998273 A	28-10-2015
		DK 1725249 T3	17-03-2014
		DK 2489364 T3	02-03-2015
		EP 1725249 A2	29-11-2006
		EP 2260858 A2	15-12-2010
		EP 2478912 A1	25-07-2012
		EP 2486933 A1	15-08-2012
		EP 2489364 A1	22-08-2012
		ES 2456325 T3	22-04-2014
		ES 2534185 T3	20-04-2015
		ES 2557389 T3	25-01-2016
		HK 1098071 A1	06-06-2014
		HK 1173684 A1	11-09-2015
		IL 175247 A	30-04-2013
		JP 5020636 B2	05-09-2012
		JP 5470288 B2	16-04-2014
		JP 5551661 B2	16-07-2014
		JP 2007514652 A	07-06-2007
		JP 2011121969 A	23-06-2011

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/063515

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		JP 2011231131 A	17-11-2011
		JP 2013151567 A	08-08-2013
		KR 20060111473 A	27-10-2006
		KR 20130119004 A	30-10-2013
		KR 20140015148 A	06-02-2014
		NZ 547633 A	27-08-2010
		NZ 583292 A	30-03-2012
		PT 1725249 E	10-04-2014
		PT 2489364 E	16-04-2015
		SG 149815 A1	27-02-2009
		SG 195524 A1	30-12-2013
		SI 1725249 T1	30-04-2014
		SI 2489364 T1	30-04-2015
		US 2005238649 A1	27-10-2005
		US 2008226657 A1	18-09-2008
		US 2008248051 A1	09-10-2008
		US 2008248053 A1	09-10-2008
		US 2009047296 A1	19-02-2009
		US 2012003247 A1	05-01-2012
		US 2012003248 A1	05-01-2012
		US 2012027783 A1	02-02-2012
		US 2012027784 A1	02-02-2012
		US 2012034246 A1	09-02-2012
		US 2012034247 A1	09-02-2012
		US 2012141508 A1	07-06-2012
		US 2012141509 A1	07-06-2012
		US 2012141510 A1	07-06-2012
		US 2012148608 A1	14-06-2012
		US 2012148610 A1	14-06-2012
		US 2014220047 A1	07-08-2014
		WO 2005081711 A2	09-09-2005
		ZA 200603619 A	29-10-2008
WO 2005082023 A2	09-09-2005	AU 2005216251 A1	09-09-2005
		CA 2556752 A1	09-09-2005
		EP 1718667 A2	08-11-2006
		JP 5064037 B2	31-10-2012
		JP 2007527421 A	27-09-2007
		US 2005256030 A1	17-11-2005
		US 2009041791 A1	12-02-2009
		US 2010273843 A1	28-10-2010
		WO 2005082023 A2	09-09-2005
WO 2008141044 A2	20-11-2008	AR 066476 A1	19-08-2009
		AU 2008251608 A1	20-11-2008
		CA 2683568 A1	20-11-2008
		CL 2008001334 A1	22-09-2008
		CN 101687037 A	31-03-2010
		EP 2144628 A2	20-01-2010
		JP 5290276 B2	18-09-2013
		JP 2010526821 A	05-08-2010
		PE 02452009 A1	17-03-2009
		TW 200902554 A	16-01-2009
		US 2008311134 A1	18-12-2008
		WO 2008141044 A2	20-11-2008
WO 2007096703 A2	30-08-2007	AU 2006339058 A1	30-08-2007
		BR PI0614758 A2	12-04-2011

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/063515

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		CA 2618741 A1	30-08-2007
		CN 101282981 A	08-10-2008
		EA 200800570 A1	27-02-2009
		EP 1928890 A2	11-06-2008
		IL 189270 A	31-05-2015
		JP 5368092 B2	18-12-2013
		JP 2009505974 A	12-02-2009
		KR 20080035687 A	23-04-2008
		US 2011263534 A1	27-10-2011
		WO 2007096703 A2	30-08-2007
WO 03045319 A2	05-06-2003	AU 2002360416 A1	10-06-2003
		CA 2465846 A1	05-06-2003
		EP 1453837 A2	08-09-2004
		JP 2005510534 A	21-04-2005
		US 2004063718 A1	01-04-2004
		WO 03045319 A2	05-06-2003
WO 2014194247 A1	04-12-2014	AU 2014273939 A1	26-11-2015
		CA 2910029 A1	04-12-2014
		WO 2014194247 A1	04-12-2014
WO 2014193722 A1	04-12-2014	CA 2913011 A1	04-12-2014
		US 2015366985 A1	24-12-2015
		WO 2014193722 A1	04-12-2014

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International Bureau

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2 December 2015 (02.12.2015)

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(26) Publication Language: English

(30) Priority Data:
62/087,213 3 December 2014 (03.12.2014) US

(71) Applicant *(for all designated States except AL, AT, BE, BG, CH, CN, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IN, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR)*: **GENENTECH, INC.** [US/US]; 1 DNA Way, South San Francisco, California 94080 (US).

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[Continued on next page]

(54) Title: ANTI-STAPHYLOCOCCUS AUREUS ANTIBODY RIFAMYCIN CONJUGATES AND USES THEREOF

(57) Abstract: The invention provides rF1 antibody antibiotic conjugates and methods of using same.

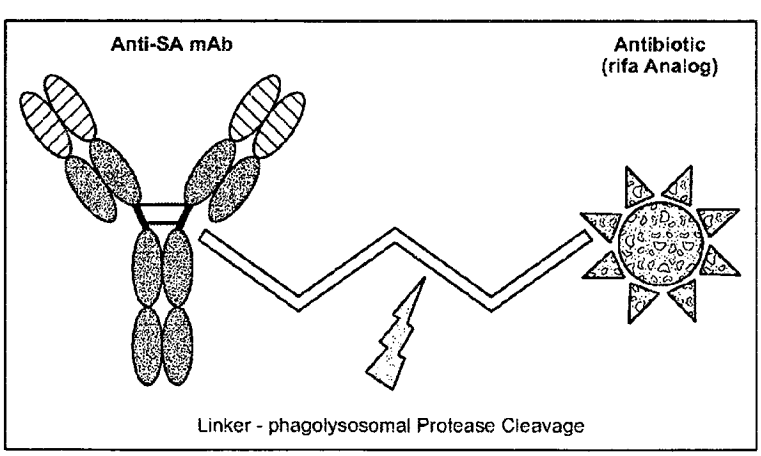

FIG. 2

WO 2016/090040 A9

(US). **XU, Min**; c/o Genentech, Inc., 1 DNA Way, South San Francisco, California 94080 (US).

(74) **Agents**: **AUSENHUS, Scott L.** et al.; Genentech, Inc., 1 DNA Way, MS-49, South San Francisco, California 94080 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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see Notice of 29 June 2017

ANTI-STAPHYLOCOCCUS AUREUS ANTIBODY RIFAMYCIN CONJUGATES AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 62/087,213, filed December 3, 2014, which is incorporated herein by reference in its entirety for all purposes.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on December 1, 2015, is named P32350WO_PCTSequenceListing.txt and is 26,747 bytes in size.

TECHNICAL FIELD

The invention relates to anti-Staphylococcus antibodies conjugated to rifamycin-type antibiotics and to use of the resultant antibody-antibiotic conjugates in the treatment of Staphylococcus infections.

BACKGROUND OF THE INVENTION

Staphylococcus aureus and *S. epidermidis* are successful human commensals that primarily colonize the nares and skin. *Staphylococcus aureus* (*S. aureus*; SA) can also invade a variety of tissues, leading to life-threatening infections; it is the leading cause of bacterial infections in humans worldwide. Recently emerged strains of *S. aureus* show increased virulence and enhanced ability to cause disease in otherwise healthy individuals. Over the last several decades, infection with *S. aureus* has become increasingly difficult to treat due to the emergence and rapid spread of methicillin-resistant *S. aureus* (MRSA) that is resistant to all known beta-lactam antibiotics (Boucher, H.W., *et al.* (2009) *Clin Infect Dis* 48, 1-12). Currently, the most prevalent and most virulent clinical strain of methicillin resistant *S. aureus* (MRSA) is USA300, which has the capacity to produce a large number of virulence factors and cause mortality in infected individuals (Chambers, HF and Deleo FR (2009) Nature Reviews Microbiology 7:629-641). The most serious infections such as endocarditis, osteomyelitis, necrotizing pneumonia and sepsis occur following dissemination of the bacteria into the bloodstream (Lowy, F.D. (1998) *N ENGL J MED* **339**, 520-532). *S. epidermidis*, which is

closely related to *S. aureus*, is often associated with hospital-acquired infections, and represents the most common source of infections on indwelling medical devices.

Important for staphylococcal adhesion to and successful colonization of host tissues, is a family of cell wall proteins, characterized by a large stretch of serine-aspartate dipeptide (SDR) repeats adjacent to an adhesive A-domain, that is present in staphylococci (Foster TJ, Hook M (1998) Trends Microbiol 6: 484-488). Such proteins important for adherence include clumping factor (Clf)A and ClfB (Foster TJ, supra). In addition to ClfA and ClfB, *S. aureus* also expresses three SDR-proteins, SdrC, SdrD and SdrE, which are organized in tandem in the genome. These proteins are also thought to be involved in tissue colonization, and elimination of any of them decreases bacterial virulence (Cheng AG, et al. (2009) FASEB Journal 23: 3393-3404). Three additional members of this family, SrdF, SdrG and SdrH, are present in most *S. epidermidis* strains (McCrea KW, et al. (2000) The serine-aspartate repeat (Sdr) protein family in *Staphylococcus epidermidis*. Microbiology 146 (Pt 7): 1535-1546). In each of these proteins, the SDR-region, which contains between 25 and 275 SD-dipeptide repeats (SEQ ID NO: 24), is located between the N-terminal ligand-binding A-domain and a C-terminal LPXTG-motif (SEQ ID NO: 25), which mediates anchoring to the cell wall by the transpeptidase sortase A. The function of the SDR-domain remains unknown, although it has been proposed to act as a cell wall spanning domain allowing exposure of the N terminal ligand binding sites of these proteins (Hartford O, et al. (1997) Mol Microbiol 25: 1065-1076).

It was found that the SDR-domains of all SDR-proteins of *S. aureus* and *S. epidermidis* are heavily glycosylated by two novel glycosyltransferases, SdgA and SdgB, which are responsible for glycosylation in two steps (Hazenbos et al. (2013) PLOS Pathogens 9 (10):1-18). These glycosylation events prevent degradation of these proteins by host proteases, thereby preserving bacterial host tissue interactions. Hazenbos et al. (2013) also showed that the SdgB-mediated glycosylation creates an immunodominant epitope for highly opsonic antibodies in humans. These antibodies account for a significant proportion of the total anti-staphylococcal IgG response.

Invasive MRSA infections are hard to treat, with a mortality rate of ~20% and are the leading cause of death by an infectious agent in the USA. Vancomycin, linezolid and daptomycin have thus become the few antibiotics of choice for treating invasive MRSA infections (Boucher, H., Miller, L.G. & Razonable, R.R. (2010) *Clin Infect Dis* 51 Suppl 2, S183-197). However, reduced susceptibility to vancomycin and cross-resistance to linezolid and daptomycin have already been reported in MRSA clinical strains (Nannini, E., Murray, B.E. & Arias, C.A. (2010) *Curr Opin Pharmacol* **10**, 516-521). Over time, the vancomycin dose

necessary to overcome resistance has crept upward to levels where nephrotoxicity occurs. Thus, mortality and morbidity from invasive MRSA infections remains high despite these antibiotics.

Investigations have revealed that *S. aureus* is able to invade and survive inside mammalian cells including the phagocytic cells that are responsible for bacterial clearance (Thwaites, G.E. & Gant, V. (2011) *Nat Rev Microbiol* **9**, 215-222); Rogers, D.E., Tompsett, R. (1952) *J. Exp. Med* **95**, 209-230); Gresham, H.D., *et al.* (2000) *J Immunol* **164**, 3713-3722); Kapral, F.A. & Shayegani, M.G. (1959) *J Exp Med* **110**, 123-138; Anwar, S., et al. (2009) *Clin Exp Immunol* **157**, 216-224); Fraunholz, M. & Sinha, B. (2012) *Front Cell Infect Microbiol* **2**, 43); Garzoni, C. & Kelley, W.L. (2011) *EMBO Mol Med* **3**, 115-117). *S. aureus* is taken up by host phagocytic cells, primarily neutrophils and macrophages, within minutes following intravenous infection (Rogers, D.E. (1956) *JEM* **103**, 713). While the majority of the bacteria are effectively killed by these cells, incomplete clearance of *S. aureus* inside blood borne phagocytes can allow these infected cells to act as "Trojan horses" for dissemination of the bacteria away from the initial site of infection. Indeed, patients with normal neutrophil counts may be more prone to disseminated disease than those with reduced neutrophil counts (Thwaites, G.E. & Gant, V. (2011) supra). Once delivered to the tissues, *S. aureus* can invade various non-phagocytic cell types, and intracellular *S. aureus* in tissues is associated with chronic or recurrent infections. Furthermore, exposure of intracellular bacteria to suboptimal antibiotic concentrations may encourage the emergence of antibiotic resistant strains, thus making this clinical problem more acute. Consistent with these observations, treatment of patients with invasive MRSA infections such as bacteremia or endocarditis with vancomycin or daptomycin was associated with failure rates greater than 50% (Kullar, R., Davis, S.L., Levine, D.P. & Rybak, M.J. Impact of vancomycin exposure on outcomes in patients with methicillin-resistant Staphylococcus aureus bacteremia: support for consensus guidelines suggested targets. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 52, 975-981 (2011); Fowler, V.G., Jr. et al. Daptomycin versus standard therapy for bacteremia and endocarditis caused by Staphylococcus aureus. *The New England journal of medicine* 355, 653-665 (2006); Yoon, Y.K., Kim, J.Y., Park, D.W., Sohn, J.W. & Kim, M.J. Predictors of persistent methicillin-resistant Staphylococcus aureus bacteraemia in patients treated with vancomycin. *The Journal of antimicrobial chemotherapy* 65:1015-1018 (2010)). Therefore, a more successful anti-staphylococcal therapy should include the elimination of intracellular bacteria.

Ansamycins are a class of antibiotics, including rifamycin, rifampin, rifampicin, rifabutin, rifapentine, rifalazil, ABI-1657, and analogs thereof, that inhibit bacterial RNA

polymerase and have exceptional potency against gram-positive and selective gram-negative bacteria (Rothstein, D.M., et al (2003) Expert Opin. Invest. Drugs 12(2):255-271; US 7342011; US 7271165).

Immunotherapies have been reported for preventing and treating *S. aureus* (including MRSA) infections. US2011/0262477 concerns uses of bacterial adhesion proteins Eap, Emp and AdsA as vaccines to stimulate immune response against MRSA. WO2000071585 describes isolated monoclonal antibodies reactive to specific *S. aureus* strain isolates. US20110059085A1 suggests an Ab-based strategy utilizing IgM Abs specific for one or more SA capsular antigens, although no actual antibodies were described.

Antibody-drug conjugates (ADC), also known as immunoconjugates, are targeted chemotherapeutic molecules which combine ideal properties of both antibodies and cytotoxic drugs by targeting potent cytotoxic drugs to antigen-expressing tumor cells (Teicher, B.A. (2009) *Curr. Cancer Drug Targets* 9:982-1004), thereby enhancing the therapeutic index by maximizing efficacy and minimizing off-target toxicity (Carter, P.J. and Senter P.D. (2008) *The Cancer J.*. 14(3):154-169; Chari, R.V. (2008) *Acc. Chem. Res.* 41:98-107. ADC comprise a targeting antibody covalently attached through a linker unit to a cytotoxic drug moiety. Immunoconjugates allow for the targeted delivery of a drug moiety to a tumor, and intracellular accumulation therein, where systemic administration of unconjugated drugs may result in unacceptable levels of toxicity to normal cells as well as the tumor cells sought to be eliminated (Polakis P. (2005) *Curr. Opin. Pharmacol.* 5:382-387).

Non-specific immunoglobulin-antibiotic conjugates are described that bind to the surface of target bacteria via the antibiotic for treating sepsis (US 5545721; US 6660267). Antibiotic-conjugated antibodies are described that have an antigen-binding portion specific for a bacterial antigen (such as SA capsular polysaccharide), but lack a constant region that reacts with a bacterial Fc-binding protein, e.g., staphylococcal protein A (US 7569677).

In view of the alarming rate of resistance of MRSA to conventional antibiotics and the resultant mortality and morbidity from invasive MRSA infections, there is a high unmet need for new therapeutics to treat S. aureus infections. The present invention satisfies this need and by providing compositions and methods that overcome the limitations of current therapeutic compositions as well as offer additional advantages that will be apparent from the detailed description below.

SUMMARY OF THE INVENTION

The present invention provides a unique therapeutic that includes the elimination of intracellular bacteria. The present invention demonstrates that such a therapeutic is efficacious in-vivo where conventional antibiotics like vancomycin fail.

The invention provides compositions referred to as "antibody-antibiotic conjugates," or "AAC") comprising an antibody conjugated by a covalent attachment to one or more rifamycin-type antibiotic moieties.

An aspect of the invention is an antibody-antibiotic conjugate compound comprising an rF1 antibody, covalently attached by a protease-cleavable, non-peptide linker to a rifamycin-type antibiotic.

An exemplary embodiment of the invention is an antibody-antibiotic conjugate having the formula:

$$Ab–(PML–abx)_p$$

wherein:

Ab is the rF1 antibody;

PML is the protease-cleavable, non-peptide linker having the formula:

$$–Str–PM–Y–$$

where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit;

abx is the rifamycin-type antibiotic; and

p is an integer from 1 to 8.

The antibody-antibiotic conjugate compounds of any of the preceding embodiments can comprise any one of the anti-SDR Abs and specifically rF1 antibodies described herein. These rF1 antibodies bind to Staphylococcus aureus. In exemplary rF1 antibodies, the Ab is a monoclonal antibody comprising a light (L) chain and a heavy (H) chain, the L chain comprising CDR L1, CDR L2, and CDR L3 and the H chain comprising CDR H1, CDR H2 and CDR H3, wherein the CDR H1, CDR H2 and CDR H3 and the CDR L1, CDR L2, and CDR L3 and comprise the amino acid sequences of the CDRs of each of Abs F1 (SEQ ID NO. 1-6), rF1 (SEQ ID NO. 1-5,7), rF1.v1 (SEQ ID NO. 1,8,3,4-6), respectively, as indicated in Tables 4A and 4B.

In some embodiments, the rF1 antibody comprises a heavy chain variable region (VH), wherein the VH comprises at least 95% sequence identity over the length of the VH region selected from the VH sequence of SEQ ID NO.13. The antibodies may further comprise a L chain variable region (VL) wherein the VL comprises at least 95% sequence identity over the length of the VL region selected from the VL sequence of SEQ ID NO.14 and SEQ ID NO.15, of antibodies rF1 and rF1.v6, respectively.

In specific embodiments, the rF1 antibody comprises L and H chain pairs as follows: a L chain comprising the sequence of SEQ ID NO. 9 paired with a H chain comprising the sequence of SEQ ID NO.10; L chain comprising the sequence of SEQ ID NO. 11paired with a H chain comprising the sequence of SEQ ID NO.10; a L chain comprising the sequence of SEQ ID NO. 11 paired with a H chain comprising the sequence of SEQ ID NO.12.

In any one of the preceding embodiments, the antibody may be an antigen-binding fragment lacking a Fc region. In some embodiments, the antibody is a F(ab) or F(ab')$_2$. In some embodiments, the antibody further comprises a heavy chain constant region and/or a light chain constant region, wherein the heavy chain constant region and/or the light chain constant region comprise one or more amino acids that are substituted with cysteine residues. In some embodiments, the heavy chain constant region comprises amino acid substitution A118C and/or S400C, and/or the light chain constant region comprises amino acid substitution V205C, wherein the numbering system is according to EU numbering.

In some embodiments of any of the antibodies described above, the antibody is not an IgM isotype. In some embodiments of any of the antibodies described above, the antibody is an IgG (e.g., IgG1, IgG2, IgG3, IgG4), IgE, IgD, or IgA (e.g., IgA1 or IgA2) isotype.

An exemplary embodiment of the invention is a pharmaceutical composition comprising the antibody-antibiotic conjugate compound, and a pharmaceutically acceptable carrier, glidant, diluent, or excipient.

Another aspect of the invention is a method of treating a bacterial infection comprising administering to an infected patient a therapeutically-effective amount of the antibody-antibiotic conjugate of any of the preceding embodiments. Another aspect of the invention is a method of treating a Staphylococcal infection in a patient comprising administering to the patient a therapeutically-effective amount of an antibody-antibiotic conjugate of the invention. In one embodiment, the patient is a human. In one embodiment the patient is infected with a *Staphylococcus aureus* and/or a *Staphylococcus epidermidis* infection. In some embodiments,

the patient has been diagnosed with a S. aureus infection. In some embodiments, treating the bacterial infection comprises reducing the bacterial load or counts.

Another aspect of the invention is a method of treating a Staphylococcal infection in an infected patient comprising administering to the patient a therapeutically-effective amount of an antibody-antibiotic conjugate of any one of the preceding embodiments. In one embodiment, the patient is a human. In one embodiment the bacterial infection is a *Staphylococcus aureus* infection. In some embodiments, the patient has been diagnosed with a S. aureus infection. In some embodiments, treating the bacterial infection comprises reducing the bacterial load or counts.

In one embodiment of any of the preceding methods of treatment, the is administered to patients where the bacterial infection including S. aureus has led to bacteremia. In specific embodiments the method is used to treat Staphylococcal endocarditis or osteomyelitis. In one embodiment, the antibody-antibiotic conjugate compound is administered to the infected patient at a dose in the range of about 50mg/kg to 100mg/kg.

Also provided is method of killing intracellular *S. aureus* in the cells of a *S. aureus* infected patient without killing the host cells by administering a rF1 antibiotic conjugate compound of any of the above embodiments. Another method is provided for killing persister Staphylococcal bacterial cells (e.g, *S. aureus*) in vivo by contacting the persister bacteria with an AAC of any of the preceding embodiments.

In another embodiment, the method of treatment further comprises administering a second therapeutic agent. In a further embodiment, the second therapeutic agent is an antibiotic including an antibiotic against Staph aureus in general or MRSA in particular.

In one embodiment, the second antibiotic administered in combination with the antibody-antibiotic conjugate compound of the invention is selected from the structural classes: (i) aminoglycosides; (ii) beta-lactams; (iii) macrolides/cyclic peptides; (iv) tetracyclines; (v) fluoroquinolines/fluoroquinolones; (vi) and oxazolidinones.

In one embodiment, the second antibiotic administered in combination with the antibody-antibiotic conjugate compound of the invention is selected from clindamycin, novobiocin, retapamulin, daptomycin, GSK-2140944, CG-400549, sitafloxacin, teicoplanin, triclosan, napthyridone, radezolid, doxorubicin, ampicillin, vancomycin, imipenem, doripenem, gemcitabine, dalbavancin, and azithromycin.

In some embodiments herein, the bacterial load in the infected patient has been reduced to an undetectable level after the treatment. In one embodiment, the patient's blood culture is negative after treatment as compared to a positive blood culture before treatment. In some

embodiments herein, the bacterial resistance in the subject is undetectable or low. In some embodiments herein, the patient is not responsive to treatment with methicillin or vancomycin.

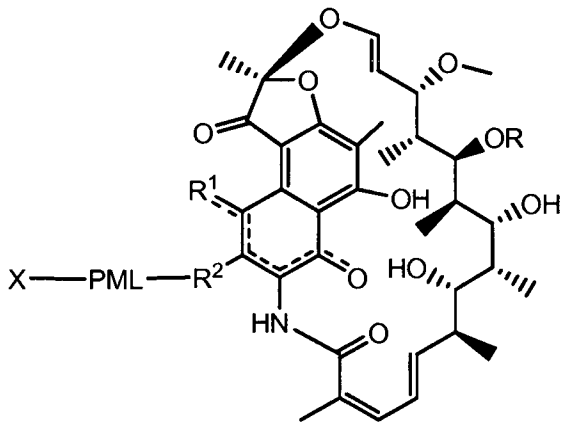
An exemplary embodiment of the invention is a process for making the antibody-antibiotic conjugate comprising conjugating a rifamycin-type antibiotic to an rF1 antibody.

An exemplary embodiment of the invention is a kit for treating a bacterial infection, comprising:

a) the pharmaceutical composition comprising the antibody-antibiotic conjugate compound, and a pharmaceutically acceptable carrier, glidant, diluent, or excipient; and

b) instructions for use.

An aspect of the invention is an antibiotic-linker intermediate having Formula II:



II

wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

R^1 is OH;

R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH;

PML is a protease-cleavable, non-peptide linker attached to R^2 or the fused heteroaryl or heterocyclyl formed by R^1 and R^2; and having the formula:

–Str–PM–Y–

where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit; and

X is a reactive functional group selected from maleimide, thiol, amino, bromide, bromoacetamido, iodoacetamido, p-toluenesulfonate, iodide, hydroxyl, carboxyl, pyridyl disulfide, and N-hydroxysuccinimide.

It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present invention. These and other aspects of the invention will become apparent to one of skill in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1F: Intracellular stores of MRSA are protected from vancomycin in vivo and in vitro. Figure 1A shows a schematic of the experimental design for generating free bacteria (planktonic) vs. intracellular bacteria. Four cohorts of mice were infected by intravenous injection with roughly equivalent doses of viable free bacteria or intracellular bacteria and selected groups were treated with vancomycin immediately after infection and then once per day (see Example 2). Figure 1B and Figure 1C show bacterial loads in kidney and brain, respectively of infected mice 4 days post infection. The dashed line indicates the limit of detection for the assay. Figure 1D shows that MRSA is protected from vancomycin when cultured on a monolayer of infectable cells. (ND = none detected). Figure 1E and Figure 1F show that MRSA is able to grow in the presence of vancomyicn when cultured on a monolayer of infectable cells. MRSA (free bacteria) was seeded in media, media + vancomycin, or media + vancomycin and plated on a monolayer of MG63 osteoblasts (Figure 1E) or Human Brain Microvascular Endothelial Cells (HBMEC, Figure 1F). Extracellular bacteria (free bacteria) grew well in media alone, but were killed by vancomycin. In wells containing a monolayer of mammalian cells (Intracellular + vanco) a fraction of the bacteria were protected from vancomycin during the first 8 hours after infection and were able to expand within the intracellular compartment over 24 hours. Error bars show standard deviation for triplicate wells.

Figure 2: shows the concept of an Antibody Antibiotic Conjugate (AAC). In one example, the AAC consists of an antibody directed against an epitope on the surface of S. aureus linked to a potent rifamycin-type antibiotic (e.g. Rifalog) via a linker that is cleaved by lysosomal proteases.

Figure 3 shows a possible mechanism of drug activation for antibody-antibiotic conjugates (AAC). AACs bind to extracellular bacteria via the antigen binding domain (Fab) of the antibody and promote uptake of the opsonized bacteria via Fc-mediated phagocytosis. The

linker is cleaved by lysosomal proteases such as cathepsin B. Following cleavage of the linker, the linker is hydrolyzed releasing free antibiotic inside the phagolysosome. The free antibiotic kills the opsonized and phagocytosed bacteria along with any previously internalized bacteria residing in the same compartment.

Figures 4A and 4B show aspects of serine-aspartate (SDR) proteins. Figure 4A shows alignment of SDR proteins revealed by mass-spectrometry from *S. aureus* and *S. epidermidis*. SDR-regions are indicated by hatches. The rF1 epitope is expressed in abundance since there are multiple SDR proteins on S. aureus and multiple epitopes per protein. Figure 4A discloses 'SDSDSDSD' as SEQ ID NO: 27. Figure 4B is a model showing the step-wise glycosylation of SDR proteins by SdgA and SdgB. First, SdgB appends GlcNAc moieties onto the SD-region on SDR proteins, followed by additional GlcNAc modification by SdgA. The epitope for mAb rF1 includes the SdgB-dependent GlcNAc moieties. Data suggests that rF1 binds to GlcNac and parts of the SD backbone. Figure 4B discloses 'SDSDSD' as SEQ ID NO: 28.

Figures 5A, 5B and 5C show mAb rF1 exhibits robust binding to and killing of *S. aureus* bacteria. (Figures A-C) Bacteria were preopsonized with huIgG1 mAbs rF1 (squares), 4675 anti-ClfA (triangles), or anti-herpes virus gD (circles). (Figure 5A): Binding of mAbs to WT (USA300-Δ*spa*) bacteria was assessed by flow cytometry, and expressed as mean fluorescent intensity (MFI). (Figure 5B): CFSE-labeled, preopsonized WT (USA300-Δ*spa*) bacteria were incubated with human PMN. Bacterial uptake was expressed as % of CFSE-positive PMN, after gating for CD11b-positive cells by flow cytometry. (Figure 5C): Preopsonized WT (USA300-Δ*spa*) bacteria were incubated with PMN to assess bacterial killing. Numbers of viable CFU per mL are representative of at least three experiments.

Figure 6 shows flow cytometry analysis of binding of rF1 to *S. aureus* from various infected tissues. Homogenized tissues were double stained with mAb rF1 (X-axis), and with anti-peptidoglycan mAb 702 to distinguish bacteria from tissue debris (Y-axis) (left panel; gate indicated by arrow), followed by gating of bacteria to generate histogram figures (see also, Hazenbos et al. (2013) PLOS Pathogens 9 (10):1-18, Fig. 1D).

Figure 7 shows binding of rF1 to various staphylococcal and non-staphylococcal Gram-positive bacterial species by flow cytometry (see also, Hazenbos et al. (2013) PLOS Pathogens 9 (10):1-18, Fig. 1E).

Figure 8 shows selection of a potent rifamycin-type antibiotic (rifalog) dimethylpipBOR for its ability to kill non-replicating MRSA.

Figure 9: Growth inhibition assay demonstrating that intact TAC (a form of AAC) does not kill planktonic bacteria unless the antibiotic is released by treatment with cathepsin B. TAC was incubated in buffer alone (open circles) or treated with cathepsin B (closed circles). The intact TAC was not able to prevent bacterial growth after overnight incubation. Pretreatment of the TAC with cathepsin B released sufficient antibiotic activity to prevent bacterial growth at .6 ug/mL of TAC, which is predicted to contain .006 ug/mL of antibiotic.

Figure 10 shows efficacy of the rF1-AACs in an *in vitro* macrophage assay, as described in Example 19.

Figures 11A and 11B show the efficacy of the rF1-AACs *in vivo* as described in Example 20. Treatment of S. aureus infected mice with rF1-AACs greatly reduced or eradicated bacterial counts in infected organs as compared to naked antibody. Figure 11A shows treatment with AAC containing 2 antibiotic molecules per antibody (AAR2) reduced bacterial load in the kidneys by approximately 30-fold and treatment with the AAC containing 4 antibiotic molecules per antibody (AAR4) reduced bacterial burdens by more than 30,000-fold. Figure 11B shows that treatment with AAC AAR2 reduced bacterial burdens in the heart by approximately 70-fold with 6 out of 8 mice having undetectable level of bacteria in hearts; treatment with the AAC AAR4 completely eradicated infection in hearts resulting in 8 out of 8 mice having undetectable levels of bacteria.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

Reference will now be made in detail to certain embodiments of the invention, examples of which are illustrated in the accompanying structures and formulas. While the invention will be described in conjunction with the enumerated embodiments, including methods, materials and examples, such description is non-limiting and the invention is intended to cover all alternatives, modifications, and equivalents, whether they are generally known, or incorporated herein. In the event that one or more of the incorporated literature, patents, and similar materials differs from or contradicts this application, including but not limited to defined terms, term usage, described techniques, or the like, this application controls. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. One skilled in the art will recognize many

methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. The present invention is in no way limited to the methods and materials described.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

I. GENERAL TECHNIQUES

The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* 3d edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; *Current Protocols in Molecular Biology* (F.M. Ausubel, et al. eds., (2003)); the series *Methods in Enzymology* (Academic Press, Inc.): *PCR 2: A Practical Approach* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) *Antibodies, A Laboratory Manual*, and *Animal Cell Culture* (R.I. Freshney, ed. (1987)); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., 1998) Academic Press; *Animal Cell Culture* (R.I. Freshney), ed., 1987); *Introduction to Cell and Tissue Culture* (J.P. Mather and P.E. Roberts, 1998) Plenum Press; *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-8) J. Wiley and Sons; *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991); *Short Protocols in Molecular Biology* (Wiley and Sons, 1999); *Immunobiology* (C.A. Janeway and P. Travers, 1997); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V.T. DeVita et al., eds., J.B. Lippincott Company, 1993).

The nomenclature used in this Application is based on IUPAC systematic nomenclature, unless indicated otherwise. Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs, and are consistent with: Singleton et al (1994) *Dictionary of Microbiology*

and Molecular Biology, 2nd Ed., J. Wiley & Sons, New York, NY; and Janeway, C., Travers, P., Walport, M., Shlomchik (2001) *Immunobiology*, 5th Ed., Garland Publishing, New York.

II. Definitions

Staphylococcus aureus is also referred to herein as Staph A or *S. aureus* in short. Likewise, *Staphylococcus epidermidis* is also referred herein as Staph E or *S. epidermidis*.

"Antibody Antibiotic Conjugate" or AAC is a compound composed of an antibody that is chemically linked to an antibiotic by a linker. The antibody binds an antigen or epitope on a bacterial surface, for example, a bacterial cell wall component. As used in this invention, the linker is a protease-cleavable, non-peptide linker that is designed to be cleaved by proteases, including cathepsin B, a lysosomal protease found in most mammalian cell types (Dubowchik et al (2002) Bioconj. Chem. 13:855-869). A diagram of the AAC with its 3 components is depicted in Figure 2. "THIOMAB™ Antibiotic Conjugate" or "TAC" is a form of AAC in which the antibody is chemically conjugated to a linker-antibiotic unit via one or more cysteines, generally a cysteine that is recombinantly engineered into the antibody at specific site(s) on the antibody to not interfere with the antigen binding function.

When indicating the number of substituents, the term "*one or more*" refers to the range from one substituent to the highest possible number of substitution, i.e. replacement of one hydrogen up to replacement of all hydrogens by substituents. The term "*substituent*" denotes an atom or a group of atoms replacing a hydrogen atom on the parent molecule. The term "*substituted*" denotes that a specified group bears one or more substituents. Where any group may carry multiple substituents and a variety of possible substituents is provided, the substituents are independently selected and need not to be the same. The term "*unsubstituted*" means that the specified group bears no substituents. The term "*optionally substituted*" means that the specified group is unsubstituted or substituted by one or more substituents, independently chosen from the group of possible substituents. When indicating the number of substituents, the term "*one or more*" means from one substituent to the highest possible number of substitution, i.e. replacement of one hydrogen up to replacement of all hydrogens by substituents.

The term "antibiotic" (abx or Abx) includes any molecule that specifically inhibits the growth of or kill micro-organisms, such as bacteria, but is non-lethal to the host at the concentration and dosing interval administered. In a specific aspect, an antibiotic is non-toxic to the host at the administered concentration and dosing intervals. Antibiotics effective against bacteria can be broadly classified as either bactericidal (*i.e.*, directly kills) or bacteriostatic (*i.e.*,

prevents division). Anti-bactericidal antibiotics can be further subclassified as narrow-spectrum or broad-spectrum. A broad-spectrum antibiotic is one effective against a broad range of bacteria including both Gram-positive and Gram-negative bacteria, in contrast to a narrow-spectrum antibiotic, which is effective against a smaller range or specific families of bacteria. Examples of antibiotics include: (i) aminoglycosides, *e.g.*, amikacin, gentamicin, kanamycin, neomycin, netilmicin, streptomycin, tobramycin, paromycin, (ii) ansamycins, e.g., geldanamycin, herbimycin, (iii) carbacephems, e.g., loracarbef, (iv), carbapenems, *e.g.,* ertapenum, doripenem, imipenem/cilastatin, meropenem, (v) cephalosporins (first generation), *e.g.,* cefadroxil, cefazolin, cefalotin, cefalexin, (vi) cephalosporins (second generation), e.g., ceflaclor, cefamandole, cefoxitin, cefprozil, cefuroxime, (vi) cephalosporins (third generation), e.g., cefixime, cefdinir, cefditoren, cefoperazone, cefotaxime, cefpodoxime, ceftazidime, ceftibuten, ceftizoxime, ceftriaxone, (vii) cephalosporins (fourth generation), e.g., cefepime, (viii), cephalosporins (fifth generation), *e.g.*, ceftobiprole, (ix) glycopeptides, e.g., teicoplanin, vancomycin, (x) macrolides, *e.g.*, axithromycin, clarithromycin, dirithromycine, erythromycin, roxithromycin, troleandomycin, telithromycin, spectinomycin, (xi) monobactams, *e.g.*, axtreonam, (xii) penicilins, *e.g.*, amoxicillin, ampicillin, axlocillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, meticillin, nafcilin, oxacillin, penicillin, peperacillin, ticarcillin, (xiii) antibiotic polypeptides, *e.g.*, bacitracin, colistin, polymyxin B, (xiv) quinolones, e.g., ciprofloxacin, enoxacin, gatifloxacin, levofloxacin, lemefloxacin, moxifloxacin, norfloxacin, orfloxacin, trovafloxacin, (xv) sulfonamides, *e.g.*, mafenide, prontosil, sulfacetamide, sulfamethizole, sulfanilamide, sulfasalazine, sulfisoxazole, trimethoprim, trimethoprim-sulfamethoxazole (TMP-SMX), (xvi) tetracyclines, *e.g.*, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline and (xvii) others such as arspenamine, chloramphenicol, clindamycin, lincomycin, ethambutol, fosfomycin, fusidic acid, furazolidone, isoniazid, linezolid, metronidazole, mupirocin, nitrofurantoin, platensimycin, pyrazinamide, quinupristin/dalfopristin, rifampin/rifampicin or tinidazole.

The term "methicillin-resistant *Staphylococcus aureus*" (MRSA), alternatively known as multidrug resistant *Staphylococcus aureus* or *oxacillin-resistant Staphylococcus aureus* (ORSA), refers to any strain of *Staphylococcus aureus* that is resistant to beta-lactam antibiotics, which in include the penicillins (*e.g.*, methicillin, dicloxacillin, nafcillin, oxacillin, *etc.*) and the cephalosporins. "Methicillin-sensitive *Staphylococcus aureus"(MSSA)* refers to any strain of *Staphylococcus aureus* that is sensitive to beta-lactam antibiotics.

The term "minimum inhibitory concentration" ("MIC") refers to the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight

incubation. Assay for determining MIC are known. One method is as described in the Example section below.

The terms "anti-Staph a antibody" and "an antibody that binds to Staph a" refer to an antibody that is capable of binding an antigen on *Staphylococcus aureus* ("S. aureus") with sufficient affinity such that the antibody is useful as a diagnostic and/or therapeutic agent in targeting S. aureus. In one embodiment, the extent of binding of an anti-Staph a antibody to an unrelated, non-Staph a protein is less than about 10% of the binding of the antibody to MRSA as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, an antibody that binds to Staph a has a dissociation constant (Kd) of $\leq$ 1μM, $\leq$ 100 nM, $\leq$ 10 nM, , $\leq$ 5 Nm, , $\leq$ 4 nM, , $\leq$ 3 nM, , $\leq$ 2 nM, $\leq$ 1 nM, $\leq$ 0.1 nM, $\leq$ 0.01 nM, or $\leq$ 0.001 nM (e.g., 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-13} M, e.g., from 10^{-9} M to 10^{-13} M). In certain embodiments, an anti-Staph a antibody binds to an epitope of Staph a that is conserved among Staph from different species. An anti-Staph antibody herein will refer to an antibody that binds to at least one more Staphylococcal species in addition S. Aureus.

"SDR" refers to serine-aspartate repeat; SDRs are are present in a family of cell wall proteins, characterized by a large stretch of serine-aspartate dipeptide repeats adjacent to an adhesive A-domain, that is present in staphylococci (Foster TJ, Hook M (1998) Trends Microbiol 6: 484-488). Such proteins involved in adherence include clumping factor (Clf)A and ClfB. In addition to ClfA and ClfB, *S. aureus* also expresses three SDR-proteins, SdrC, SdrD and SdrE, Three additional members of this family, SrdF, SdrG and SdrH, are present in most *S. epidermidis* strains (McCrea KW, et al. (2000) The serine-aspartate repeat (Sdr) protein family in *Staphylococcus epidermidis*. Microbiology 146 (Pt 7): 1535-1546). In each of these proteins, the SDR-region, which contains between 25 and 275 SD-dipeptide repeats (SEQ ID NO: 24), is located between the N-terminal ligand-binding A-domain and a C-terminal LPXTG-motif (SEQ ID NO: 25),

The antibody designated "F1" has heavy chain and light chain variable domain sequences as depicted in Figure 1 of US 8,617,556, which is incorporated herein by reference in its entirety. The CDR sequences of F1, which in particular contribute to the antigen-binding properties of F1, are also depicted in Figure 1. Antibody F1 is fully human, is capable of specifically binding *Staphylococcus* species such as *S. aureus* and *S. epidermidis* . Importantly, antibody F1 is capable of binding whole bacteria *in vivo* as well as *in vitro*. Furthermore, antibody F1 is capable of binding to bacteria that have been grown in infected tissue of, for example, an animal. Recombinantly produced F1 is herein also called "rF1". rF1 (and F1) antibody is an anti-SDR monoclonal Ab. The epitope for mAb rF1 includes the SdgB-dependent GlcNAc moieties. Data suggests that rF1 binds to

GlcNac and parts of the SD backbone. "rF1 antibody" as used herein encompasses the F1 antibody, the rF1 antibody as well as all variants of rF1containing amino acid alterations relative to rF1. The amino acid sequences of the rF1 and variant antibodies are provided below.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies, polyclonal antibodies, dimers, multimers, multispecific antibodies (e.g., bispecific antibodies), and antigen binding antibody fragments thereof, (Miller et al (2003) J. of Immunology 170:4854-4861). Antibodies may be murine, human, humanized, chimeric, or derived from other species. An antibody is a protein generated by the immune system that is capable of recognizing and binding to a specific antigen (Janeway, C., Travers, P., Walport, M., Shlomchik (2001) Immuno Biology, 5th Ed., Garland Publishing, New York). A target antigen generally has numerous binding sites, also called epitopes, recognized by CDRs on multiple antibodies. Each antibody that specifically binds to a different epitope has a different structure. Thus, one antigen may be recognized and bound by more than one corresponding antibody. An antibody includes a full-length immunoglobulin molecule or an immunologically active portion of a full-length immunoglobulin molecule, i.e., a molecule that contains an antigen binding site that immunospecifically binds an antigen of a target of interest or part thereof, such targets including but not limited to, cancer cell or cells that produce autoimmune antibodies associated with an autoimmune disease, an infected cell or a microorganism such as a bacterium. The immunoglobulin (Ig) disclosed herein can be of any isotype except IgM (e.g., IgG, IgE, IgD, and IgA) and subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The immunoglobulins can be derived from any species. In one aspect, the Ig is of human, murine, or rabbit origin. In a specific embodiment, the Ig is of human origin.

The "class" of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively.

"Native antibodies" refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant

domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

The terms "full length antibody," "intact antibody," and "whole antibody" are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure or having heavy chains that contain an Fc region as defined herein.

An "antigen-binding fragment" of an antibody refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, $F(ab')_2$; diabodies; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation (e.g., natural variation in glycosylation), such variants generally being present in minor amounts. One such possible variant for IgG1 antibodies is the cleavage of the C-terminal lysine (K) of the heavy chain constant region. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein. In addition to their specificity, the monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies.

The term "chimeric antibody" refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

A "humanized antibody" refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A "humanized form" of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization.

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). (See, e.g., Kindt et al. Kuby Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007).) A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. See, e.g., Portolano et al., J. Immunol. 150:880-887 (1993); Clarkson et al., Nature 352:624-628 (1991).

The term "hypervariable region," "HVR," or "HV," when used herein refers to the regions of an antibody variable domain which are hypervariable in sequence ("complementarity determining regions" or "CDRs") and/or form structurally defined loops and/or contain the antigen-contacting residues ("antigen contacts"). Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, e.g., Xu et al., Immunity 13:37-45 (2000); Johnson and Wu, in Methods in Molecular Biology 248:1-25 (Lo, ed., Human Press, Totowa,

NJ, 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain (Hamers-Casterman et al., (1993) Nature 363:446-448; Sheriff et al., (1996) Nature Struct. Biol. 3:733-736).

A number of HVR delineations are in use and are encompassed herein. The Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Chothia refers instead to the location of the structural loops (Chothia and Lesk, (1987) J. Mol. Biol. 196:901-917). For antigen contacts, refer to MacCallum et al. *J. Mol. Biol.* 262: 732-745 (1996). The AbM HVRs represent a compromise between the Kabat HVRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software. The "contact" HVRs are based on an analysis of the available complex crystal structures. The residues from each of these HVRs are noted below.

Loop	Kabat	AbM	Chothia	Contact
L1	L24-L34	L24-L34	L26-L32	L30-L36
L2	L50-L56	L50-L56	L50-L52	L46-L55
L3	L89-L97	L89-L97	L91-L96	L89-L96
H1	H31-H35B	H26-H35B	H26-H32	H30-H35B (Kabat numbering)
H1	H31-H35	H26-H35	H26-H32	H30-H35 (Chothia numbering)
H2	H50-H65	H50-H58	H53-H55	H47-H58
H3	H95-H102	H95-H102	H96-H101	H93-H101

HVRs may comprise "extended HVRs" as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2) and 89-97 or 89-96 (L3) in the VL and 26-35 (H1), 50-65 or 49-65 (H2) and 93-102, 94-102, or 95-102 (H3) in the VH. Unless otherwise indicated, HVR residues, CDR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al., *supra*.

The expression "variable-domain residue-numbering as in Kabat" or "amino-acid-position numbering as in Kabat," and variations thereof, refers to the numbering system used for heavy-chain variable domains or light-chain variable domains of the compilation of antibodies in Kabat et al., supra. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FR or HVR of the variable domain. For example, a heavy-chain variable domain may include a

single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (e.g. residues 82a, 82b, and 82c, etc. according to Kabat) after heavy-chain FR residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

"Framework" or "FR" refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

An "acceptor human framework" for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments, the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al., supra. In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al., supra.

The term "Fc region" herein is used to define a C-terminal region of an immunoglobulin heavy chain. The term includes native-sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy-chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system - also called the EU index, as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National

Institutes of Health, Bethesda, MD, 1991) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue. The term "Fc receptor" or "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus. Guyer et al., J. Immunol. 117: 587 (1976) and Kim et al., J. Immunol. 24: 249 (1994). Methods of measuring binding to FcRn are known (see, e.g., Ghetie and Ward, Immunol. Today 18: (12): 592-8 (1997); Ghetie et al., Nature Biotechnology 15 (7): 637-40 (1997); Hinton et al., J. Biol. Chem. 279(8): 6213-6 (2004); WO 2004/92219 (Hinton et al.). Binding to FcRn in vivo and serum half-life of human FcRn high-affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides having a variant Fc region are administered. WO 2004/42072 (Presta) describes antibody variants which improved or diminished binding to FcRs. See also, e.g., Shields et al., J. Biol. Chem. 9(2): 6591-6604 (2001).

An "affinity matured" antibody refers to an antibody with one or more alterations in one or more hypervariable regions (HVRs), compared to a parent antibody which does not possess such alterations, such alterations resulting in an improvement in the affinity of the antibody for antigen.

The term "epitope" refers to the particular site on an antigen molecule to which an antibody binds.

An "antibody that binds to the same epitope" as a reference antibody refers to an antibody that blocks binding of the reference antibody to its antigen in a competition assay by 50% or more, and conversely, the reference antibody blocks binding of the antibody to its antigen in a competition assay by 50% or more. An exemplary competition assay is provided herein.

A "naked antibody" refers to an antibody that is not conjugated to a heterologous moiety (e.g., a cytotoxic moiety) or radiolabel. The naked antibody may be present in a pharmaceutical formulation.

"Effector functions" refer to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding;

antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

"Antibody-dependent cell-mediated cytotoxicity" or ADCC refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., natural killer (NK) cells, neutrophils and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The antibodies "arm" the cytotoxic cells and are required for killing of the target cell by this mechanism. The primary cells for mediating ADCC, NK cells, express Fcγ(gamma)RIII only, whereas monocytes express Fcγ(gamma)RI, Fcγ(gamma)RII and Fcγ(gamma)RIII. Fc expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, Annu. Rev. Immunol. 9: 457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in US 5,500,362 or US 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and natural killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes et al., PNAS USA 95:652-656 (1998).

"Phagocytosis" refers to a process by which a pathogen is engulfed or internalized by a host cell (e.g., macrophage or neutrophil). Phagocytes mediate phagocytosis by three pathways: (i) direct cell surface receptors (for example, lectins, integrins and scavenger receptors) (ii) complement enhanced - using complement receptors (including CRI, receptor for C3b, CR3 and CR4) to bind and ingest complement opsonized pathogens, and (iii) antibody enhanced - using Fc Receptors (including FcγgammaRI, FcγgammaRIIA and FcγgammaRIIIA) to bind antibody opsonized particles which then become internalized and fuse with lysosomes to become phagolysosomes. In the present invention, it is believed that pathway (iii) plays a significant role in the delivery of the anti-MRSA AAC therapeutics to infected leukocytes, e.g., neutrophils and macrophages (Phagocytosis of Microbes: complexity in Action by D. Underhill and A Ozinsky. (2002) Annual Review of Immunology, Vol 20:825).

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass) which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g., as described in Gazzano-Santoro et al., J. Immunol. Methods 202: 163 (1996), may be performed.

The carbohydrate attached to the Fc region may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, e.g., Wright et al. (1997) TIBTECH 15:26-32. The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the "stem" of the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in an IgG may be made in order to create IgGs with certain additionally improved properties. For example, antibody modifications are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. Such modifications may have improved ADCC function. See, e.g. US 2003/0157108 (Presta, L.); US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Examples of publications related to "defucosylated" or "fucose-deficient" antibody modifications include: US 2003/0157108; WO 2000/61739; WO 2001/29246; US 2003/0115614; US 2002/0164328; US 2004/0093621; US 2004/0132140; US 2004/0110704; US 2004/0110282; US 2004/0109865; WO 2003/085119; WO 2003/084570; WO 2005/035586; WO 2005/035778; WO2005/053742; WO2002/031140; Okazaki et al., J. Mol. Biol. 336: 1239-1249 (2004); Yamane-Ohnuki et al. Biotech. Bioeng. 87: 614 (2004). Examples of cell lines capable of producing defucosylated antibodies include Lee 13 CHO cells deficient in protein fucosylation (Ripka et al. Arch. Biochem. Biophys. 249:533-545 (1986); US Pat. Appl. Pub. No. 2003/0157108 Al, Presta, L; and WO 2004/056312 Al, Adams et al., especially at Example 11), and knockout cell lines, such as alpha- 1,6-fucosyltransferase gene, FUT8, knockout CHO cells (see, e.g., Yamane-Ohnuki et al., Biotech. Bioeng. 87: 614 (2004); Kanda, Y. et al, Biotechnol. Bioeng., 94(4):680-688 (2006); and WO2003/085107).

An "isolated antibody" is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman et al., J. Chromatogr. B 848:79-87 (2007).

An "isolated nucleic acid" refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

"Isolated nucleic acid encoding a rF1antibody" refers to one or more nucleic acid molecules encoding antibody heavy and light chains, including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

As use herein, the term "specifically binds to" or is "specific for" refers to measurable and reproducible interactions such as binding between a target and an antibody, which is determinative of the presence of the target in the presence of a heterogeneous population of molecules including biological molecules. For example, an antibody that specifically binds to a target (which can be an epitope) is an antibody that binds this target with greater affinity, avidity, more readily, and/or with greater duration than it binds to other targets. In one embodiment, the extent of binding of an antibody to a target unrelated to rF1 is less than about 10% of the binding of the antibody to the target as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, an antibody that specifically binds to rF1 has a dissociation constant (Kd) of $\leq 1\mu M$, ≤ 100 nM, ≤ 10 nM, ≤ 1 nM, or ≤ 0.1 nM. In certain embodiments, an antibody specifically binds to an epitope on that is conserved from different species. In another embodiment, specific binding can include, but does not require exclusive binding.

"Binding affinity" generally refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity that reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present invention. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

In one embodiment, the "Kd" or "Kd value" according to this invention is measured by a radiolabeled antigen-binding assay (RIA) performed with the Fab version of an antibody of interest and its antigen as described by the following assay. Solution-binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, e.g., Chen et al., (1999) J. Mol. Biol. 293:865-881). To

establish conditions for the assay, microtiter plates (DYNEX Technologies, Inc.) are coated overnight with 5 μg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23°C). In a non-adsorbent plate (Nunc #269620), 100 pM or 26 pM [^{125}I]-antigen are mixed with serial dilutions of a Fab of interest (e.g., consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta et al., Cancer Res. 57:4593-4599 (1997)). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (e.g., about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (e.g., for one hour). The solution is then removed and the plate washed eight times with 0.1% TWEEN-20™ surfactant in PBS. When the plates have dried, 150 μl/well of scintillant (MICROSCINT-20™; Packard) is added, and the plates are counted on a TOPCOUNT™ gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

According to another embodiment, the Kd is measured by using surface-plasmon resonance assays using a BIACORE®-2000 or a BIACORE®-3000 instrument (BIAcore, Inc., Piscataway, NJ) at 25°C with immobilized antigen CM5 chips at ~10 response units (RU). Briefly, carboxymethylated dextran biosensor chips (CM5, BIAcore Inc.) are activated with N-ethyl-N'- (3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 μg/ml (~0.2 μM) before injection at a flow rate of 5 μl/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% TWEEN 20™ surfactant (PBST) at 25°C at a flow rate of approximately 25 μl/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIAcore® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on}. See, e.g., Chen et al., J. Mol. Biol. 293:865-881 (1999). If the on-rate exceeds 10^6 M^{-1} s^{-1} by the surface-plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence-emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25 °C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow-equipped spectrophotometer (Aviv

Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

An "on-rate," "rate of association," "association rate," or "k_{on}" according to this invention can also be determined as described above using a BIACORE®-2000 or a BIACORE®-3000 system (BIAcore, Inc., Piscataway, NJ).

The terms "host cell," "host cell line," and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

The term "vector," as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors".

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the

source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y, where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described.

The term "rifamycin-type antibiotic" means the class or group of antibiotics having the structure of, or similar structure to, rifamycin.

The term "rifalazil-type antibiotic" means the class or group of antibiotics having the structure of, or similar structure to, rifalazil.

When indicating the number of substituents, the term "one or more" refers to the range from one substituent to the highest possible number of substitution, i.e. replacement of one hydrogen up to replacement of all hydrogens by substituents. The term "substituent" denotes an atom or a group of atoms replacing a hydrogen atom on the parent molecule. The term "substituted" denotes that a specified group bears one or more substituents. Where any group may carry multiple substituents and a variety of possible substituents is provided, the substituents are independently selected and need not to be the same. The term "unsubstituted" means that the specified group bears no substituents. The term "optionally substituted" means that the specified group is unsubstituted or substituted by one or more substituents, independently chosen from the group of possible substituents. When indicating the number of substituents, the term "one or more" means from one substituent to the highest possible number of substitution, i.e. replacement of one hydrogen up to replacement of all hydrogens by substituents.

The term "alkyl" as used herein refers to a saturated linear or branched-chain monovalent hydrocarbon radical of one to twelve carbon atoms (C_1–C_{12}), wherein the alkyl radical may be

optionally substituted independently with one or more substituents described below. In another embodiment, an alkyl radical is one to eight carbon atoms (C_1–C_8), or one to six carbon atoms (C_1–C_6). Examples of alkyl groups include, but are not limited to, methyl (Me, -CH_3), ethyl (Et, -CH_2CH_3), 1-propyl (n-Pr, n-propyl, -$CH_2CH_2CH_3$), 2-propyl (i-Pr, i-propyl, -$CH(CH_3)_2$), 1-butyl (n-Bu, n-butyl, -$CH_2CH_2CH_2CH_3$), 2-methyl-1-propyl (i-Bu, i-butyl, -$CH_2CH(CH_3)_2$), 2-butyl (s-Bu, s-butyl, -$CH(CH_3)CH_2CH_3$), 2-methyl-2-propyl (t-Bu, t-butyl, -$C(CH_3)_3$), 1-pentyl (n-pentyl, -$CH_2CH_2CH_2CH_2CH_3$), 2-pentyl (-$CH(CH_3)CH_2CH_2CH_3$), 3-pentyl (-$CH(CH_2CH_3)_2$), 2-methyl-2-butyl (-$C(CH_3)_2CH_2CH_3$), 3-methyl-2-butyl (-$CH(CH_3)CH(CH_3)_2$), 3-methyl-1-butyl (-$CH_2CH_2CH(CH_3)_2$), 2-methyl-1-butyl (-$CH_2CH(CH_3)CH_2CH_3$), 1-hexyl (-$CH_2CH_2CH_2CH_2CH_2CH_3$), 2-hexyl (-$CH(CH_3)CH_2CH_2CH_2CH_3$), 3-hexyl (-$CH(CH_2CH_3)(CH_2CH_2CH_3)$), 2-methyl-2-pentyl (-$C(CH_3)_2CH_2CH_2CH_3$), 3-methyl-2-pentyl (-$CH(CH_3)CH(CH_3)CH_2CH_3$), 4-methyl-2-pentyl (-$CH(CH_3)CH_2CH(CH_3)_2$), 3-methyl-3-pentyl (-$C(CH_3)(CH_2CH_3)_2$), 2-methyl-3-pentyl (-$CH(CH_2CH_3)CH(CH_3)_2$), 2,3-dimethyl-2-butyl (-$C(CH_3)_2CH(CH_3)_2$), 3,3-dimethyl-2-butyl (-$CH(CH_3)C(CH_3)_3$, 1-heptyl, 1-octyl, and the like.

The term "alkylene" as used herein refers to a saturated linear or branched-chain divalent hydrocarbon radical of one to twelve carbon atoms (C_1–C_{12}), wherein the alkylene radical may be optionally substituted independently with one or more substituents described below. In another embodiment, an alkylene radical is one to eight carbon atoms (C_1–C_8), or one to six carbon atoms (C_1–C_6). Examples of alkylene groups include, but are not limited to, methylene (-CH_2-), ethylene (–CH_2CH_2–), propylene (–$CH_2CH_2CH_2$–), and the like.

The term "alkenyl" refers to linear or branched-chain monovalent hydrocarbon radical of two to eight carbon atoms (C_2–C_8) with at least one site of unsaturation, i.e., a carbon-carbon, sp^2 double bond, wherein the alkenyl radical may be optionally substituted independently with one or more substituents described herein, and includes radicals having "cis" and "trans" orientations, or alternatively, "E" and "Z" orientations. Examples include, but are not limited to, ethylenyl or vinyl (–CH=CH_2), allyl (–CH_2CH=CH_2), and the like.

The term "alkenylene" refers to linear or branched-chain divalent hydrocarbon radical of two to eight carbon atoms (C_2–C_8) with at least one site of unsaturation, i.e., a carbon-carbon, sp^2 double bond, wherein the alkenylene radical may be optionally substituted independently with one or more substituents described herein, and includes radicals having "cis" and "trans" orientations, or alternatively, "E" and "Z" orientations. Examples include, but are not limited to, ethylenylene or vinylene (–CH=CH–), allyl (–CH_2CH=CH–), and the like.

The term "alkynyl" refers to a linear or branched monovalent hydrocarbon radical of two to eight carbon atoms (C_2–C_8) with at least one site of unsaturation, i.e., a carbon-carbon, sp triple bond, wherein the alkynyl radical may be optionally substituted independently with one or more substituents described herein. Examples include, but are not limited to, ethynyl (-C≡CH), propynyl (propargyl, -CH_2C≡CH), and the like.

The term "alkynylene" refers to a linear or branched divalent hydrocarbon radical of two to eight carbon atoms (C_2–C_8) with at least one site of unsaturation, i.e., a carbon-carbon, sp triple bond, wherein the alkynylene radical may be optionally substituted independently with one or more substituents described herein. Examples include, but are not limited to, ethynylene (-C≡C-), propynylene (propargylene, -CH_2C≡C-), and the like.

The terms "carbocycle", "carbocyclyl", "carbocyclic ring" and "cycloalkyl" refer to a monovalent non-aromatic, saturated or partially unsaturated ring having 3 to 12 carbon atoms (C_3–C_{12}) as a monocyclic ring or 7 to 12 carbon atoms as a bicyclic ring. Bicyclic carbocycles having 7 to 12 atoms can be arranged, for example, as a bicyclo [4,5], [5,5], [5,6] or [6,6] system, and bicyclic carbocycles having 9 or 10 ring atoms can be arranged as a bicyclo [5,6] or [6,6] system, or as bridged systems such as bicyclo[2.2.1]heptane, bicyclo[2.2.2]octane and bicyclo[3.2.2]nonane. Spiro moieties are also included within the scope of this definition. Examples of monocyclic carbocycles include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, 1-cyclopent-1-enyl, 1-cyclopent-2-enyl, 1-cyclopent-3-enyl, cyclohexyl, 1-cyclohex-1-enyl, 1-cyclohex-2-enyl, 1-cyclohex-3-enyl, cyclohexadienyl, cycloheptyl, cyclooctyl, cyclononyl, cyclodecyl, cycloundecyl, cyclododecyl, and the like. Carbocyclyl groups are optionally substituted independently with one or more substituents described herein.

"Aryl" means a monovalent aromatic hydrocarbon radical of 6-20 carbon atoms (C_6–C_{20}) derived by the removal of one hydrogen atom from a single carbon atom of a parent aromatic ring system. Some aryl groups are represented in the exemplary structures as "Ar". Aryl includes bicyclic radicals comprising an aromatic ring fused to a saturated, partially unsaturated ring, or aromatic carbocyclic ring. Typical aryl groups include, but are not limited to, radicals derived from benzene (phenyl), substituted benzenes, naphthalene, anthracene, biphenyl, indenyl, indanyl, 1,2-dihydronaphthalene, 1,2,3,4-tetrahydronaphthyl, and the like. Aryl groups are optionally substituted independently with one or more substituents described herein.

"Arylene" means a divalent aromatic hydrocarbon radical of 6-20 carbon atoms (C_6–C_{20}) derived by the removal of two hydrogen atom from a two carbon atoms of a parent aromatic ring system. Some arylene groups are represented in the exemplary structures as "Ar". Arylene

includes bicyclic radicals comprising an aromatic ring fused to a saturated, partially unsaturated ring, or aromatic carbocyclic ring. Typical arylene groups include, but are not limited to, radicals derived from benzene (phenylene), substituted benzenes, naphthalene, anthracene, biphenylene, indenylene, indanylene, 1,2-dihydronaphthalene, 1,2,3,4-tetrahydronaphthyl, and the like. Arylene groups are optionally substituted with one or more substituents described herein.

The terms "heterocycle," "heterocyclyl" and "heterocyclic ring" are used interchangeably herein and refer to a saturated or a partially unsaturated (i.e., having one or more double and/or triple bonds within the ring) carbocyclic radical of 3 to about 20 ring atoms in which at least one ring atom is a heteroatom selected from nitrogen, oxygen, phosphorus and sulfur, the remaining ring atoms being C, where one or more ring atoms is optionally substituted independently with one or more substituents described below. A heterocycle may be a monocycle having 3 to 7 ring members (2 to 6 carbon atoms and 1 to 4 heteroatoms selected from N, O, P, and S) or a bicycle having 7 to 10 ring members (4 to 9 carbon atoms and 1 to 6 heteroatoms selected from N, O, P, and S), for example: a bicyclo [4,5], [5,5], [5,6], or [6,6] system. Heterocycles are described in Paquette, Leo A.; "Principles of Modern Heterocyclic Chemistry" (W.A. Benjamin, New York, 1968), particularly Chapters 1, 3, 4, 6, 7, and 9; "The Chemistry of Heterocyclic Compounds, A series of Monographs" (John Wiley & Sons, New York, 1950 to present), in particular Volumes 13, 14, 16, 19, and 28; and J. Am. Chem. Soc. (1960) 82:5566. "Heterocyclyl" also includes radicals where heterocycle radicals are fused with a saturated, partially unsaturated ring, or aromatic carbocyclic or heterocyclic ring. Examples of heterocyclic rings include, but are not limited to, morpholin-4-yl, piperidin-1-yl, piperazinyl, piperazin-4-yl-2-one, piperazin-4-yl-3-one, pyrrolidin-1-yl, thiomorpholin-4-yl, S-dioxothiomorpholin-4-yl, azocan-1-yl, azetidin-1-yl, octahydropyrido[1,2-a]pyrazin-2-yl, [1,4]diazepan-1-yl, pyrrolidinyl, tetrahydrofuranyl, dihydrofuranyl, tetrahydrothienyl, tetrahydropyranyl, dihydropyranyl, tetrahydrothiopyranyl, piperidino, morpholino, thiomorpholino, thioxanyl, piperazinyl, homopiperazinyl, azetidinyl, oxetanyl, thietanyl, homopiperidinyl, oxepanyl, thiepanyl, oxazepinyl, diazepinyl, thiazepinyl, 2-pyrrolinyl, 3-pyrrolinyl, indolinyl, 2H-pyranyl, 4H-pyranyl, dioxanyl, 1,3-dioxolanyl, pyrazolinyl, dithianyl, dithiolanyl, dihydropyranyl, dihydrothienyl, dihydrofuranyl, pyrazolidinylimidazolinyl, imidazolidinyl, 3-azabicyco[3.1.0]hexanyl, 3-azabicyclo[4.1.0]heptanyl, azabicyclo[2.2.2]hexanyl, 3H-indolyl quinolizinyl and N-pyridyl ureas. Spiro moieties are also included within the scope of this definition. Examples of a heterocyclic group wherein 2 ring atoms are substituted with oxo (=O) moieties are

pyrimidinonyl and 1,1-dioxo-thiomorpholinyl. The heterocycle groups herein are optionally substituted independently with one or more substituents described herein.

The term "heteroaryl" refers to a monovalent aromatic radical of 5-, 6-, or 7-membered rings, and includes fused ring systems (at least one of which is aromatic) of 5-20 atoms, containing one or more heteroatoms independently selected from nitrogen, oxygen, and sulfur. Examples of heteroaryl groups are pyridinyl (including, for example, 2-hydroxypyridinyl), imidazolyl, imidazopyridinyl, pyrimidinyl (including, for example, 4-hydroxypyrimidinyl), pyrazolyl, triazolyl, pyrazinyl, tetrazolyl, furyl, thienyl, isoxazolyl, thiazolyl, oxadiazolyl, oxazolyl, isothiazolyl, pyrrolyl, quinolinyl, isoquinolinyl, tetrahydroisoquinolinyl, indolyl, benzimidazolyl, benzofuranyl, cinnolinyl, indazolyl, indolizinyl, phthalazinyl, pyridazinyl, triazinyl, isoindolyl, pteridinyl, purinyl, oxadiazolyl, triazolyl, thiadiazolyl, thiadiazolyl, furazanyl, benzofurazanyl, benzothiophenyl, benzothiazolyl, benzoxazolyl, quinazolinyl, quinoxalinyl, naphthyridinyl, and furopyridinyl. Heteroaryl groups are optionally substituted independently with one or more substituents described herein.

The heterocycle or heteroaryl groups may be carbon (carbon-linked), or nitrogen (nitrogen-linked) bonded where such is possible. By way of example and not limitation, carbon bonded heterocycles or heteroaryls are bonded at position 2, 3, 4, 5, or 6 of a pyridine, position 3, 4, 5, or 6 of a pyridazine, position 2, 4, 5, or 6 of a pyrimidine, position 2, 3, 5, or 6 of a pyrazine, position 2, 3, 4, or 5 of a furan, tetrahydrofuran, thiofuran, thiophene, pyrrole or tetrahydropyrrole, position 2, 4, or 5 of an oxazole, imidazole or thiazole, position 3, 4, or 5 of an isoxazole, pyrazole, or isothiazole, position 2 or 3 of an aziridine, position 2, 3, or 4 of an azetidine, position 2, 3, 4, 5, 6, 7, or 8 of a quinoline or position 1, 3, 4, 5, 6, 7, or 8 of an isoquinoline.

By way of example and not limitation, nitrogen bonded heterocycles or heteroaryls are bonded at position 1 of an aziridine, azetidine, pyrrole, pyrrolidine, 2-pyrroline, 3-pyrroline, imidazole, imidazolidine, 2-imidazoline, 3-imidazoline, pyrazole, pyrazoline, 2-pyrazoline, 3-pyrazoline, piperidine, piperazine, indole, indoline, 1H-indazole, position 2 of a isoindole, or isoindoline, position 4 of a morpholine, and position 9 of a carbazole, or β-carboline.

A "metabolite" is a product produced through metabolism in the body of a specified compound or salt thereof. Metabolites of a compound may be identified using routine techniques known in the art and their activities determined using tests such as those described herein. Such products may result for example from the oxidation, reduction, hydrolysis, amidation, deamidation, esterification, deesterification, enzymatic cleavage, and the like, of the administered compound. Accordingly, the invention includes metabolites of compounds of the

invention, including compounds produced by a process comprising contacting a Formula I compound of this invention with a mammal for a period of time sufficient to yield a metabolic product thereof.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

A "sterile" formulation is aseptic or free from all living microorganisms and their spores.

A "stable" formulation is one in which the protein therein essentially retains its physical and chemical stability and integrity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, New York, Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10: 29-90 (1993). Stability can be measured at a selected temperature for a selected time period. For rapid screening, the formulation may be kept at 40 °C for 2 weeks to 1 month, at which time stability is measured. Where the formulation is to be stored at 2-8 °C, generally the formulation should be stable at 30 °C or 40 °C for at least 1 month and/or stable at 2-8°C for at least 2 years. Where the formulation is to be stored at 30 °C, generally the formulation should be stable for at least 2 years at 30 °C and/or stable at 40 °C for at least 6 months. For example, the extent of aggregation during storage can be used as an indicator of protein stability. Thus, a "stable" formulation may be one wherein less than about 10% and preferably less than about 5% of the protein are present as an aggregate in the formulation. In other embodiments, any increase in aggregate formation during storage of the formulation can be determined.

An "isotonic" formulation is one which has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 250 to 350 mOsm. The term "hypotonic" describes a formulation with an osmotic pressure below that of human blood. Correspondingly, the term "hypertonic" is used to describe a formulation with an osmotic pressure above that of human blood. Isotonicity can be measured using a vapor pressure or ice-freezing type osmometer, for example. The formulations of the present invention are hypertonic as a result of the addition of salt and/or buffer.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH

buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONICS™.

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative. A "pharmaceutically acceptable acid" includes inorganic and organic acids which are nontoxic at the concentration and manner in which they are formulated. For example, suitable inorganic acids include hydrochloric, perchloric, hydrobromic, hydroiodic, nitric, sulfuric, sulfonic, sulfinic, sulfanilic, phosphoric, carbonic, etc. Suitable organic acids include straight and branched-chain alkyl, aromatic, cyclic, cycloaliphatic, arylaliphatic, heterocyclic, saturated, unsaturated, mono, di- and tri-carboxylic, including for example, formic, acetic, 2-hydroxyacetic, trifluoroacetic, phenylacetic, trimethylacetic, t-butyl acetic, anthranilic, propanoic, 2-hydroxypropanoic, 2-oxopropanoic, propandioic, cyclopentanepropionic, cyclopentane propionic, 3-phenylpropionic, butanoic, butandioic, benzoic, 3-(4-hydroxybenzoyl)benzoic, 2-acetoxy-benzoic, ascorbic, cinnamic, lauryl sulfuric, stearic, muconic, mandelic, succinic, embonic, fumaric, malic, maleic, hydroxymaleic, malonic, lactic, citric, tartaric, glycolic, glyconic, gluconic, pyruvic, glyoxalic, oxalic, mesylic, succinic, salicylic, phthalic, palmoic, palmeic, thiocyanic, methanesulphonic, ethanesulphonic, 1,2-ethanedisulfonic, 2-hydroxyethanesulfonic, benzenesulphonic, 4-chorobenzenesulfonic, napthalene-2-sulphonic, p-toluenesulphonic, camphorsulphonic, 4-methylbicyclo[2.2.2]-oct-2-ene-1-carboxylic, glucoheptonic, 4,4'-methylenebis-3-(hydroxy-2-ene-1-carboxylic acid), hydroxynapthoic.

"Pharmaceutically-acceptable bases" include inorganic and organic bases which are non-toxic at the concentration and manner in which they are formulated. For example, suitable bases include those formed from inorganic base forming metals such as lithium, sodium, potassium, magnesium, calcium, ammonium, iron, zinc, copper, manganese, aluminum, N-methylglucamine, morpholine, piperidine and organic nontoxic bases including, primary, secondary and tertiary amines, substituted amines, cyclic amines and basic ion exchange resins,

[e.g., $N(R')_4^+$ (where R' is independently H or C_{1-4} alkyl, e.g., ammonium, Tris)], for example, isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, ethanolamine, 2-diethylaminoethanol, trimethamine, dicyclohexylamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, methylglucamine, theobromine, purines, piperazine, piperidine, N-ethylpiperidine, polyamine resins and the like. Particularly preferred organic non-toxic bases are isopropylamine, diethylamine, ethanolamine, trimethamine, dicyclohexylamine, choline, and caffeine.

Additional pharmaceutically acceptable acids and bases useable with the present invention include those which are derived from the amino acids, for example, histidine, glycine, phenylalanine, aspartic acid, glutamic acid, lysine and asparagine.

"Pharmaceutically acceptable" buffers and salts include those derived from both acid and base addition salts of the above indicated acids and bases. Specific buffers and/ or salts include histidine, succinate and acetate.

A "pharmaceutically acceptable sugar" is a molecule which, when combined with a protein of interest, significantly prevents or reduces chemical and/or physical instability of the protein upon storage. When the formulation is intended to be lyophilized and then reconstituted, "pharmaceutically acceptable sugars" may also be known as a "lyoprotectant". Exemplary sugars and their corresponding sugar alcohols include: an amino acid such as monosodium glutamate or histidine; a methylamine such as betaine; a lyotropic salt such as magnesium sulfate; a polyol such as trihydric or higher molecular weight sugar alcohols, e.g. glycerin, dextran, erythritol, glycerol, arabitol, xylitol, sorbitol, and mannitol; propylene glycol; polyethylene glycol; PLURONICS®; and combinations thereof. Additional exemplary lyoprotectants include glycerin and gelatin, and the sugars mellibiose, melezitose, raffinose, mannotriose and stachyose. Examples of reducing sugars include glucose, maltose, lactose, maltulose, iso-maltulose and lactulose. Examples of non-reducing sugars include non-reducing glycosides of polyhydroxy compounds selected from sugar alcohols and other straight chain polyalcohols. Preferred sugar alcohols are monoglycosides, especially those compounds obtained by reduction of disaccharides such as lactose, maltose, lactulose and maltulose. The glycosidic side group can be either glucosidic or galactosidic. Additional examples of sugar alcohols are glucitol, maltitol, lactitol and iso-maltulose. The preferred pharmaceutically-acceptable sugars are the non-reducing sugars trehalose or sucrose. Pharmaceutically acceptable sugars are added to the formulation in a "protecting amount" (e.g. pre-lyophilization) which means that the protein essentially retains its physical and chemical stability and integrity during storage (e.g., after reconstitution and storage).

The "diluent" of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a liquid formulation, such as a formulation reconstituted after lyophilization. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution. In an alternative embodiment, diluents can include aqueous solutions of salts and/or buffers.

A "preservative" is a compound which can be added to the formulations herein to reduce bacterial activity. The addition of a preservative may, for example, facilitate the production of a multi-use (multiple-dose) formulation. Examples of potential preservatives include octadecyldimethylbenzyl ammonium chloride, hexamethonium chloride, benzalkonium chloride (a mixture of alkylbenzyldimethylammonium chlorides in which the alkyl groups are long-chain compounds), and benzethonium chloride. Other types of preservatives include aromatic alcohols such as phenol, butyl and benzyl alcohol, alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, 3-pentanol, and m-cresol. The most preferred preservative herein is benzyl alcohol.

An "individual" or "subject" or "patient" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human.

As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention designed to alter the natural course of the individual, tissue or cell being treated during the course of clinical pathology. Desirable effects of treatment include, but are not limited to, decreasing the rate of disease progression, ameliorating or palliating the disease state, and remission or improved prognosis, all measurable by one of skill in the art such as a physician. In one embodiment, treatment can mean alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, decreasing the rate of infectious disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, the AACs and TACs of the invention are used to delay development of a disease or to slow the progression of an infectious disease or reduce the bacterial load in the blood stream and/or in infected tissues and organs.

As used herein, "in conjunction with" refers to administration of one treatment modality in addition to another treatment modality. As such, "in conjunction with" refers to administration of one treatment modality before, during or after administration of the other treatment modality to the individual.

The term "bacteremia" refers to the presence of bacteria in the bloodstream which is most commonly detected through a blood culture. Bacteria can enter the bloodstream as a severe complication of infections (like pneumonia or meningitis), during surgery (especially when involving mucous membranes such as the gastrointestinal tract), or due to catheters and other foreign bodies entering the arteries or veins. Bacteremia can have several consequences. The immune response to the bacteria can cause sepsis and septic shock, which has a relatively high mortality rate. Bacteria can also use the blood to spread to other parts of the body, causing infections away from the original site of infection. Examples include endocarditis or osteomyelitis.

A "therapeutically effective amount" is the minimum concentration required to effect a measurable improvement of a particular disorder. A therapeutically effective amount herein may vary according to factors such as the disease state, age, sex, and weight of the patient, and the ability of the antibody to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody are outweighed by the therapeutically beneficial effects. In one embodiment, a therapeutically effective amount is an amount effective to reduce bacteremia in an *in vivo* infection. In one aspect, a "therapeutically effective amount" is at least the amount effective to reduce the bacterial load or colony forming units (CFU) isolated from a patient sample such as blood by at least one log relative to prior to drug administration. In a more specific aspect, the reduction is at least 2 logs. In another aspect, the reduction is at least 3, 4, 5 logs. In yet another aspect, the reduction is to below detectable levels using assays known in the art including assays exemplified herein. In another embodiment, a therapeutically effective amount is the amount of an AAC in one or more doses given over the course of the treatment period, that achieves a negative blood culture (i.e., does not grow out the bacteria that is the target of the AAC) as compared to the positive blood culture before or at the start of treatment of the infected patient.

A "prophylactically effective amount" refers to an amount effective, at the dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to, at the earlier stage of disease, or even prior to exposure to conditions where the risk of infection is elevated, the prophylactically effective amount can be less than the therapeutically effective amount. In one embodiment, a prophylactically effective amount is at least an amount effective to reduce, prevent the occurrence of or spread of infection from one cell to another.

"Chronic" administration refers to administration of the medicament(s) in a continuous as opposed to acute mode, so as to maintain the initial therapeutic effect (activity) for an

extended period of time. "Intermittent" administration is treatment that is not consecutively done without interruption, but rather is cyclic in nature.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

The term "chiral" refers to molecules which have the property of non-superimposability of the mirror image partner, while the term "achiral" refers to molecules which are superimposable on their mirror image partner.

The term "stereoisomers" refers to compounds which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space.

"Diastereomer" refers to a stereoisomer with two or more centers of chirality and whose molecules are not mirror images of one another. Diastereomers have different physical properties, e.g. melting points, boiling points, spectral properties, and reactivities. Mixtures of diastereomers may separate under high resolution analytical procedures such as electrophoresis and chromatography.

"Enantiomers" refer to two stereoisomers of a compound which are non-superimposable mirror images of one another.

Stereochemical definitions and conventions used herein generally follow S. P. Parker, Ed., McGraw-Hill Dictionary of Chemical Terms (1984) McGraw-Hill Book Company, New York; and Eliel, E. and Wilen, S., Stereochemistry of Organic Compounds (1994) John Wiley & Sons, Inc., New York. Many organic compounds exist in optically active forms, i.e., they have the ability to rotate the plane of plane-polarized light. In describing an optically active compound, the prefixes D and L, or R and S, are used to denote the absolute configuration of the molecule about its chiral center(s). The prefixes d and l or (+) and (-) are employed to designate the sign of rotation of plane-polarized light by the compound, with (-) or 1 meaning that the compound is levorotatory. A compound prefixed with (+) or d is dextrorotatory. For a given chemical structure, these stereoisomers are identical except that they are mirror images of one another. A specific stereoisomer may also be referred to as an enantiomer, and a mixture of such isomers is often called an enantiomeric mixture. A 50:50 mixture of enantiomers is referred to as a racemic mixture or a racemate, which may occur where there has been no stereoselection or stereospecificity in a chemical reaction or process. The terms "racemic mixture" and "racemate" refer to an equimolar mixture of two enantiomeric species, devoid of optical activity.

The term "protecting group" refers to a substituent that is commonly employed to block or protect a particular functionality while other functional groups react on the compound. For example, an "amino-protecting group" is a substituent attached to an amino group that blocks or protects the amino functionality in the compound. Suitable amino-protecting groups include, but are not limited to, acetyl, trifluoroacetyl, t-butoxycarbonyl (BOC), benzyloxycarbonyl (CBZ) and 9-fluorenylmethylenoxycarbonyl (Fmoc). For a general description of protecting groups and their use, see T. W. Greene, Protective Groups in Organic Synthesis, John Wiley & Sons, New York, 1991, or a later edition.

The term "about" as used herein refers to the usual error range for the respective value readily known to the skilled person in this technical field. Reference to "about" a value or parameter herein includes (ad describes) embodiments that are directed to that value or parameter per se.

As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly indicates otherwise. For example, reference to an "antibody" is a reference to from one to many antibodies, such as molar amounts, and includes equivalents thereof known to those skilled in the art, and so forth.

III. Compositions and Methods

ANTIBODY-ANTIBIOTIC CONJUGATES (AAC)

The experimental results herein are a strong indication that therapies aimed at eliminating intracellular bacteria will improve clinical success. Towards this aim, the present invention provides a unique therapeutic that selectively kills *S. aureus* organisms that have invaded intracellular compartments of host cells. The present invention demonstrates that such a therapeutic is efficacious in in-vivo models where conventional antibiotics like vancomycin fail.

The invention provides an antibacterial therapy that aims to prevent antibiotic escape by targeting populations of bacteria that evade conventional antibiotic therapy. The novel antibacterial therapy is achieved with an Antibody Antibiotic Conjugate (AAC) in which an rF1 antibody specific for cell wall components found on *S. aureus* (including MRSA) is chemically linked to a potent rifamycin-type antibiotic (a derivative of rifamycin). The rifamycin-type antibiotic is joined to the antibody via a protease-cleavable, non-peptide linker that is designed to be cleaved by proteases, including cathepsin B, a lysosomal protease found in most mammalian cell types (Dubowchik et al (2002) Bioconj. Chem. 13:855-869). A diagram of the AAC with its 3 components is depicted in FIG. 2. Not to be limited by any one theory, one

mechanism of action of the AAC is schematized in FIG. 3. The AAC acts as a pro-drug in that the rifamycin-type antibiotic is inactive (due to the large size of the antibody) until the linker is cleaved. Since a significant proportion of *S. aureus* found in a natural infection is taken up by host cells, primarily neutrophils and macrophages, at some point during the course of infection in the host, the time spent inside host cells provides a significant opportunity for the bacterium to evade antibiotic activity. The AACs of the invention are designed to bind to the Staph bacteria and release the antibiotic inside the phagolysosome after bacteria are taken up by host cells. By this mechanism, AAC are able to concentrate the active antibiotic specifically in a location where S. aureus is poorly treated by conventional antibiotics. While the invention is not limited or defined by an particular mechanism of action, the AAC improve antibiotic activity via three potential mechanisms: (1) The AAC delivers antibiotic inside mammalian cells that take up the bacteria, thereby increasing the potency of antibiotics that diffuse poorly into the phagolysosomes where bacteria are sequestered. (2) AAC opsonize bacteria thereby increasing uptake of free bacteria by phagocytic cells, and release the antibiotic locally to kill the bacteria while they are sequestered in the phagolysosome. Since thousands of AACs can bind to a single bacterium, this platform releases sufficient antibiotics in these intracellular niches to sustain maximal antimicrobial killing. Furthermore, as more bacteria are released from pre-existing intracellular reservoirs, the fast on-rate of this antibody-based therapy ensures immediate "tagging" of these bacteria before they can escape to neighboring or distant cells, thus mitigating further spread of the infection. (3) AAC improve the half-life of antibiotics *in vivo* (improved pharmacokinetics) by linking the antibiotic to an antibody, as compared to antibiotics which are cleared rapidly from serum. Improved pharmacokinetics of AAC enable delivery of sufficient antibiotic in regions where *S. aureus* is concentrated while limiting the overall dose of antibiotic that needs to be administered systemically. This property should permit long-term therapy with AAC to target persistent infection with minimal antibiotic side effects.

An antibody-antibiotic conjugate compound of the invention comprises an anti-SDR antibody covalently attached by a protease-cleavable, non-peptide linker via a recombinantly introduced cysteine, to a rifamycin-type antibiotic.

In an exemplary embodiment, the anti-SDR antibody (e.g. rF1 antibody) is a cysteine-engineered antibody comprising a recombinantly introduced cysteine amino acid.

In an exemplary embodiment, the protease-cleavable, non-peptide linker is covalently attached via a recombinantly introduced cysteine on the rF1, anti-SDR antibody, to the rifamycin-type antibiotic

An exemplary embodiment is the antibody-antibiotic conjugate having the formula:

$$Ab–(PML–abx)_p$$

wherein:

Ab is the rF1 antibody;

PML is the protease-cleavable, non-peptide linker having the formula:

$$–Str–PM–Y–$$

where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit;

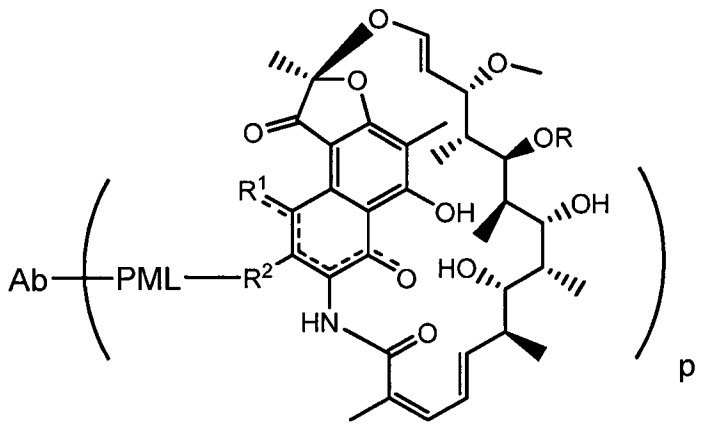
abx is the rifamycin-type antibiotic; and

p is an integer from 1 to 8.

The rifamycin-type antibiotic may be a rifalazil-type antibiotic.

The rifamycin-type antibiotic may comprise a quaternary amine attached to the protease-cleavable, non-peptide linker.

An exemplary embodiment of the antibody-antibiotic conjugate has Formula I:



I

wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

R^1 is OH;

R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH;

PML is the protease-cleavable, non-peptide linker attached to R^2 or the fused heteroaryl or heterocyclyl formed by R^1 and R^2; and

Ab is the rF1 antibody.

The number of antibiotic moieties which may be conjugated via a reactive linker moiety to an antibody molecule may be limited by the number of free cysteine residues, which are introduced by the methods described herein. Exemplary AAC comprise antibodies which have 1, 2, 3, or 4 engineered cysteine amino acids (Lyon, R. et al (2012) *Methods in Enzym.* 502:123-138).

To be effective target on MRSA, the epitope is preferably highly abundant, stably expressed during infection and highly conserved in all clinical MRSA strains. The rF1 antibody fulfills these requirements and additionally, also binds to Staph epidermidis as well.

ANTI-SDR AND rF1 ANTIBODIES

Anti-SDR antibodies can be produced as described below for the generation of F1 antibody. Several examples of anti-SDR antibodies are provided herein including rF1, SD2, SD3 and SD4.

The rF1 Abs will be described in detail here.

rF1 antibody is a fully human is capable of specifically binding *Staphylococcus* species such as *S. aureus* and *S. epidermidis*. Importantly, rF1 is capable of binding whole bacteria *in vivo* as well as *in vitro*. Furthermore, antibody rF1 is capable of binding to bacteria that have been grown in infected tissue of, for example, an animal. The rF1 Abs provided herein or functional equivalents thereof are capable of binding to *S. aureus* surface proteins ClfA, ClfB, SdrC, SdrD and SdrE.

Table 4A and Table 4B show an alignment of the H chain and L chain CDR sequences of the parent antibody F1, rF1 antibody and its variants. F1 and rF1 differ in sequence in FW1 and LC CDR3 (QHYXRFPYT, where X can be I or M (SEQ ID NO: 26); F1 is I (SEQ ID NO: 6) and rF1 is M (SEQ ID NO: 7)).

5

Table 4A: Heavy chain CDR sequences

Antibody	HC CDR1	HC CDR2	HC CDR3
F1	RFAMS (SEQ ID NO:1)	SINNGNNPYYARSVQY (SEQ ID NO: 2)	DHPSSGWPTFDS (SEQ ID NO: 3)
rF1	RFAMS (SEQ ID NO:1)	SINNGNNPYYARSVQY (SEQ ID NO: 2)	DHPSSGWPTFDS (SEQ ID NO: 3)
rF1.v1	RFAMS (SEQ ID NO:1)	SINSGNNPYYARSVQY (SEQ ID NO: 8)	DHPSSGWPTFDS (SEQ ID NO: 3)

Table 4B Light chain CDR sequences

Antibody	LC CDR1	LC CDR2	LC CDR3
F1	RASENVGDWLA (SEQ ID NO: 4)	KTSILES (SEQ ID NO:5)	QHYIRFPYT (SEQ ID NO:6)
rF1	RASENVGDWLA (SEQ ID NO: 4)	KTSILES (SEQ ID NO:5)	QHYMRFPYT (SEQ ID NO:7)
rF1.v6	RASENVGDWLA (SEQ ID NO: 4)	KTSILES (SEQ ID NO:5)	QHYIRFPYT (SEQ ID NO:6)

In one embodiment, the H and L chain Framework (FR) sequences are as follows:

HC FW1 EVQLVESGGGLVQPGGSLRLSCAASGFTLS (SEQ ID NO. 16)

HC FW2 WVRQAPGRGLEWVA (SEQ ID NO. 17)

HC FW3 RFTVSRDVSQNTVSLQMNNLRAEDSATYFCAK (SEQ ID NO. 18)

HC FW4 WGPGTLVTVSS (SEQ ID NO. 19)

LC FW1 DIQLTQSPSALPASVGDRVSITC (SEQ ID NO. 20)

LC FW2 WYRQKPGKAPNLLIY (SEQ ID NO. 21)

LC FW3 GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC (SEQ ID NO. 22)

LC FW4 FGQGTKVEIKRTV (SEQ ID NO. 23)

Various amino acid modifications were made to rF1 to improve stability and function. In the HC CDR2, the NG deamindation site was eliminated by changing the 4th residue N to S, thus improving the stability of the antibody. A repair of TV was made to the LC backbone to eliminate the severe antibody aggregation present in rF1.

For conjugation to form the therapeutic AACs of the invention, the following pairings of H and L chain can be made to form the full tetrameric antibody. Boxed are the CDR1, CDR2, CDR3 sequences. The introduced Cysteine (C) is underlined. Residues in bold are amino acid changes over the parent F1. In the Lchain, the A after the bolded "RTV" is the first residue of the Constant region. The underlined C at Kabat position 114 in the H chain starts the Constant region.

In 1A and 2A, the full length (FL) L chain of SEQ ID NO. 9 with an engineered Cys at aa 205 near the end of C kappa is paired with the FL IgG1 H chain of SEQ ID NO. 10 (no Cys). This antibody will have 2 Cys sites, one on each L chain, for conjugation to the linker-antibiotic unit to form the AAC.

1A. rF1-V205C FL Light chain

DIQLTQSPSALPASVGDRVSITC[RASENVGDWLA]WYRQKPGKAPNLLIY[KTSILES]GVPSRFSG
SGSGTEFTLTISSLQPDDFATYYC[QHY*M*RFPYT]FGQGTK**V**EIK**RTV**AAPSVFIFPPSDEQLKSG
TASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVY
ACEVTHQGLSSPCTKSFNRGEC (SEQ ID NO.9)

2A. rF1.v1 FL Heavy chain (No Cys), pair of rF1-V205C Light Chain with Cys205

EVQL**V**ESGGGLVQPGGSLRLSCAASGFTLS[RFAMS]WVRQAPGRGLEWVA[SIN**S**GNNPYYARSVQ
[Y]RFTVSRDVSQNTVSLQMNNLRAEDSATYFCAK[DHPSSGWPTFDS]WGPGTLVTVSSASTKGPSV
FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI
SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK

EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G (SEQ ID NO: 10)

In 1B with 2A, rF1.v6 L chain of SEQ ID NO. 11 with an engineered Cys 205 is paired with the FL IgG1 H chain of SEQ ID NO. 10 (no Cys). This antibody will have 2 Cys sites, one on each L chain, for conjugation to the linker-antibiotic unit.

1B. rF1.v6-V205C Light chain

DIQLTQSPSALPASVGDRVSITCRASENVGDWLAWYRQKPGKAPNLLIYKTSILESGVPSRFSG
SGSGTEFTLTISSLQPDDFATYYCQHYIRFPYTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSG
TASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVY
ACEVTHQGLSSPCTKSFNRGEC (FL SEQ ID NO. 11)

In 1B with 2B, each of L and H chains has an engineered Cys, thus the tetramer antibody can have up to 4 AAR (Antibiotic: antibody ratio).

2B. rF1.v1 FL Heavy chain, with Cys114 (114 Kabat numbering, or 118 -Eu numbering)

EVQLVESGGGLVQPGGSLRLSCAASGFTLSRFAMSWVRQAPGRGLEWVASINSGNNPYYARSVQ
YRFTVSRDVSQNTVSLQMNNLRAEDSATYFCAKDHPSSGWPTFDSWGPGTLVTVSS
CSTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS
LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT
QKSLSLSPG (SEQ ID NO. 12)

rF1.v1 H chain Variable region

EVQL**V**ESGGGLVQPGGSLRLSCAASGFTLSRFAMSWVRQAPGRGLEWVASIN**S**GNNPYY
ARSVQYRFTVSRDVSQNTVSLQMNNLRAEDSATYFCAKDHPSSGWPTFDSWGPGTLVTVSS
(SEQ ID NO. 13)

rF1 L chain Variable region

DIQLTQSPSALPASVGDRVSITCRASENVGDWLAWYRQKPGKAPNLLIYKTSILESGVP SRFSGSGSGTEFTLTISSLQPDDFATYYCQHYMRFPYTFGQGTKVEIKRTV (SEQ ID NO. 14)

rF1.v6 L chain Variable region

DIQLTQSPSALPASVGDRVSITCRASENVGDWLAWYRQKPGKAPNLLIYKTSILESGVP SRFSGSGSGTEFTLTISSLQPDDFATYYCQHYIRFPYTFGQGTKVEIKRTV (SEQ ID NO. 15)

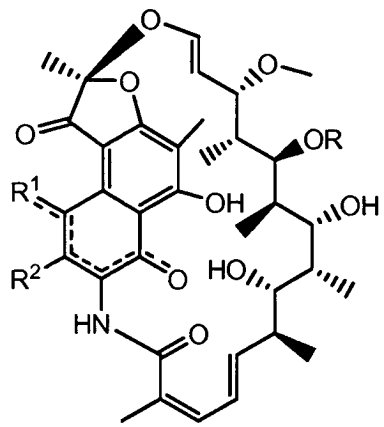
The anti-SDR Abs including rF1 may comprise at least one amino acid other than cysteine has been replaced with cysteine. In some embodiments, the at least one amino acid other than cysteine is valine at light chain position 205 and/or valine at light chain position 110, and/or alanine at heavy chain position 114, whereby the amino acid numbering is according to Kabat (1991), which is the same as position 118 according to the Eu numbering convention.

RIFAMYCIN-TYPE ANTIBIOTIC MOIETIES

The antibiotic moiety (abx) of the antibody-antibiotic conjugates (AAC) of the invention is a rifamycin-type antibiotic or group that has a cytotoxic or cytostatic effect. The rifamycins are a group of antibiotics that are obtained either naturally by the bacterium, *Nocardia mediterranei, Amycolatopsis mediterranei* or artificially. They are a subclass of the larger Ansamycin family which inhibit bacterial RNA polymerase (Fujii et al (1995) Antimicrob. Agents Chemother. 39:1489-1492; Feklistov, et al (2008) Proc Natl Acad Sci USA, 105(39): 14820-5) and have potency against gram-positive and selective gram-negative bacteria. Rifamycins are particularly effective against mycobacteria, and are therefore used to treat tuberculosis, leprosy, and mycobacterium avium complex (MAC) infections. The rifamycin-type group includes the "classic" rifamycin drugs as well as the rifamycin derivatives rifampicin (rifampin, CA Reg. No. 13292-46-1), rifabutin (CA Reg. No. 72559-06-9; US 2011/0178001), rifapentine and rifalazil (CA Reg. No. 129791-92-0, Rothstein et al (2003) Expert Opin. Investig. Drugs 12(2):255-271; Fujii et al (1994) Antimicrob. Agents Chemother. 38:1118-1122. Many rifamycin-type antibiotics share the detrimental property of resistance development (Wichelhaus et al (2001) J. Antimicrob. Chemother. 47:153-156). Rifamycins were first isolated in 1957 from a fermentation culture of *Streptomyces mediterranei*. About seven rifamycins

were discovered, named Rifamycin A, B, C, D, E, S, and SV (US 3150046). Rifamycin B was the first introduced commercially and was useful in treating drug-resistant tuberculosis in the 1960s. Rifamycins have been used for the treatment of many diseases, the most important one being HIV-related Tuberculosis. Due to the large number of available analogues and derivatives, rifamycins have been widely utilized in the elimination of pathogenic bacteria that have become resistant to commonly used antibiotics. For instance, Rifampicin is known for its potent effect and ability to prevent drug resistance. It rapidly kills fast-dividing bacilli strains as well as "persisters" cells, which remain biologically inactive for long periods of time that allow them to evade antibiotic activity. In addition, rifabutin and rifapentine have both been used against tuberculosis acquired in HIV-positive patients.

Antibiotic moieties (abx) of the Formula I antibody-antibiotic conjugates are rifamycin-type moieties having the structure:



wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

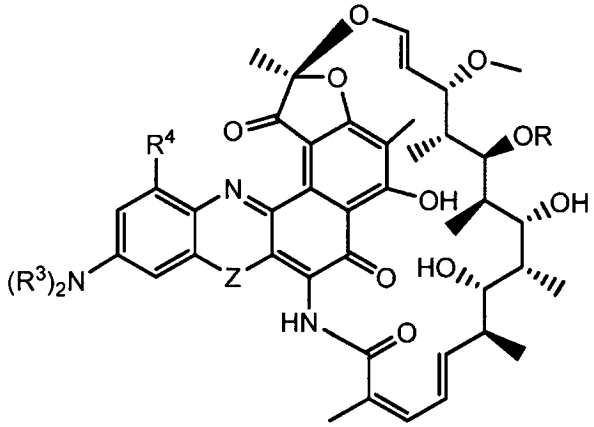
R^1 is OH;

R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH; and

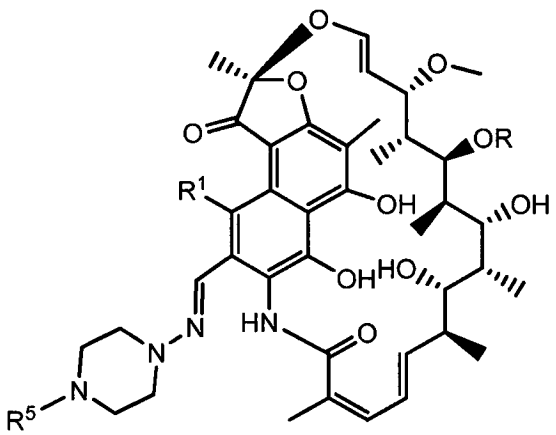
where the non-peptide linker PML is covalently attached to R^2.

An embodiment of a rifamycin-type moiety is:



wherein R^3 is independently selected from H and C_1–C_{12} alkyl; R^4 is selected from H, F, Cl, Br, I, C_1–C_{12} alkyl, and OH; and Z is selected from NH, N(C_1–C_{12} alkyl), O and S; and where the non-peptide linker PML is covalently attached to the nitrogen atom of $N(R^3)_2$.

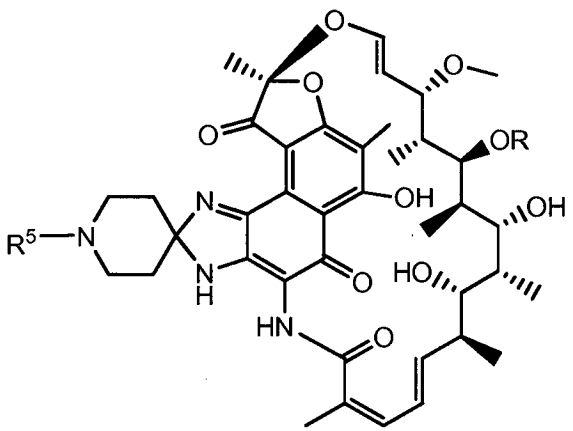
An embodiment of a rifampicin-type moiety is:



wherein

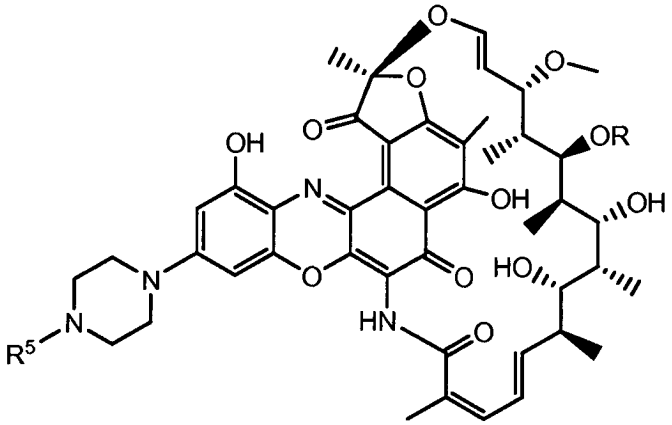
R^5 is selected from H and C_1–C_{12} alkyl; and where the non-peptide linker PML is covalently attached to the nitrogen atom of NR^5.

An embodiment of a rifabutin-type moiety is:



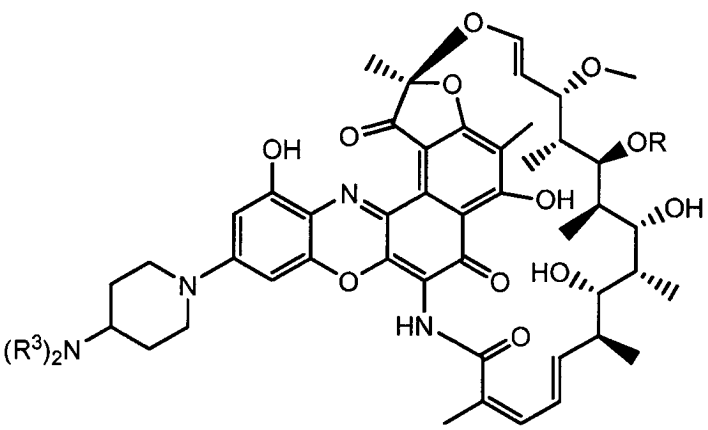
wherein R^5 is selected from H and C_1–C_{12} alkyl; and where the non-peptide linker PML is covalently attached to the nitrogen atom of NR^5.

An embodiment of a benzoxazinorifamycin-type moiety is:



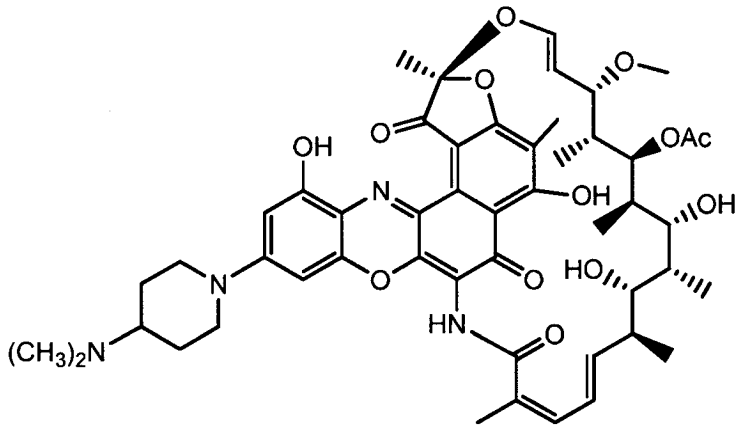
wherein R^5 is selected from H and C_1–C_{12} alkyl; and where the non-peptide linker PML is covalently attached to the nitrogen atom of NR^5.

An embodiment of a benzoxazinorifamycin-type moiety, referred to herein as pipBOR, is:



wherein R^3 is independently selected from H and C_1–C_{12} alkyl; and where the non-peptide linker PML is covalently attached to the nitrogen atom of $N(R^3)_2$.

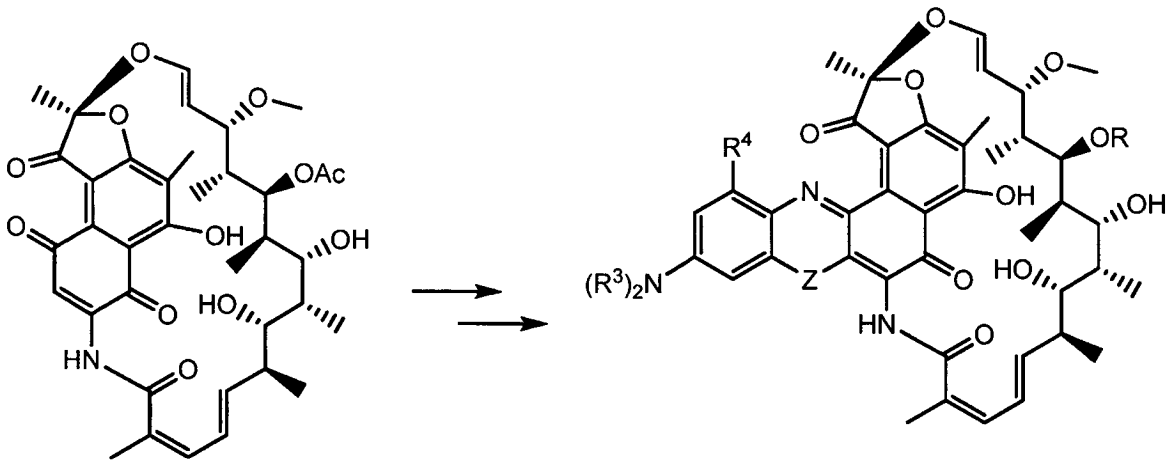
An embodiment of a benzoxazinorifamycin-type moiety, referred to herein as dimethylpipBOR, is:



where the non-peptide linker PML is covalently attached to the nitrogen atom of $N(CH_3)_2$.

The semi-synthetic derivative rifamycin S, or the reduced, sodium salt form rifamycin SV, can be converted to Rifalazil-type antibiotics in several steps, where R is H, or Ac, R^3 is independently selected from H and C_1–C_{12} alkyl; R^4 is selected from H, F, Cl, Br, I, C_1–C_{12} alkyl, and OH; and Z is selected from NH, N(C_1–C_{12} alkyl), O and S (see, e.g., Fig. 23A and B, and Fig. 25A and B in WO 2014/194247). Benzoxazino (Z = O), benzthiazino (Z = S), benzdiazino (Z = NH, N(C_1–C_{12} alkyl) rifamycins may be prepared (US 7271165). Benzoxazinorifamycin (BOR), benzthiazinorifamycin (BTR), and benzdiazinorifamycin (BDR) analogs that contain substituents are numbered according to the numbering scheme provided in formula A at column 28 in US 7271165, which is incorporated by reference for this purpose. By "25-O-deacetyl" rifamycin is meant a rifamycin analog in which the acetyl group at the 25-position has been removed. Analogs in which this position is further derivatized are referred to as a "25-O-deacetyl-25-(substituent) rifamycin", in which the nomenclature for the derivatizing group replaces "substituent" in the complete compound name.

Rifamycin-type antibiotic moieties can be synthesized by methods analogous to those disclosed in US 4610919; US 4983602; US 5786349; US5981522; US 4859661; US 7271165; US 2011/0178001; Seligson, et al., (2001) Anti-Cancer Drugs 12:305-13; Chem. Pharm. Bull., (1993) 41:148, and in WO 2014/194247, each of which is hereby incorporated by reference). Rifamycin-type antibiotic moieties can be screened for antimicrobial activity by measuring their minimum inhibitory concentration (MIC), using standard MIC in vitro assays (Tomioka et al., (1993) Antimicrob. Agents Chemother. 37:67).



rifamycin-S benzoxazinorifamycin

PROTEASE-CLEAVABLE NON-PEPTIDE LINKERS

A "protease-cleavable, non-peptide linker" (PML) is a bifunctional or multifunctional moiety which is covalently attached to one or more antibiotic moieties (abx) and an antibody unit (Ab) to form antibody-antibiotic conjugates (AAC) of Formula I. Protease-cleavable, non-peptide linkers in AAC are substrates for cleavage by intracellular proteases, including under lysosomal conditions. Proteases includes various cathepsins and caspases. Cleavage of the non-peptide linker of an AAC inside a cell may release the rifamycin-type antibiotic with anti-bacterial effects.

Antibody-antibiotic conjugates (AAC) can be conveniently prepared using a linker reagent or linker-antibiotic intermediate having reactive functionality for binding to the antibiotic (abx) and to the antibody (Ab). In one exemplary embodiment, a cysteine thiol of a cysteine engineered antibody (Ab) can form a bond with a functional group of a linker reagent, an antibiotic moiety or antibiotic-linker intermediate.

The PML moiety of an AAC may comprise one amino acid residue.

The PML moiety of an AAC comprises a peptidomimetic unit.

In one aspect, a linker reagent or linker-antibiotic intermediate has a reactive site which has an electrophilic group that is reactive to a nucleophilic cysteine present on an antibody. The cysteine thiol of the antibody is reactive with an electrophilic group on a linker reagent or linker-antibiotic, forming a covalent bond. Useful electrophilic groups include, but are not limited to, maleimide and haloacetamide groups.

Cysteine engineered antibodies react with linker reagents or linker-antibiotic intermediates, with electrophilic functional groups such as maleimide or α-halo carbonyl,

according to the conjugation method at page 766 of Klussman, et al (2004), *Bioconjugate Chemistry* 15(4):765-773, and according to the protocol of Example 18.

In another embodiment, the reactive group of a linker reagent or linker-antibiotic intermediate contains a thiol-reactive functional group that can form a bond with a free cysteine thiol of an antibody. Examples of thiol-reaction functional groups include, but are not limited to, maleimide, α-haloacetyl, activated esters such as succinimide esters, 4-nitrophenyl esters, pentafluorophenyl esters, tetrafluorophenyl esters, anhydrides, acid chlorides, sulfonyl chlorides, isocyanates and isothiocyanates.

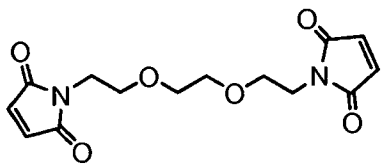
In another embodiment, a linker reagent or antibiotic-linker intermediate has a reactive functional group which has a nucleophilic group that is reactive to an electrophilic group present on an antibody. Useful electrophilic groups on an antibody include, but are not limited to, pyridyl disulfide, aldehyde and ketone carbonyl groups. The heteroatom of a nucleophilic group of a linker reagent or antibiotic-linker intermediate can react with an electrophilic group on an antibody and form a covalent bond to an antibody unit. Useful nucleophilic groups on a linker reagent or antibiotic-linker intermediate include, but are not limited to, hydrazide, oxime, amino, thiol, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide. The electrophilic group on an antibody provides a convenient site for attachment to a linker reagent or antibiotic-linker intermediate.

A PML moiety may comprise one or more linker components. Exemplary linker components include a single amino acid such as citrulline ("cit"), 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), and p-aminobenzyloxycarbonyl ("PAB"), N-succinimidyl 4-(2-pyridylthio) pentanoate ("SPP"), and 4-(N-maleimidomethyl) cyclohexane-1 carboxylate ("MCC"). Various linker components are known in the art, some of which are described below.

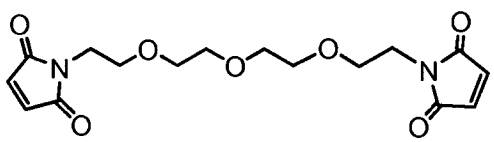
In another embodiment, the linker may be substituted with groups that modulate solubility or reactivity. For example, a charged substituent such as sulfonate ($-SO_3^-$) or ammonium, may increase water solubility of the reagent and facilitate the coupling reaction of the linker reagent with the antibody or the antibiotic moiety, or facilitate the coupling reaction of Ab-L (antibody-linker intermediate) with abx, or abx-L (antibiotic-linker intermediate) with Ab, depending on the synthetic route employed to prepare the AAC.

The AAC of the invention expressly contemplate, but are not limited to, those prepared with linker reagents: BMPEO, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, sulfo-SMPB, SVSB (succinimidyl-(4-vinylsulfone)benzoate),

and bis-maleimide reagents such as DTME, BMB, BMDB, BMH, BMOE, $BM(PEG)_2$, and $BM(PEG)_3$. Bis-maleimide reagents allow the attachment of the thiol group of a cysteine engineered antibody to a thiol-containing antibiotic moiety, label, or linker intermediate, in a sequential or convergent fashion. Other functional groups besides maleimide, which are reactive with a thiol group of a cysteine engineered antibody, antibiotic moiety, or linker-antibiotic intermediate include iodoacetamide, bromoacetamide, vinyl pyridine, disulfide, pyridyl disulfide, isocyanate, and isothiocyanate.



$BM(PEG)_2$



$BM(PEG)_3$

Useful linker reagents can also be obtained via other commercial sources, such as Molecular Biosciences Inc.(Boulder, CO), or synthesized in accordance with procedures described in Toki et al (2002) J. Org. Chem. 67:1866-1872; Dubowchik, et al. (1997) Tetrahedron Letters, 38:5257-60; Walker, M.A. (1995) J. Org. Chem. 60:5352-5355; Frisch et al (1996) Bioconjugate Chem. 7:180-186; US 6214345; WO 02/088172; US 2003130189; US2003096743; WO 03/026577; WO 03/043583; and WO 04/032828.

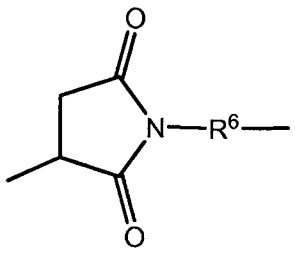
In another embodiment, the PML moiety of an AAC comprises a dendritic type linker for covalent attachment of more than one antibiotic moiety through a branching, multifunctional linker moiety to an antibody (Sun et al (2002) *Bioorganic & Medicinal Chemistry Letters* 12:2213-2215; Sun et al (2003) *Bioorganic & Medicinal Chemistry* 11:1761-1768). Dendritic linkers can increase the molar ratio of antibiotic to antibody, i.e. loading, which is related to the potency of the AAC. Thus, where a cysteine engineered antibody bears only one reactive cysteine thiol group, a multitude of antibiotic moieties may be attached through a dendritic linker.

In certain embodiments of Formula I AAC, the protease-cleavable, non-peptide linker PML has the formula:

$$-Str-PM-Y-$$

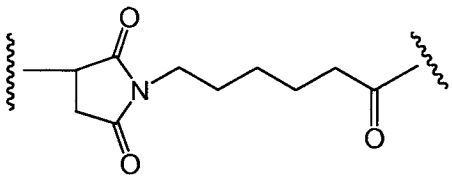
where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit;
abx is the rifamycin-type antibiotic; and
p is an integer from 1 to 8.

In one embodiment, a stretcher unit "Str" has the formula:

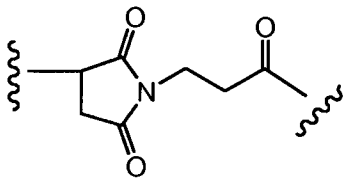


wherein R^6 is selected from the group consisting of C_1-C_{12} alkylene, C_1-C_{12} alkylene-C(=O), C_1-C_{12} alkylene-NH, $(CH_2CH_2O)_r$, $(CH_2CH_2O)_r$-C(=O), $(CH_2CH_2O)_r$-CH_2, and C_1-C_{12} alkylene-NHC(=O)CH_2CH(thiophen-3-yl), where r is an integer ranging from 1 to 10.

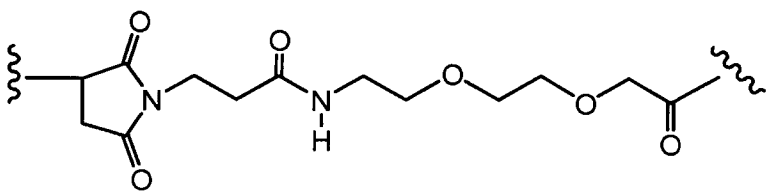
Exemplary stretcher units are shown below (wherein the wavy line indicates sites of covalent attachment to an antibody):

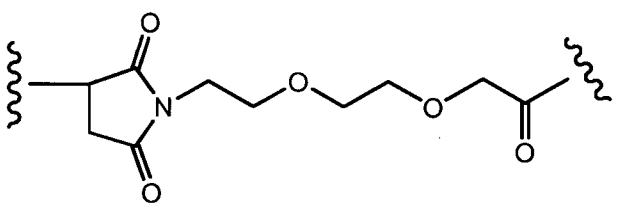


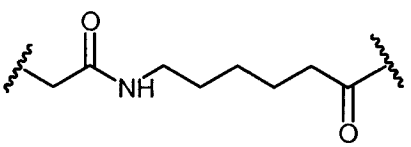
MC



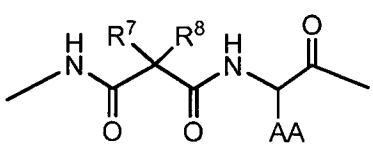
MP







In one embodiment, PM has the formula:



where R^7 and R^8 together form a C_3-C_7 cycloalkyl ring, and

AA is an amino acid side chain selected from H, $-CH_3$, $-CH_2(C_6H_5)$, $-CH_2CH_2CH_2CH_2NH_2$, $-CH_2CH_2CH_2NHC(NH)NH_2$, $-CHCH(CH_3)CH_3$, and $-CH_2CH_2CH_2NHC(O)NH_2$.

In one embodiment, spacer unit Y comprises para-aminobenzyl (PAB) or para-aminobenzyloxycarbonyl (PABC).

A spacer unit allows for release of the antibiotic moiety without a separate hydrolysis step. A spacer unit may be "self-immolative" or a "non-self-immolative." In certain embodiments, a spacer unit of a linker comprises a p-aminobenzyl unit (PAB). In one such embodiment, a p-aminobenzyl alcohol is attached to an amino acid unit via an amide bond, a carbamate, methylcarbamate, or carbonate between the p-aminobenzyl group and the antibiotic moiety (Hamann et al. (2005) *Expert Opin. Ther. Patents* (2005) 15:1087-1103). In one embodiment, the spacer unit is p-aminobenzyloxycarbonyl (PAB).

In one embodiment, the antibiotic comprises a quaternary amine, such as the dimethylaminopiperidyl group, when attached to the PAB spacer unit of the non-peptide linker. Examples of such quaternary amines are linker-antibiotic intermediates (PLA) are PLA-1 to 4 from Table 2. The quaternary amine group may modulate cleavage of the antibiotic moiety to optimize the antibacterial effects of the AAC. In another embodiment, the antibiotic is linked to the PABC spacer unit of the non-peptide linker, forming a carbamate functional group in the AAC. Such carbamate functional group may also optimize the antibacterial effects of the AAC. Examples of PABC carbamate linker-antibiotic intermediates (PLA) are PLA-5 and PLA-6 from Table 2.

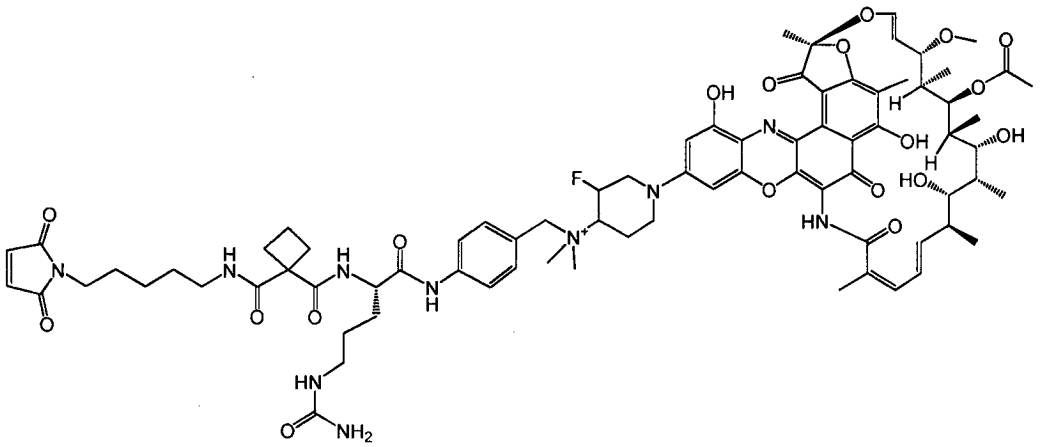
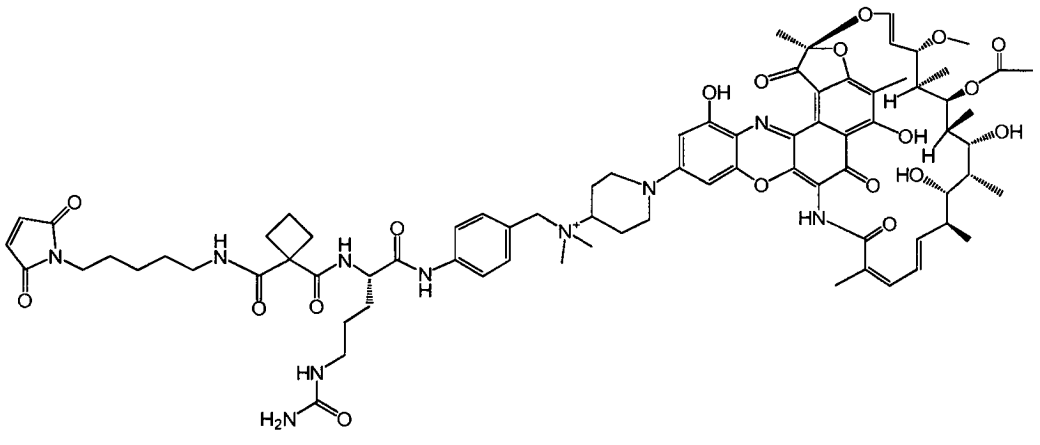
Other examples of self-immolative spacers include, but are not limited to, aromatic compounds that are electronically similar to the PAB group such as 2-aminoimidazol-5-methanol derivatives (US 7375078; Hay et al. (1999) *Bioorg. Med. Chem. Lett.* 9:2237) and ortho- or para-aminobenzylacetals. Spacers can be used that undergo cyclization upon amide bond hydrolysis, such as substituted and unsubstituted 4-aminobutyric acid amides (Rodrigues et al (1995) Chemistry Biology 2:223), appropriately substituted bicyclo[2.2.1] and bicyclo[2.2.2] ring systems (Storm et al (1972) *J. Amer. Chem. Soc.* 94:5815) and 2-aminophenylpropionic acid amides (Amsberry, et al (1990) *J. Org. Chem.* 55:5867). Elimination of amine-containing drugs that are substituted at glycine (Kingsbury et al (1984) *J. Med. Chem.* 27:1447) is also exemplary of self-immolative spacers useful in AAC.

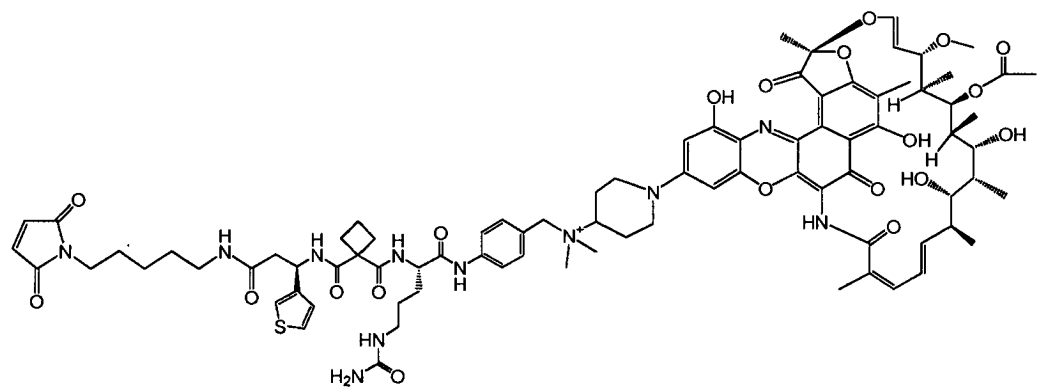
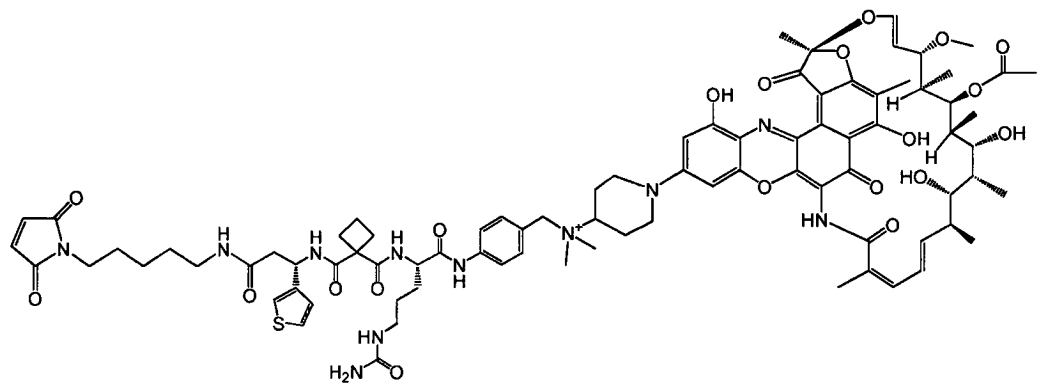
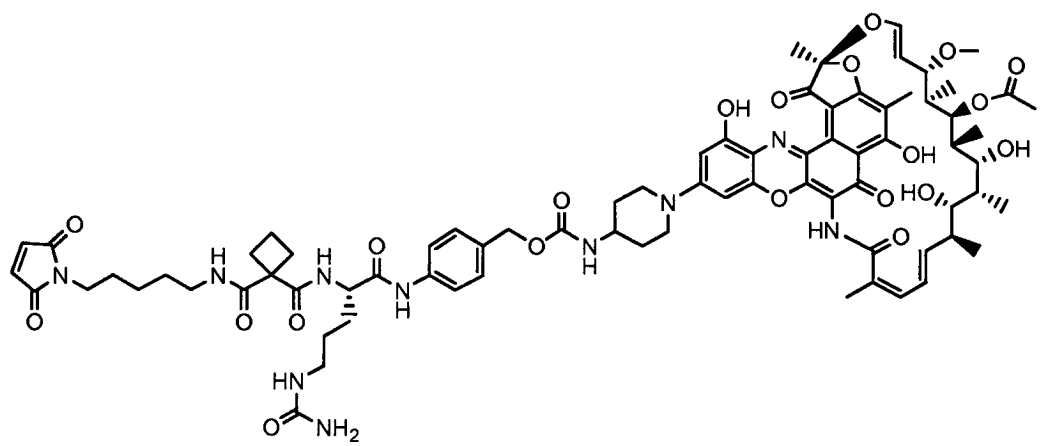
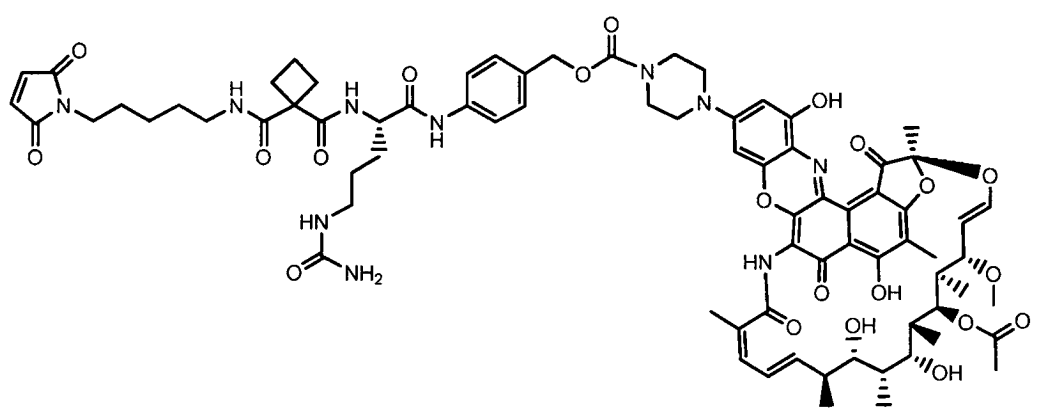
The amount of active antibiotic released from cleavage of AAC can be measured by a caspase release assay.

LINKER-ANTIBIOTIC INTERMEDIATES USEFUL FOR AAC

PML Linker-antibiotic intermediates (PLA) of Formula II and Table 2 were prepared by coupling a rifamycin-type antibiotic moiety with a linker reagent, Examples 7-17. Linker reagents were prepared by methods described in WO 2012/113847; US 7659241; US 7498298; US 20090111756; US 2009/0018086; US 6214345; Dubowchik et al (2002) Bioconjugate Chem. 13(4):855-869

Table 2 PML Linker-antibiotic intermediates

LA No.	Structure
PLA-1	
PLA-2	

PLA-3	
PLA-4	
PLA-5	
PLA-6	

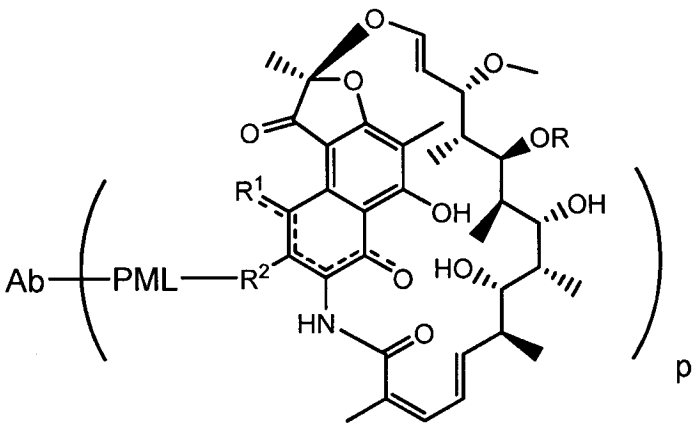
EMBODIMENTS OF ANTIBODY-ANTIBIOTIC CONJUGATES

Cysteine engineered, rF1 antibodies were linked via the free cysteine thiol group to derivatives of rifamycin, termed pipBOR and others, via a protease cleavable, non-peptide linker to form the antibody-antibiotic conjugate compounds (AAC) in Table 3. The linker is designed to be cleaved by lysosomal proteases including cathepsins B, D and others, Generation of the linker-antibiotic intermediate consisting of the antibiotic and the PML linker and others, is described in detail in Examples 7-17. The linker is designed such that cleavage of the amide bond at the PAB moiety separates the antibody from the antibiotic in an active state.

The AAC named "dimethylpipBOR" is identical to the "pipBOR" AAC except for the dimethylated amino on the antibiotic and the oxycarbonyl group on the linker.

Figure 3 shows a possible mechanism of drug activation for antibody-antibiotic conjugates (AAC). Active antibiotic (Ab) is only released after internalization of the AAC inside mammalian cells. The Fab portion of the antibody in AAC binds S. aureus whereas the Fc portion of the AAC enhances uptake of the bacteria by Fc-receptor mediated binding to phagocytic cells including neutrophils and macrophages. After internalization into the phagolysosome, the linker may be cleaved by lysosomal proteases releasing the active antibiotic inside the phagolysosome.

An embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes Formula I:



I

wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

R^1 is OH;

R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

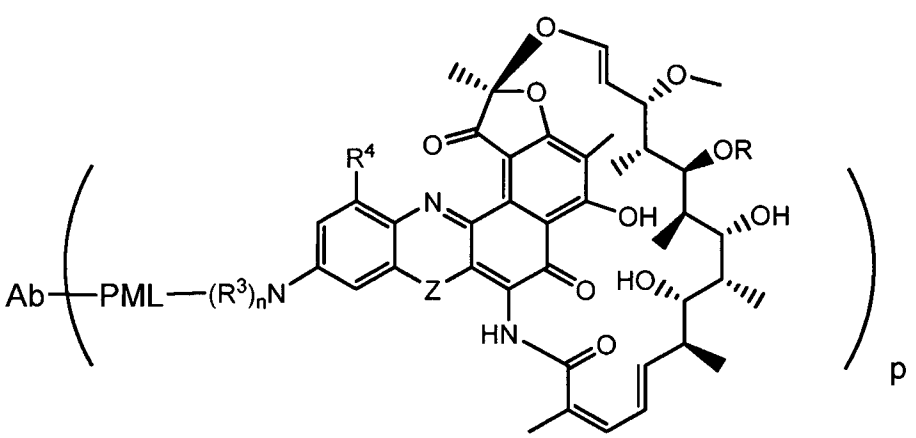
or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH;

PML is the protease-cleavable, non-peptide linker attached to R^2 or the fused heteroaryl or heterocyclyl formed by R^1 and R^2;

Ab is the rF1 antibody; and

p is an integer from 1 to 8.

Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



wherein

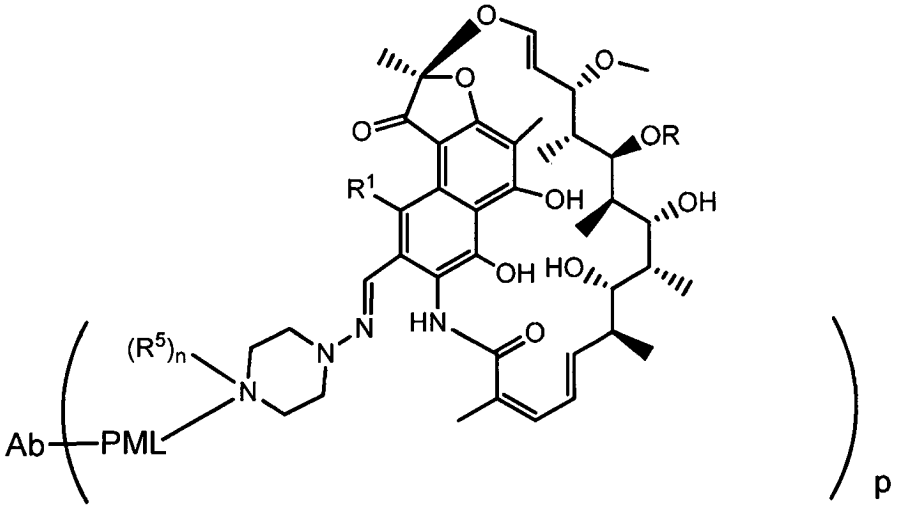
R^3 is independently selected from H and C_1–C_{12} alkyl;

n is 1 or 2;

R^4 is selected from H, F, Cl, Br, I, C_1–C_{12} alkyl, and OH; and

Z is selected from NH, N(C_1–C_{12} alkyl), O and S.

Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:

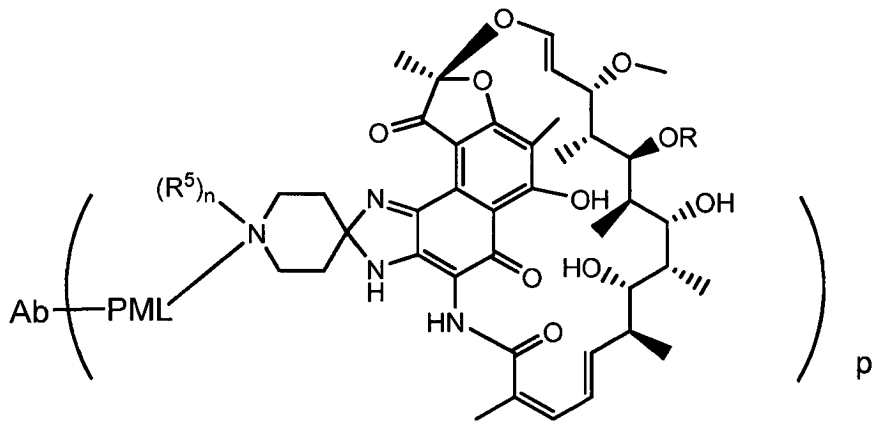


wherein

R^5 is selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:

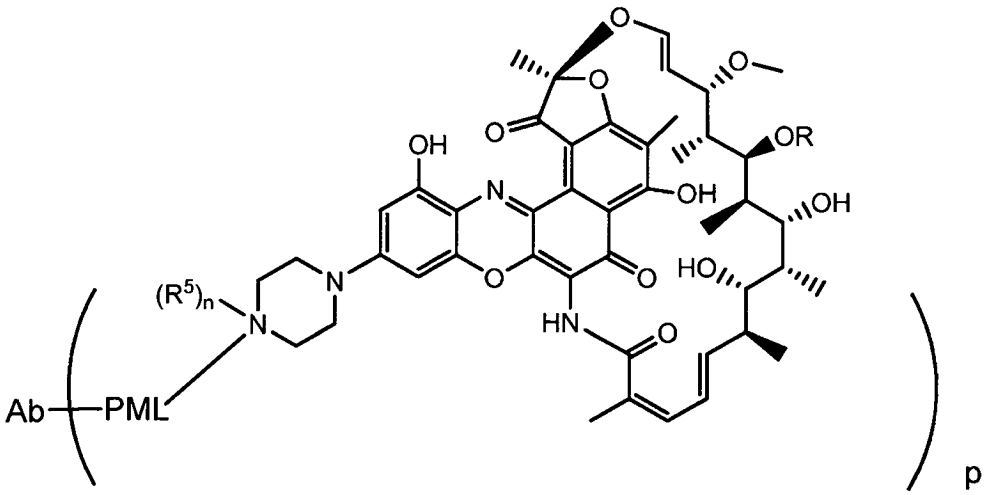


wherein

R^5 is selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:

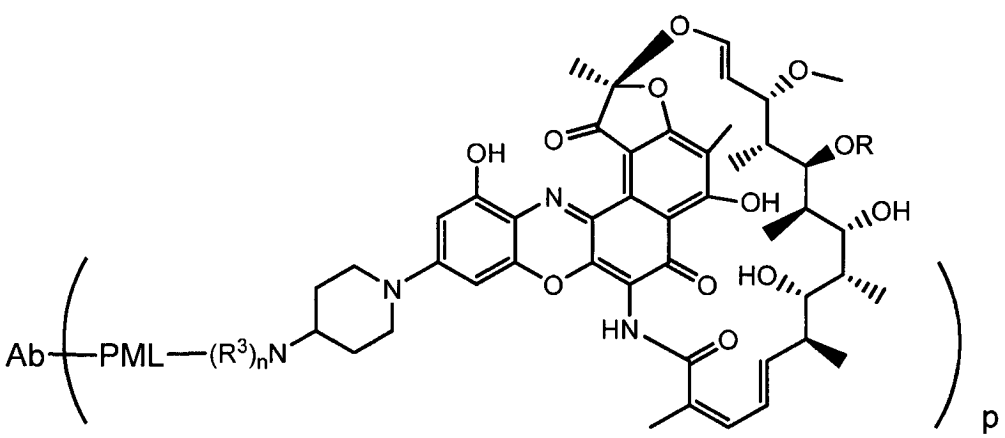


wherein

R^5 is independently selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:

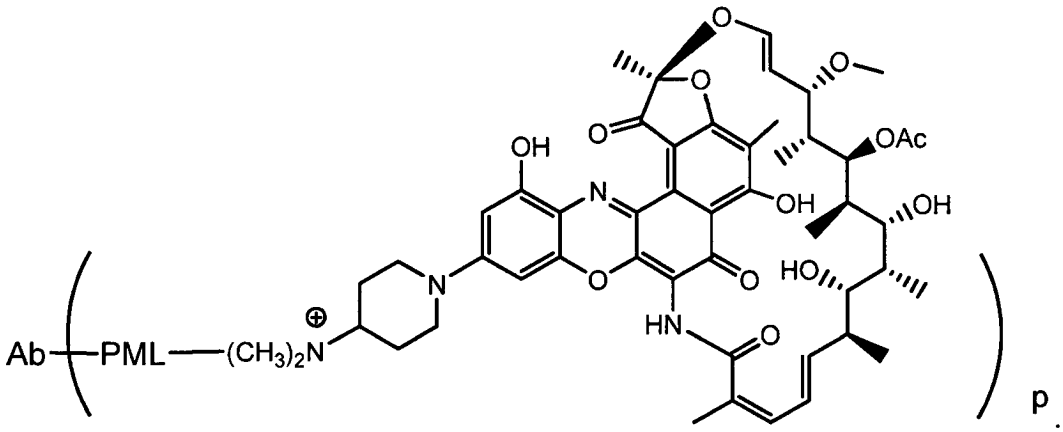


wherein

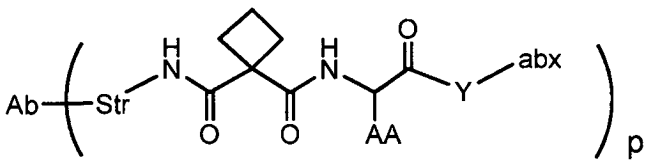
R^3 is independently selected from H and C_1–C_{12} alkyl; and

n is 1 or 2.

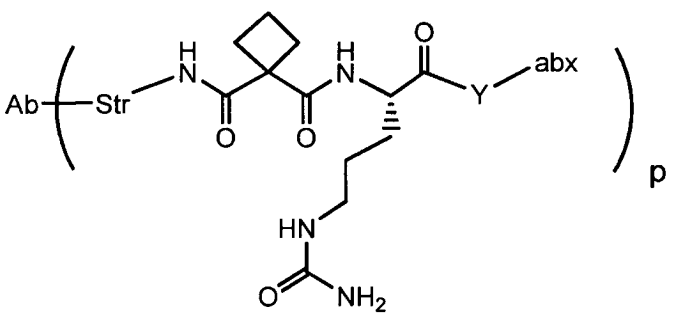
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



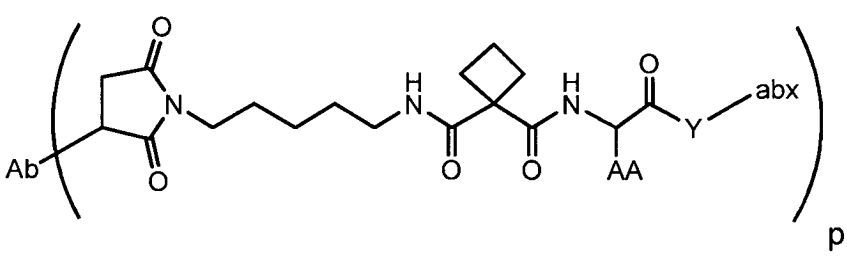
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



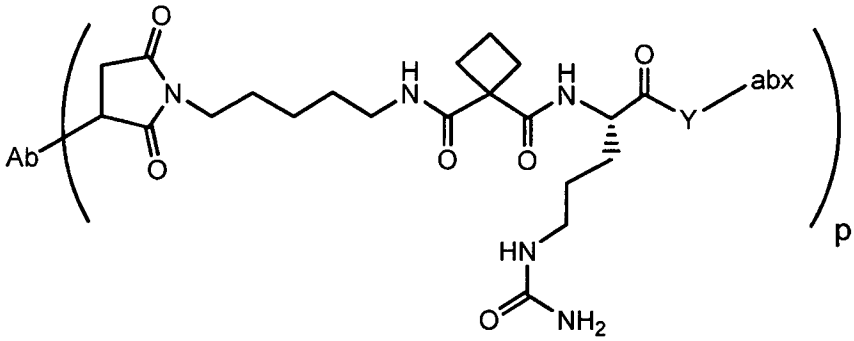
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



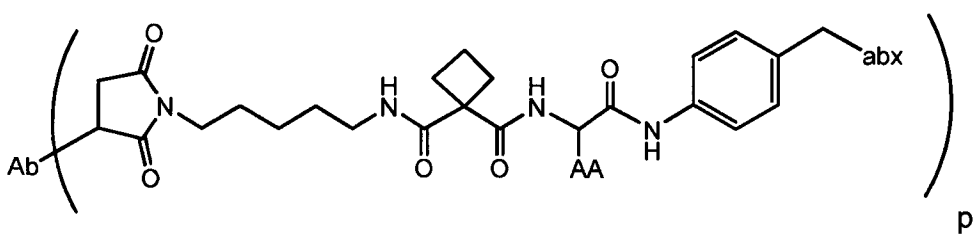
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



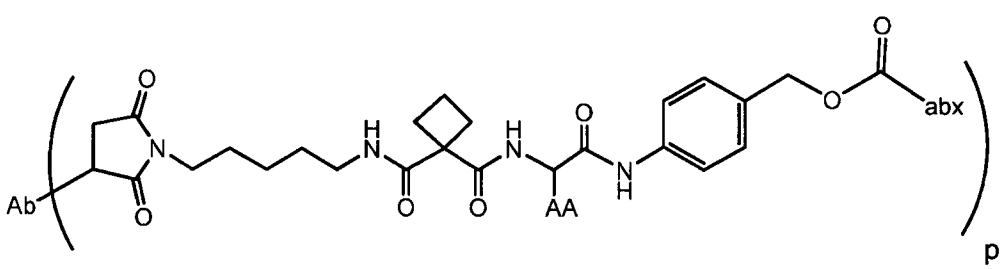
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formula:



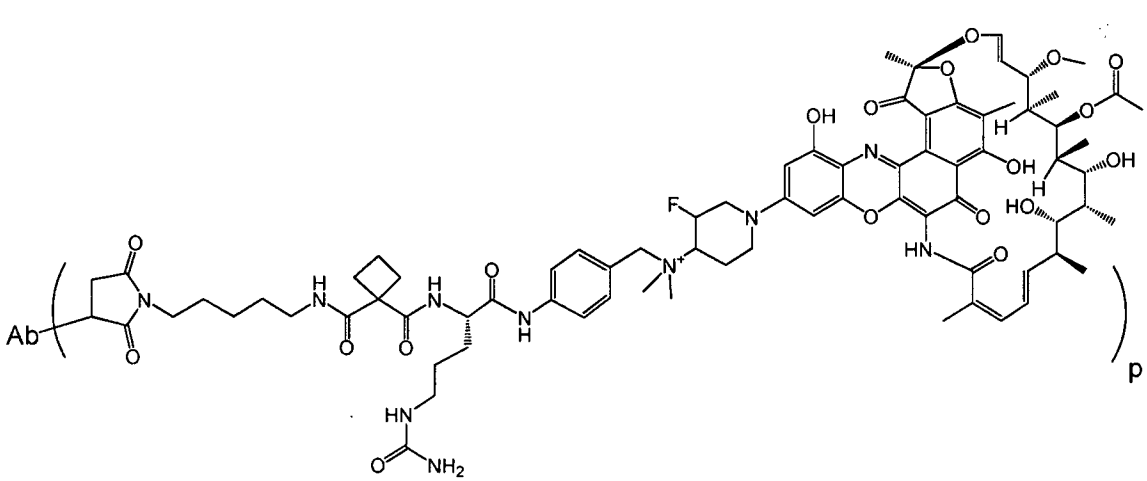
Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formulas:



and



Another embodiment of the antibody-antibiotic conjugate (AAC) compounds of the invention includes the formulas:



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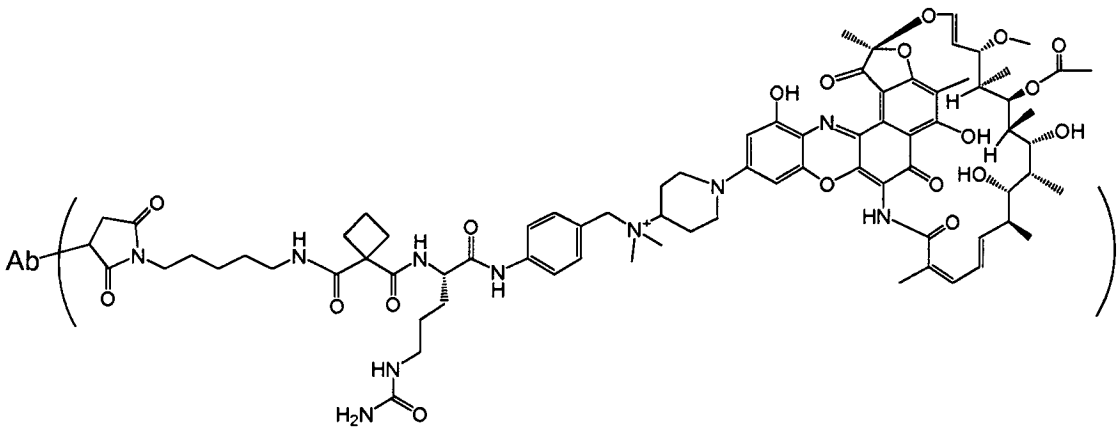

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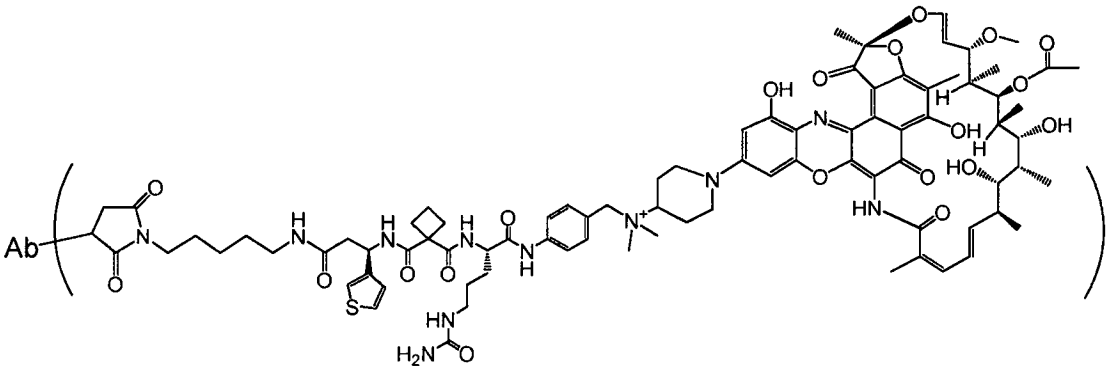

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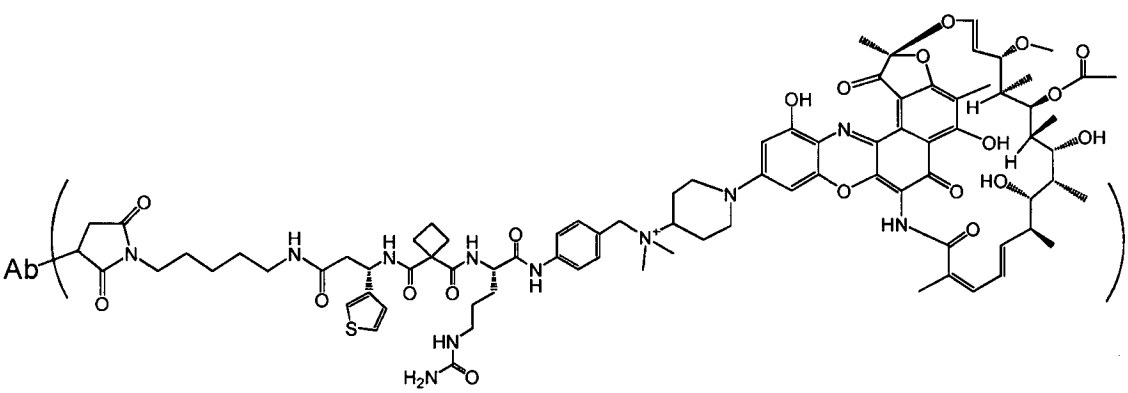

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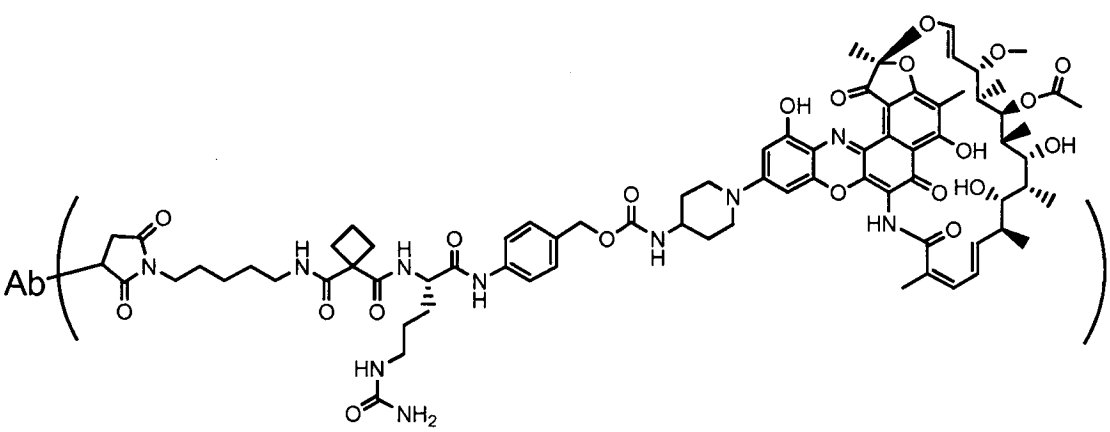
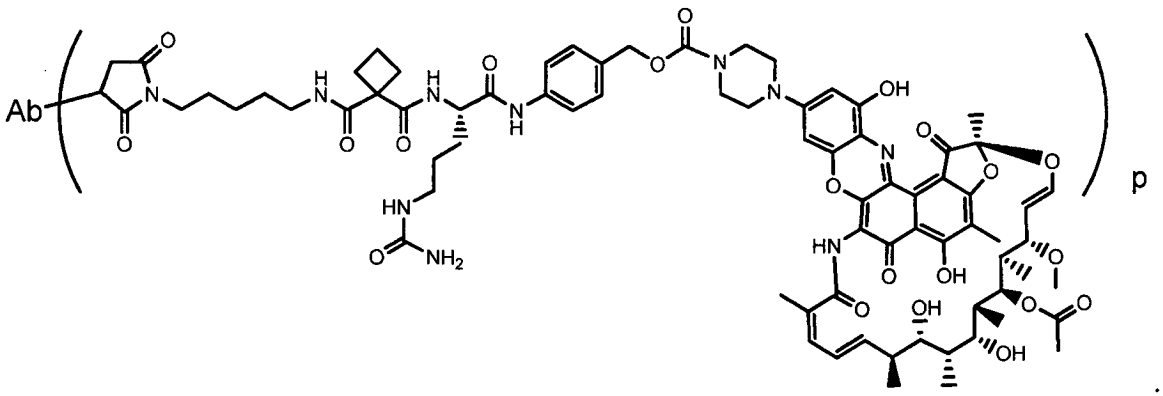


;

and



ANTIBIOTIC LOADING OF AAC

Antibiotic loading is represented by p, the average number of antibiotic (abx) moieties per antibody in a molecule of Formula I. Antibiotic loading may range from 1 to 20 antibiotic moieties (D) per antibody. The AAC of Formula I include collections or a pool of antibodies conjugated with a range of antibiotic moieties, from 1 to 20. The average number of antibiotic moieties per antibody in preparations of AAC from conjugation reactions may be characterized by conventional means such as mass spectroscopy, ELISA assay, and HPLC. The quantitative distribution of AAC in terms of p may also be determined. In some instances, separation, purification, and characterization of homogeneous AAC where p is a certain value from AAC with other antibiotic loadings may be achieved by means such as reverse phase HPLC or electrophoresis.

For some antibody-antibiotic conjugates, p may be limited by the number of attachment sites on the antibody. For example, where the attachment is a cysteine thiol, as in the exemplary embodiments above, an antibody may have only one or several cysteine thiol groups, or may have only one or several sufficiently reactive thiol groups through which a linker may be attached. In certain embodiments, higher antibiotic loading, e.g. p >5, may cause aggregation, insolubility, toxicity, or loss of cellular permeability of certain antibody-antibiotic conjugates. In certain embodiments, the antibiotic loading for an AAC of the invention ranges from 1 to about 8; from about 2 to about 6; from about 2 to about 4; or from about 3 to about 5; about 4; or about 2.

In certain embodiments, fewer than the theoretical maximum of antibiotic moieties are conjugated to an antibody during a conjugation reaction. An antibody may contain, for example, lysine residues that do not react with the antibiotic-linker intermediate or linker reagent, as discussed below. Generally, antibodies do not contain many free and reactive cysteine thiol

groups which may be linked to an antibiotic moiety; indeed most cysteine thiol residues in antibodies exist as disulfide bridges. In certain embodiments, an antibody may be reduced with a reducing agent such as dithiothreitol (DTT) or tricarbonylethylphosphine (TCEP), under partial or total reducing conditions, to generate reactive cysteine thiol groups. In certain embodiments, an antibody is subjected to denaturing conditions to reveal reactive nucleophilic groups such as lysine or cysteine.

The loading (antibiotic/antibody ratio, "AAR") of an AAC may be controlled in different ways, e.g., by: (i) limiting the molar excess of antibiotic-linker intermediate or linker reagent relative to antibody, (ii) limiting the conjugation reaction time or temperature, and (iii) partial or limiting reductive conditions for cysteine thiol modification. "DAR" if referred to herein or in the figures shall mean the same as "AAR".

It is to be understood that where more than one nucleophilic group reacts with an antibiotic-linker intermediate or linker reagent followed by antibiotic moiety reagent, then the resulting product is a mixture of AAC compounds with a distribution of one or more antibiotic moieties attached to an antibody. The average number of antibiotics per antibody may be calculated from the mixture by a dual ELISA antibody assay, which is specific for antibody and specific for the antibiotic. Individual AAC molecules may be identified in the mixture by mass spectroscopy and separated by HPLC, e.g. hydrophobic interaction chromatography (*see, e.g.*, McDonagh et al (2006) Prot. Engr. Design & Selection 19(7):299-307; Hamblett et al (2004) Clin. Cancer Res. 10:7063-7070; Hamblett, K.J., et al. "Effect of drug loading on the pharmacology, pharmacokinetics, and toxicity of an anti-CD30 antibody-drug conjugate," Abstract No. 624, American Association for Cancer Research, 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004; Alley, S.C., et al. "Controlling the location of drug attachment in antibody-drug conjugates," Abstract No. 627, American Association for Cancer Research, 2004 Annual Meeting, March 27-31, 2004, Proceedings of the AACR, Volume 45, March 2004). In certain embodiments, a homogeneous AAC with a single loading value may be isolated from the conjugation mixture by electrophoresis or chromatography. Cysteine-engineered antibodies of the invention enable more homogeneous preparations since the reactive site on the antibody is primarily limited to the engineered cysteine thiol. In one embodiment, the average number of antibiotic moieties per antibody is in the range of about 1 to about 20. In some embodiments the range is selected and controlled from about 1 to 4.

METHODS OF PREPARING ANTIBODY-ANTIBIOTIC CONJUGATES

An AAC of Formula I may be prepared by several routes employing organic chemistry reactions, conditions, and reagents known to those skilled in the art, including: (1) reaction of a nucleophilic group of an antibody with a bivalent linker reagent to form Ab-L via a covalent bond, followed by reaction with an antibiotic moiety (abx); and (2) reaction of a nucleophilic group of an antibiotic moiety with a bivalent linker reagent, to form L-abx, via a covalent bond, followed by reaction with a nucleophilic group of an antibody. Exemplary methods for preparing an AAC of Formula I via the latter route are described in US 7498298, which is expressly incorporated herein by reference.

Nucleophilic groups on antibodies include, but are not limited to: (i) N-terminal amine groups, (ii) side chain amine groups, e.g. lysine, (iii) side chain thiol groups, e.g. cysteine, and (iv) sugar hydroxyl or amino groups where the antibody is glycosylated. Amine, thiol, and hydroxyl groups are nucleophilic and capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups. Certain antibodies have reducible interchain disulfides, i.e. cysteine bridges. Antibodies may be made reactive for conjugation with linker reagents by treatment with a reducing agent such as DTT (dithiothreitol) or tricarbonylethylphosphine (TCEP), such that the antibody is fully or partially reduced. Each cysteine bridge will thus form, theoretically, two reactive thiol nucleophiles. Additional nucleophilic groups can be introduced into antibodies through modification of lysine residues, e.g., by reacting lysine residues with 2-iminothiolane (Traut's reagent), resulting in conversion of an amine into a thiol. Reactive thiol groups may be introduced into an antibody by introducing one, two, three, four, or more cysteine residues (e.g., by preparing variant antibodies comprising one or more non-native cysteine amino acid residues).

Antibody-antibiotic conjugates of the invention may also be produced by reaction between an electrophilic group on an antibody, such as an aldehyde or ketone carbonyl group, with a nucleophilic group on a linker reagent or antibiotic. Useful nucleophilic groups on a linker reagent include, but are not limited to, hydrazide, oxime, amino, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide. In one embodiment, an antibody is modified to introduce electrophilic moieties that are capable of reacting with nucleophilic substituents on the linker reagent or antibiotic. In another embodiment, the sugars of glycosylated antibodies may be oxidized, e.g. with periodate oxidizing reagents, to form aldehyde or ketone groups which may react with the amine group of linker reagents or antibiotic

moieties. The resulting imine Schiff base groups may form a stable linkage, or may be reduced, e.g. by borohydride reagents to form stable amine linkages. In one embodiment, reaction of the carbohydrate portion of a glycosylated antibody with either galactose oxidase or sodium meta-periodate may yield carbonyl (aldehyde and ketone) groups in the antibody that can react with appropriate groups on the antibiotic (Hermanson, Bioconjugate Techniques). In another embodiment, antibodies containing N-terminal serine or threonine residues can react with sodium meta-periodate, resulting in production of an aldehyde in place of the first amino acid (Geoghegan & Stroh, (1992) *Bioconjugate Chem.* 3:138-146; US 5362852). Such an aldehyde can be reacted with an antibiotic moiety or linker nucleophile.

Nucleophilic groups on an antibiotic moiety include, but are not limited to: amine, thiol, hydroxyl, hydrazide, oxime, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide groups capable of reacting to form covalent bonds with electrophilic groups on linker moieties and linker reagents including: (i) active esters such as NHS esters, HOBt esters, haloformates, and acid halides; (ii) alkyl and benzyl halides such as haloacetamides; (iii) aldehydes, ketones, carboxyl, and maleimide groups.

The antibody-antibiotic conjugates (AAC) in Table 3 were prepared by conjugation of the described rF1 antibodies and linker-antibiotic intermediates of Table 2, and according to the described methods in Example 18. AAC were tested for efficacy by *in vitro* macrophage assay (Example 19) and *in vivo* mouse kidney model (Example 20).

Table 3 rF1 Antibody-PML-antibiotic conjugates (AAC)

AAC No.	AAC formula	linker-abx PLA No.	AAR *
101	thio-rF1-LC-V205C-MC-(CBDK-cit)-PAB-(dimethyl, fluoropipBOR)	PLA-1	2.0
102	thio-rF1-HC-121C, LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR)	PLA-2	3.9
103	thio-rF1-LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR)	PLA-2	1.9
104	thio-rF1-HC-A121C, LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR)	PLA-2	3.7

* AAR = antibiotic/antibody ratio average

Wild-type ("WT"), cysteine engineered mutant antibody ("thio"), light chain ("LC"), heavy chain ("HC"), 6-maleimidocaproyl ("MC"), maleimidopropanoyl ("MP"), cyclobutyldiketo ("CBDK"), citrulline ("cit"), cysteine ("cys"), p-aminobenzyl ("PAB"), and p-aminobenzyloxycarbonyl ("PABC")

METHODS OF TREATING AND PREVENTING INFECTIONS WITH ANTIBODY-ANTIBIOTIC CONJUGATES

The rF1-AAC of the invention are useful as antimicrobial agents effective against human and veterinary Staphylococci, for example S. aureus, S. saprophyticus and S. simulans. In a specific aspect, the AAC of the invention are useful to treat *S. aureus* infections.

Following entry into the bloodstream, *S. aureus* can cause metastatic infection in almost any organ. Secondary infections occur in about one-third of cases before the start of therapy (Fowler et al., (2003) Arch. Intern. Med. 163:2066-2072), and even in 10% of patients after the start of therapy (Khatib et al., (2006) Scand. J. Infect. Dis., 38:7-14). Hallmarks of infections are large reservoirs of pus, tissue destruction, and the formation of abcesses (all of which contain large quantities of neutrophils). About 40% of patients develop complications if the bacteremia persists beyond three days.

The proposed mechanism of action of an AAC has been described above (under subheading Antibody-Antibiotic Conjugates). The rF1 antibody-antibiotic conjugates (AAC) of the invention have significant therapeutic advantages for treating intracellular pathogens. The AAC linker is cleaved by exposure to phagolysosomal enzymes, releasing an active antibiotic. Due to the confined space and relatively high local antibiotic concentration (about 10^4 per bacterium), the result is that the phagolysosome no longer supports the survival of the intracellular pathogen. Because the AAC is essentially an inactive prodrug, the therapeutic index of the antibiotic can be extended relative to the free (unconjugated) form. The antibody provides pathogen specific targeting, while the cleavable linker is cleaved under conditions specific to the intracellular location of the pathogen. The effect can be both directly on the opsonized pathogen as well as other pathogens that are co-localized in the phagolysosome. Antibiotic tolerance is the ability of a disease-causing pathogen to resist killing by antibiotics and other antimicrobials and is mechanistically distinct from multidrug resistance (Lewis K (2007). "Persister cells, dormancy and infectious disease". *Nature Reviews Microbiology* 5 (1): 48–56. doi:10.1038/nrmicro1557). Rather, this form of tolerance is caused by a small sub-population of microbial cells called persisters (Bigger JW (14 October 1944). "Treatment of staphylococcal infections with penicillin by intermittent sterilization". *Lancet* 244 (6320): 497–500). These cells are not multidrug resistant in the classical sense, but rather are dormant cells that are tolerant to antibiotic treatment that can kill their genetically identical siblings. This antibiotic tolerance is induced by a non-or extremely slow dividing physiological state. When antimicrobial treatment fails to eradicate these persister cells, they become a reservoir for

recurring chronic infections. The antibody-antibiotic conjugates of the invention possess a unique property to kill these persister cells and suppress the emergence of multidrug tolerant bacterial populations.

In another embodiment, the rF1-AAC of the invention may be used to treat infection regardless of the intracellular compartment in which the pathogen survives.

In another embodiment, rF1-AACs of the invention could also be used to target Staphylococci bacteria in planktonic or biofilm form. Bacterial infections treatable with antibody-antibiotic conjugates (AAC) of the invention include treating bacterial pulmonary infections, such as S. aureus pneumonia, osteomyelitis, recurrent rhinosinusitis, bacterial endocarditis, bacterial ocular infections, such as trachoma and conjunctivitis, heart, brain or skin infections, infections of the gastrointestinal tract, such as travellers' diarrhea, ulcerative colitis, irritable bowel syndrome (IBS), Crohn's disease, and IBD (inflammatory bowel disease) in general, bacterial meningitis, and abscesses in any organ, such as muscle, liver, meninges, or lung. The bacterial infections can be in other parts of the body like the urinary tract, the bloodstream, a wound or a catheter insertion site. The AACs of the invention are useful for difficult-to-treat infections that involve biofilms, implants or sanctuary sites (e.g., osteomyelitis and prosthetic joint infections), and high mortality infections such as hospital acquired pneumonia and bacteremia. Vulnerable patient groups that can be treated to prevent Staphylococcal aureus infection include hemodialysis patients, immune-compromised patients, patients in intensive care units, and certain surgical patients. In another aspect, the invention provides a method of killing, treating, or preventing a microbial infection in an animal, preferably a mammal, and most preferably a human, that includes administering to the animal an rF1 AAC or pharmaceutical formulation of an AAC of the invention. The invention further features treating or preventing diseases associated with or which opportunistically result from such microbial infections. Such methods of treatment or prevention may include the oral, topical, intravenous, intramuscular, or subcutaneous administration of a composition of the invention. For example, prior to surgery or insertion of an IV catheter, in ICU care, in transplant medicine, with or post cancer chemotherapy, or other activities that bear a high risk of infection, the AAC of the invention may be administered to prevent the onset or spread of infection.

The bacterial infection may be caused by bacteria with an active and inactive form, and the AAC is administered in an amount and for a duration sufficient to treat both the active and the inactive, latent form of the bacterial infection, which duration is longer than is needed to treat the active form of the bacterial infection.

An aspect of the invention is a method of treating a patient infected with S. aureus and/or Listeria monocytogenes by administering a therapeutically effective amount of an rF1-AAC of the invention. The invention also contemplates a method of preventing infections by one or more of S. aureus or S. Epidermidis, or S. saprophyticus or S. simulans by administering a therapeutically effective amount of an rF1-AAC of the invention in hospital settings such as surgery, burn patient, and organ transplantation.

The patient needing treatment for a bacterial infection as determined by a physician of skill in the art may have already been, but does not need to be diagnosed with the kind of bacteria that he/she is infected with. Since a patient with a bacterial infection can take a turn for the worse very quickly, in a matter of hours, the patient upon admission into the hospital can be administered the rF1-AACs of the invention along with one or more standard of care Abx such as vancomycin or ciprofloxacin. When the diagnostic results become available and indicate the presence of, e.g., S. aureus in the infection, the patient can continue with treatment with the rF1 AAC. Therefore, in one embodiment of the method of treating a bacterial infection or specifically a S. aureus infection, the patient is administered a therapeutically effective amount of an rF1 AAC. In the methods of treatment or prevention of the present invention, an AAC of the invention can be administered as the sole therapeutic agent or in conjunction with other agents such as those described below. The AACs of the invention show superiority to vancomycin in the treatment of MRSA in pre-clinical models. Comparison of AACs to SOC can be measured, e.g., by a reduction in mortality rate. The patient being treated would be assessed for responsiveness to the AAC treatment by a variety of measurable factors. Examples of signs and symptoms that clinicians might use to assess improvement in their patients includes the following: normalization of the white blood cell count if elevated at diagnosis, normalization of body temperature if elevated (fever) at the time of diagnosis, clearance of blood cultures, visual improvement in wound including less erythema and drainage of pus, reduction in ventilator requirements such as requiring less oxygen or reduced rate of ventilation in a patient who is ventilated, coming off of the ventilator entirely if the patient is ventilated at the time of diagnosis, use of less medications to support a stable blood pressure if these medications were required at the time of diagnosis, normalization of lab abnormalities that suggest end-organ failure such as elevated creatinine or liver function tests if they were abnormal at the time of diagnosis, and improvement in radiologic imaging (e.g. chest x-ray that previously suggested pneumonia showing resolution). In a patient in the ICU, these factors might be measured at least daily. Fever is monitored closely as is white blood cell count including absolute neutrophil

counts as well as evidence that a "left shift" (appearance of blasts indicating increased neutrophil production in response to an active infection) has resolved.

In the context of the present methods of treatment of the invention, a patient with a bacterial infection is considered to be treated if there is significant measurable improvement as assessed by the physician of skill in the art, in at least two or more of the preceding factors compared to the values, signs or symptoms before or at the start of treatment or at the time of diagnosis. In some embodiments, there is measurable improvement in 3, 4, 5, 6 or more of the aforementioned factors. If some embodiments, the improvement in the measured factors is by at least 50%, 60%, 70%, 80%, 90%, 95% or 100% compared to the values before treatment. Typically, a patient can be considered completely treated of the bacterial infection (e.g., S. aureus infection) if the patient's measurable improvements include the following: i) repeat blood or tissue cultures (typically several) that do not grow out the bacteria that was originally identified ; ii) fever is normalized; iii) WBC is normalized; and iv) evidence that end-organ failure (heart, lungs, liver, kidneys, vascular collapse) has resolved either fully or partially given the pre-existent co-morbidities that the patient had.

Dosing. In any of the foregoing aspects, in treating an infected patient, the dosage of an AAC is normally about 0.001 to 1000 mg/kg/day. In one embodiment the patient with a bacterial infection is treated at an AAC dose in the range of about 1 mg/kg to about 150mg/kg, typically about 5mg/kg to about 150mg/kg, more specifically, 25mg/kg to 125 mg/kg, 50mg/kg to 125mg/kg, even more specifically at about 50mg/kg to 100mg/kg. The AAC may be given daily (e.g., a single dose of 5 to 50 mg/kg/day) or less frequently (e.g., a single dose of 5, 10, 25 or 50 mg/kg/week). One dose may be split over 2 days, for example, 25mg/kg on one day and 25mg/kg the next day. The patient can be administered a dose once every 3 days (q3D), once a week to every other week (qOW), for a duration of 1-8 weeks. In one embodiment, the patient is administered an AAC of the invention via IV once a week for 2-6 weeks with standard of care (SOC) to treat the bacterial infection such as a staph A infection. Treatment length would be dictated by the condition of the patient or the extent of the infection, e.g. a duration of 2 weeks for uncomplicated bacteremia, or 6 weeks for bacteremia with endocarditis.

In one embodiment, an AAC administered at an initial dose of 2.5 to 100 mg/kg for one to seven consecutive days, followed by a maintenance dose of 0.005 to 10 mg/kg once every one to seven days for one month.

Route of administration. For treating the bacterial infections, the AACs of the invention can be administered at any of the preceding dosages intravenously (i.v.) or subcutaneously. In one embodiment, the rF1-AAC is administered intravenously. In a specific embodiment, the rF1-AAC is administered via i.v., wherein the rF1 antibody is one selected from the group of Abs with amino acid sequences as disclosed under SDR and rF1Abs and Tables 4A and 4B.

Combination therapy. An AAC may be administered in conjunction with one or more additional, e.g. second, therapeutic or prophylactic agents as appropriate as determined by the physician treating the patient.

In one embodiment, the second antibiotic administered in combination with the antibody-antibiotic conjugate compound of the invention is selected from the structural classes: : (i) aminoglycosides; (ii) beta-lactams; (iii) macrolides/cyclic peptides; (iv) tetracyclines; (v) fluoroquinolines/fluoroquinolones; (vi) and oxazolidinones. See: Shaw, K. and Barbachyn, M. (2011) Ann. N.Y. Acad. Sci. 1241:48-70; Sutcliffe, J. (2011) Ann. N.Y. Acad. Sci. 1241:122-152.

In one embodiment, the second antibiotic administered in combination with the antibody-antibiotic conjugate compound of the invention is selected from clindamycin, novobiocin, retapamulin, daptomycin, GSK-2140944, CG-400549, sitafloxacin, teicoplanin, triclosan, napthyridone, radezolid, doxorubicin, ampicillin, vancomycin, imipenem, doripenem, gemcitabine, dalbavancin, and azithromycin.

Additional examples of these additional therapeutic or prophylactic agents are anti-inflammatory agents (e.g., non-steroidal anti-inflammatory drugs (NSAIDs; e.g., detoprofen, diclofenac, diflunisal, etodolac, fenoprofen, flurbiprofen, ibuprofen, indomethacin, ketoprofen, meclofenameate, mefenamic acid, meloxicam, nabumeone, naproxen sodium, oxaprozin, piroxicam, sulindac, tolmetin, celecoxib, rofecoxib, aspirin, choline salicylate, salsalte, and sodium and magnesium salicylate) and steroids (e.g., cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, prednisone, triamcinolone)), antibacterial agents (e.g., azithromycin, clarithromycin, erythromycin, gatifloxacin, levofloxacin, amoxicillin, metronidazole, penicillin G, penicillin V, methicillin, oxacillin, cloxacillin, dicloxacillin, nafcillin, ampicillin, carbenicillin, ticarcillin, mezlocillin, piperacillin, azlocillin, temocillin, cepalothin, cephapirin, cephradine, cephaloridine, cefazolin, cefamandole, cefuroxime, cephalexin, cefprozil, cefaclor, loracarbef, cefoxitin, cefmatozole, cefotaxime, ceftizoxime, ceftriaxone, cefoperazone, ceftazidime, cefixime, cefpodoxime, ceftibuten, cefdinir, cefpirome, cefepime, BAL5788, BAL9141, imipenem, ertapenem, meropenem, astreonam, clavulanate,

sulbactam, tazobactam, streptomycin, neomycin, kanamycin, paromycin, gentamicin, tobramycin, amikacin, netilmicin, spectinomycin, sisomicin, dibekalin, isepamicin, tetracycline, chlortetracycline, demeclocycline, minocycline, oxytetracycline, methacycline, doxycycline, telithromycin, ABT-773, lincomycin, clindamycin, vancomycin, oritavancin, dalbavancin, teicoplanin, quinupristin and dalfopristin, sulphanilamide, para-aminobenzoic acid, sulfadiazine, sulfisoxazole, sulfamethoxazole, sulfathalidine, linezolid, nalidixic acid, oxolinic acid, norfloxacin, perfloxacin, enoxacin, ofloxacin, ciprofloxacin, temafloxacin, lomefloxacin, fleroxacin, grepafloxacin, sparfloxacin, trovafloxacin, clinafloxacin, moxifloxacin, gemifloxacin, sitafloxacin, daptomycin, garenoxacin, ramoplanin, faropenem, polymyxin, tigecycline, AZD2563, or trimethoprim), antibacterial antibodies including antibodies to the same or different antigen from the AAC targeted Ag, platelet aggregation inhibitors (e.g., abciximab, aspirin, cilostazol, clopidogrel, dipyridamole, eptifibatide, ticlopidine, or tirofiban), anticoagulants (e.g., dalteparin, danaparoid, enoxaparin, heparin, tinzaparin, or warfarin), antipyretics (e.g., acetaminophen), or lipid lowering agents (e.g., cholestyramine, colestipol, nicotinic acid, gemfibrozil, probucol, ezetimibe, or statins such as atorvastatin, rosuvastatin, lovastatin simvastatin, pravastatin, cerivastatin, and fluvastatin). In one embodiment the AAC of the invention is administered in combination with standard of care (SOC) for S. aureus (including methicillin-resistant and methicillin-sensitive strains). MSSA is usually typically treated with nafcillin or oxacillin and MRSA is typically treated with vancomycin or cefazolin.

These additional agents may be administered within 14 days, 7 days, 1 day, 12 hours, or 1 hour of administration of an AAC, or simultaneously therewith. The additional therapeutic agents may be present in the same or different pharmaceutical compositions as an AAC. When present in different pharmaceutical compositions, different routes of administration may be used. For example, an AAC may be administered intravenous or subcutaneously, while a second agent may be administered orally.

PHARMACEUTICAL FORMULATIONS

The present invention also provides pharmaceutical compositions containing the rF1-AAC, and to methods of treating a bacterial infection using the pharmaceutical compositions containing AAC. Such compositions may further comprise suitable excipients, such as pharmaceutically acceptable excipients (carriers) including buffers, acids, bases, sugars, diluents, glidants, preservatives and the like, which are well known in the art and are described herein. The present methods and compositions may be used alone or in combinations with other conventions methods and/or agents for treating infectious diseases. In some embodiments, a

pharmaceutical formulation comprises 1) a rF1-AAC of the invention, and 2) a pharmaceutically acceptable carrier. In some embodiments, a pharmaceutical formulation comprises 1) an AAC of the invention and optionally, 2) at least one additional therapeutic agent.

Pharmaceutical formulations comprising an AAC of the invention are prepared for storage by mixing the AAC having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)) in the form of aqueous solutions or lyophilized or other dried formulations. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, histidine and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride); phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.,* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG). Pharmaceutical formulations to be used for *in vivo* administration are generally sterile, readily accomplished by filtration through sterile filtration membranes.

Active ingredients may also be entrapped in microcapsule prepared, for example, by co-acervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacrylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody or AAC of the invention, which matrices are in the form of shaped articles, *e.g.,* films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No.

3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies or AAC remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37 °C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

An AAC may be formulated in any suitable form for delivery to a target cell/tissue. For example, AACs may be formulated as liposomes, a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., (1985) *Proc. Natl. Acad. Sci. USA* 82:3688; Hwang et al., (1980) *Proc. Natl Acad. Sci. USA* 77:4030; US 4485045; US 4544545; WO 97/38731; US 5013556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter.

MATERIALS AND METHODS

Bacterial strains and culture:

All experiments were done with MRSA-USA300 NRS384 obtained from NARSA (http://www.narsa.net/control/member/repositories) unless noted otherwise.

Bacteria were grown on tryptic soy agar plates supplemented with 5% sheep blood (TSA plates) for 18 h at 37 °C. For liquid cultures, single colonies from TSA plates were inoculated into tryptic soy broth (TSB) and incubated at 37 °C while shaking at 200 rpm for 18 h; 100 fold dilutions of these cultures in fresh TSB were further subcultured for various times.

MIC determinations for extracellular bacteria

The MIC for extracellular bacteria was determined by preparing serial 2-fold dilutions of the antibiotic in Tryptic Soy Broth. Dilutions of the antibiotic were made in quadruplicate in 96 well culture dishes. MRSA (NRS384 strain of USA300) was taken from an exponentially growing culture and diluted to $1x10^4$ CFU/mL. The bacteria was cultured in the presence of antibiotic for 18-24 hours with shaking at 37°C and bacterial growth was determined by reading the Optical Density (OD) at 630 nM. The MIC was determined to be the dose of antibiotic that inhibited bacterial growth by >90%.

MIC determinations for intracellular bacteria

Intracellular MIC was determined on bacteria that were sequestered inside mouse peritoneal macrophages (see below for generation of murine peritoneal macrophages). Macrophages were plated in 24 well culture dishes at a density of $4x10^5$ cells/mL and infected with MRSA at a ratio of 10-20 bacteria per macrophage. Macrophage cultures were maintained in growth media supplemented with 50 ug/mL of gentamycin (an antibiotic that is active only on extracellular bacteria) to inhibit the growth of extracellular bacteria and test antibiotics were added to the growth media 1 day after infection. The survival of intracellular bacteria was assessed 24 hours after addition of the antibiotics. Macrophages were lysed with Hanks Buffered Saline Solution supplemented with .1% Bovine Serum Albumin and .1% Triton-X, and serial dilutions of the lysate were made in Phosphate Buffered Saline solution containing .05% Tween-20. The number of surviving intracellular bacteria was determined by plating on Tryptic Soy Agar plates with 5% defibrinated sheep blood.

Bacterial cell wall preparations (CWP), immunoblotting, and ELISA

CWP were generated by incubating 40 mg of pelleted *S. aureus* or *S. epidermidis* per mL of 10 mM Tris-HCl (pH 7.4) supplemented with 30% raffinose, 100 μg/ml of lysostaphin (Cell Sciences, Canton, MA), and EDTA-free protease inhibitor cocktail (Roche, Pleasanton, CA), for 30 min at 37°C. The lysates were centrifuged at 11,600 x g for 5 min, and the supernatants containing cell wall components were collected. For immunoprecipitation, CWP were diluted 4 times in NP-40 buffer (120 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40, complete protease inhibitor cocktail (Roche) and 2 mM dithiothreitol) containing 1 μg/mL of indicated primary antibodies and incubated for 2 h at 4°C, followed by a 1 h incubation with Protein A/G agarose (Thermo, Waltham, MA). Whole cell lysates (WCL) were generated by a 30 min incubation at 37°C in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 100 μg/ml of lysostaphin, 1% Triton-X100

(Thermo) and EDTA-free protease inhibitor cocktail. For immunoblot analysis, proteins were separated on a 4-12% Tris-glycine gel, and transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA), followed by blotting with indicated primary antibodies (1 μg/mL). Antibodies used are listed in Table 1. Lectin studies were performed by immunoprecipitating filtered (0.2 micron) overnight culture supernatants with concanavalin A (ConA)- or sWGA-agarose beads (Vector Labs, Burlingame, CA) supplemented with 0.1 mM $CaCl_2$ and 0.01 mM $MnCl_2$.

ELISA experiments were performed using standard protocols. Briefly, plates which were pre-coated with CWP were reacted with human IgG preparations, ie. purified human IgG (Sigma), intravenous immunoglobulin Gammagard Liquid (Baxter, Westlake Village, CA), pooled serum from healthy donors or from MRSA patients (both generated in-house). The concentrations of anti-staphylococcal IgG present in the serum or purified IgG were calculated by using a calibration curve that was generated with known concentrations of mAb 28.9.9 against peptidoglycan.

Treatment of bacteria with human neutrophil proteases or lysosomal extracts from human neutrophils and cultured cells

Lysosomal extracts were isolated from human neutrophils, THP-1 cells, and RAW cells, using a Lysosome Enrichment kit (Thermo). A total of $5x10^7$ cells was used to obtain 300 to 500 microgram of total proteins in the lysosomes. Protease inhibitors were omitted from all steps to maintain protease activity in the lysosomes. The plasma membranes of the cells were disrupted by 30 strokes using a dounce homogenizer (Wheaton, Millville, NJ). The homogenate was centrifuged at 500 x g for 5 min to obtain postnuclear supernatant, which was loaded onto the top of a gradient of 8%, 20%, 23%, 27% and 30% (from top to bottom) of iodixanol. After ultracentrifugation at 145,000 x g for 2 h at 4°C, we obtained the lysosomes layered between 8% and 20% iodixanol. This lysosomal fraction was diluted into PBS and pelleted by centrifugation at 18,000 x g for 30 min at 4 °C. The lysosomal pellets were washed with PBS and lysed in 2% CHAPS with Tris-buffered saline to obtain lysosomal extracts.

To analyze the cleavage of SDR proteins by host proteases, *S. aureus* bacteria were treated with 50 nM of purified human neutrophil serine proteases or 0.1 mg/ml of neutrophil lysosomal extracts in 50 mM Tris (pH 8.0) with 150 mM NaCl and 2mM $CaCl_2$; or with 0.1 mg/ml of RAW or THP-1 lysosomal extracts in 50 mM NaCitrate with 100 mM NaCl and 2 mM DTT (pH 5.5). Cathepsin G inhibitor (Calbiochem, Billerica, MA) was added at 100 g/ml. These mixtures were incubated at 37°C for 30 minutes when using purified proteases or for 1 h when using lysosomal lysates, and centrifuged to pellet bacteria. The supernatants were analyzed

by immunoblotting to detect cleavage products. In some experiments, cell wall preparations were obtained from the remaining bacterial pellets and also analyzed by immunoblotting.

EXAMPLES

Example 1 Intracellular MRSA are protected from conventional antibiotics

To confirm the hypothesis that mammalian cells provide a protective niche for *S. aureus* in the presence of antibiotic therapy, the efficacy was compared of three major antibiotics that are currently used as standard of care (SOC) for invasive MRSA infections (vancomycin, daptomycin and linezolid) against extracellular planktonic bacteria versus bacteria sequestered inside murine macrophages (Table 1).

For extracellular bacteria, MRSA was cultured overnight in Tryptic Soy Broth, and the MIC was determined to be the minimum antibiotic dose that prevented growth. For intracellular bacteria, murine peritoneal macrophages were infected with MRSA and cultured in the presence of gentamycin to kill extracellular bacteria. Test antibiotics were added to the culture medium one day post infection, and the total number of surviving intracellular bacteria was determined 24 hours later. The expected serum concentrations for clinically relevant antibiotics was reported in Antimicrobial Agents, Andre Bryskier. ASM Press, Washington DC (2005).

Table 1: Minimum inhibitory concentrations (MIC) for several antibiotics on extracellular bacteria grown in liquid culture *vs.* intracellular bacteria sequestered inside murine macrophages.

Antibiotics (Abx)	Extracellular MRSA MIC (μg/mL)	Intracellular MRSA MIC (μg/mL)	Serum Cmax (μg/mL)
Vancomycin	1	>100	50
Daptomycin	4	>100	60
Linezolid	0.3	>20	20
Rifampicin	0.004	50	20

This analysis with a highly virulent community-acquired MRSA strain USA300 revealed that although extracellular MRSA is highly susceptible to growth inhibition by low

concentrations of vancomycin, daptomycin, and linezolid in liquid culture, all three antibiotics failed to kill the same strain of MRSA sequestered inside macrophages exposed to clinically achievable concentrations of the antibiotics. Even rifampicin, thought to be relatively effective at eliminating intracellular pathogens(Vandenbroek, P.V. (1989) Antimicrobial Drugs, Microorganisms, and Phagocytes. *Reviews of Infectious Diseases* **11**, 213-245), required a 6,000-fold higher dose to eliminate intracellular MRSA compared to the dose required to inhibit growth (MIC) of planktonic bacteria (Table 1), consistent with other studies showing that the majority of existing antibiotics are inefficient at killing intracellular *S. aureus* both in vitro and in vivo(Sandberg, A., Hessler, J.H., Skov, R.L., Blom, J. & Frimodt-Moller, N. (2009) "Intracellular activity of antibiotics against Staphylococcus aureus in a mouse peritonitis model" *Antimicrob Agents Chemother* **53**, 1874-1883).

Example 2 Dissemination of infection with intracellular MRSA

These experiments compared the virulence of intracellular bacteria *versus* an equivalent dose of free-living planktonic bacteria, and determined whether the intracellular bacteria are able to establish infection in the presence of vancomycin in vivo. Four cohorts of mice were infected by intravenous injection with roughly equivalent doses of *S. aureus* viable free bacteria (2.9 x 10^6) taken directly from broth culture or intracellular bacteria (1.8 x 10^6) sequestered inside host macrophages and neutrophils that were generated by peritoneal infection of donor mice (Fig. 1A) and selected groups were treated with vancomycin immediately after infection and then once per day. Mice were examined 4 days after infection for bacterial colonization in the kidney, an organ that is consistently colonized by *S. aureus* in mice [23]. In three independent experiments, equivalent or higher bacterial burdens in the kidneys of mice infected with intracellular bacteria compared to those infected with an equivalent dose of planktonic bacteria was observed (Fig. 1B). Surprisingly, it was found that infection with intracellular bacteria resulted in more consistent colonization of the brain, an organ that is not efficiently colonized following infection with planktonic bacteria in this model (Fig. 1C). Furthermore, intracellular bacteria, but not planktonic bacteria, were able to establish infection in the face of vancomycin therapy in this model (Fig. 1B, Fig.1C)

Further analyses in vitro addressed more quantitatively the extent to which intracellular survival facilitates antibiotic evasion. To this end, MG63 osteoblasts were infected with either planktonic MRSA or intracellular MRSA, in the presence of vancomycin.

Infection of osteoblasts or HBMEC. MG63 cell line was obtained from ATCC (CRL-1427) and maintained in RPMI 1640 tissue culture media supplemented with 10 mM Hepes and

10 % Fetal Calf Serum (RPMI-10). HBMEC cells (Catalog #1000) and ECM media (catalog# 1001) were obtained from SciencCell Research Labs (Carlsbad, CA). Cells were plated in 24 well tissue culture plates and cultured to obtain a confluent layer. On the day of the experiment, the cells were washed once in RPMI (without supplements). MRSA or infected peritoneal cells were diluted in complete RPMI-10 and vancomycin was added at 5 ug/mL immediately prior to infection. Peritoneal cells were added to the osteoblasts at $1x10^6$ peritoneal cells/mL. A sample of the cells was lysed with .1% triton-x to determine the actual concentration of live intracellular bacteria at the time of infection. The actual titer for all infections was determined by plating serial dilutions of the bacteria on Tryptic Soy Agar with 5% defibrinated sheep blood.

MRSA (free bacteria) was seeded in media, media + vancomycin, or media + vancomycin and plated on a monolayer of MG63 osteoblasts (Fig.1E) or Human Brain Microvascular Endothelial Cells (HBMEC, Fig.1F). Plates were centrifuged to promote contact of the bacteria with the monolayer. At each time point, the culture supernatant was collected to recover extracellular bacteria or adherent cells were lysed to release intracellular bacteria.

Planktonic bacteria exposed to vancomycin alone were efficiently killed. Surviving bacteria were not recovered after one day in culture (Fig. 1D). When a similar number of planktonic bacteria were plated on MG63 osteoblasts, a small number of surviving bacteria (approximately 0.06% of input) associated with the MG63 cells one day after infection, which had been protected from vancomycin by invasion of the osteoblasts, was recovered.

MRSA that were sequestered inside peritoneal cells showed a dramatic increase in both survival and efficiency of infection in the presence of vancomycin. About 15% of intracellular MRSA in the leukocytes survived under identical conditions where vancomycin had sterilized the cultures of planktonic bacteria. Intracellular bacteria also were better able to infect the monolayer of MG63 osteoblasts in the presence of vancomycin, resulting in a doubling of the bacteria recovered one day after exposure to vancomycin (Fig. 1D). Moreover, intracellular *S. aureus* were able to increase by almost 10-fold over a 24 hour period in MG63 cells (Fig. 1E), primary human brain endothelial cells (Fig. 1F), and A549 bronchial epithelial cells (not shown) under constant exposure to a concentration of vancomycin that killed free living bacteria. Although protected from antibiotic killing, bacterial growth did not occur in cultures of infected peritoneal macrophages and neutrophils (not shown). Together these data support that intracellular reservoirs of MRSA in myeloid cells can promote dissemination of infection to new sites, even in the presence of active antibiotic treatment, and intracellular growth can occur in endothelial and epithelial cells, even under conditions of constant antibiotic therapy.

Example 3 Generation of anti-SDR and other antibodies

For generation of mAb rF1, $CD19^+CD3^-CD27^+IgD^-IgA^-$ memory B cells were isolated from peripheral blood of an MRSA-infected donor using a FACSAria cell sorter (BD, San Jose, CA). Before viral transduction with B-cell lymphoma (Bcl)-xL and Bcl-6 genes, the memory cells were activated on CD40L-expressing mouse L fibroblasts in the presence of interleukin-21, as described previously in Kwakkenbos MJ, et al. (2010) Nat Med 16: 123-128. Transduced B cells were maintained in the same culture system. The use of donor blood was approved by the institutional committee. Monoclonal antibody (mAb) rF1 was selected from culture supernatants by reactivity with lysates of MSSA strain Newman by ELISA; positive wells were subcloned and re-tested by ELISA twice. Recombinant rF1 was generated by cloning the heavy and light chain variable regions with human IgG1 kappa constant regions using pcDNA3.1 (Invitrogen) and transfection into 293T cells (ATCC). Purified IgG was obtained from culture supernatants using protein A-coupled SEPHAROSE® (Invitrogen). The generation of mAb rF1 and its variants are described in US 8,617,556 (Beaumont et al.) and Hazenbos et al. (2103) *PLOS Pathogens* 9(10): 1-18, incorporated by reference herein in their entirety.

The human IgG1 mAbs SD2, SD3 and SD4 (all against glycosylated SDR proteins) and 4675 (human IgG1 anti-ClfA),were cloned from peripheral B cells from patients post *S. aureus* infection using the Symplex™ technology which conserves the cognate pairing of antibody heavy and light chains [34]. Both plasma and memory B-cells were used as genetic source for the recombinant full length IgG repertoires (manuscript in preparation). Individual antibody clones were expressed by transfection of mammalian cells [35]. Supernatants containing full length IgG1 antibodies were harvested after seven days and used to screen for antigen binding by ELISA. Antibodies 4675, SD2, SD3 and SD4 were positive for binding to cell wall preparations from USA300 or Newman *S. aureus* strains. Antibodies were subsequently produced in 200-ml transient transfections and purified with Protein A chromatography (MabSelect SuRe, GE Life Sciences, Piscataway, NJ) for further testing. Isolation and usage of these antibodies were approved by the regional ethical review board. rF1 variants were generated.

Mouse mAb against ClfA (9E10), ClfB, (10D2), SdrD (17H4), IsdA (2D3) and non-modified SDR proteins (9G4) were generated by immunizing mice with the respective recombinant proteins, which were purified after expression in *E. coli*, using standard protocols; hybridoma supernatants were purified by protein A affinity chromatography. Rabbit mAb 28.9.9 was generated by immunizing rabbits with peptidoglycan (PGN)-derived peptide CKKGGG-(L-Ala)-(D-gamma-Glu)-(L-Lys)-(D-Ala)-D-Ala) followed by cloning of the IgG.

Example 4 Characterization of a highly opsonic monoclonal antibody (rF1) isolated from an MRSA infected donor

Several *S. aureus*-reactive monoclonal antibodies (mAb) from memory B cells from peripheral blood of MRSA-infected donors were isolated as described above. When characterizing these antibodies, one IgG1 mAb (hereafter referred to as rF1) was identified with broad reactivity to a panel of *S. aureus* strains that induced robust opsonophagocytic killing (OPK) by human polymorphonuclear leukocytes (PMN).

Maximum binding of mAb rF1 to bacteria from clinical MRSA strain USA300 was approximately 10 fold higher than that of an isotype-matched anti-ClfA mAb (Figure 5A). Consistent with increased binding, opsonization with rF1 resulted in increased uptake (Figure 5B) and killing (Figure 5C) of USA300 by PMN. In contrast, preopsonization with human anti-ClfA had no effect on bacterial viability (Figure 5C). The rF1 antibody did not affect viability of USA300 in the absence of PMN. Thus, rF1 is a mAb with the capacity to bind MRSA and induce potent killing of MRSA by PMN.

Example 5 Binding of rF1 to Staphylococcus strains

FACS analysis of rF1 binding to whole bacteria from culture or infected tissues

Whole bacteria were harvested from TSA plates or TSB cultures and washed with HBSS without phenol red supplemented with 0.1% IgG free BSA (Sigma) and 10 mM Hepes, pH 7.4 (HB buffer) Bacteria ($20x10^8$ CFU/mL) were incubated with 300 μg/mL of rabbit IgG (Sigma) in HB buffer for 1 h at room temperature (RT) to block nonspecific IgG binding. Bacteria were stained with 2 μg/mL of primary antibodies, including rF1 or isotype control IgG1 mAb gD:5237 (Nakamura GR, et al. (1993) J Virol 67: 6179-6191), and next with fluorescent anti-human IgG secondary antibodies (Jackson Immunoresearch, West Grove, PA). The bacteria were washed and analyzed by FACSCalibur® (BD).

For antibody staining of bacteria from infected mouse tissues, 6-8 weeks old female C57Bl/6 mice (Charles River, Wilmington, MA) were injected intravenously with 10^8 CFU of logphase-grown USA300 in PBS. Mouse organs were harvested two days after infection. Rabbit infective endocarditis (IE) was established as described in Tattevin P, et al. (2010) Antimicrobial agents and chemotherapy 54: 610-613. Rabbits were injected intravenously with $5x10^7$ CFU of stationary-phase grown MRSA strain COL, and heart vegetations were harvested eighteen hours later. Treatment with 30 mg/kg of vancomycin was given intravenously b.i.d. 18 h after infection with $7x10^7$ CFU stationary-phase COL.

To lyse mouse or rabbit cells, tissues were homogenized in M tubes (Miltenyi, Auburn, CA) using a gentleMACS® cell dissociator (Miltenyi), followed by incubation for 10 min at RT in PBS containing 0.1% Triton-X100 (Thermo), 10 μg/mL of DNAseI (Roche) and Complete Mini protease inhibitor cocktail (Roche). The suspensions were passed through a 40 micron filter (BD) and bacteria were stained with mAbs as described above. Bacteria were differentiated from mouse organ debris by double staining with 20 μg/mL mouse mAb 702 anti-*S. aureus* peptidoglycan (abcam, Cambridge, MA) and a fluorochrome-labeled anti-mouse IgG secondary antibody (Jackson Immunoresearch). During flow cytometry analysis, bacteria were gated for positive staining with mAb 702 from double fluorescence plots. All animal experiments were approved by the Institutional Review Boards of Genentech and the University of California, San Francisco.

Flow cytometry (FCM) analysis showed potent binding activity of rF1 to all 15 *S. aureus* strains tested (Figure 7). These strains were broadly distributed across the *S. aureus* phylogeny [8]. As expression levels of bacterial cell surface antigens might differ between *in vitro* and *in vivo* growth, we also tested the ability of rF1 to recognize USA300 isolated from various mouse tissues after systemic infection. The rF1 mAb strongly bound to USA300 derived from infected mouse kidneys, livers and lungs (Figure 6). The binding rF1 to USA300 from mouse kidneys was sustained until at least 8 days after infection (not shown), suggesting robust long-term expression of the rF1 epitope during infection. In addition, rF1 strongly bound to MRSA COL bacteria from heart vegetations in a rabbit model of infectious endocarditis. Treatment with vancomycin did not affect the reactivity of rF1 with MRSA (Figure 6). Thus, the antigen recognized by rF1 is conserved across various strains and stably expressed in various growth and infection conditions.

Given the ubiquitous nature of rF1-reactivity across all *S. aureus* strains, experiments were performed to see if such reactivity is extended to other gram-positive bacteria. Notably, rF1 binding was detectable only for the coagulase-negative human pathogen *S. epidermidis* (Figure 7). The rF1 mAb did not bind to any other staphylococcal species tested, including *S. saprophyticus*, *S. lugdunensis*, *S. simulans* and *S. carnosus*, or other Gram-positive species such as *Streptococcus pyogenes, Bacillus subtilis*, *Enterococcus faecalis*, and *Listeria monocytogenes* (Figure 7). Thus, rF1 is a human antibody that binds to stably-expressed surface antigen(s) on human-adapted staphylococcal pathogens and promotes bacterial killing by human PMNs.

Example 6 Amino acid modifications of rF1 antibodies

In summary, the VH region of each of the rF1 Abs were cloned out and linked to human H chain gamma1 constant region and the VL linked to kappa constant region to express the Abs as IgG1. Wild-type sequences were altered at certain positions to improve the antibody stability while maintaining antigen binding as described below. Cysteine engineered Abs (ThioMabs, also referred to as THIOMAB™) were then generated.

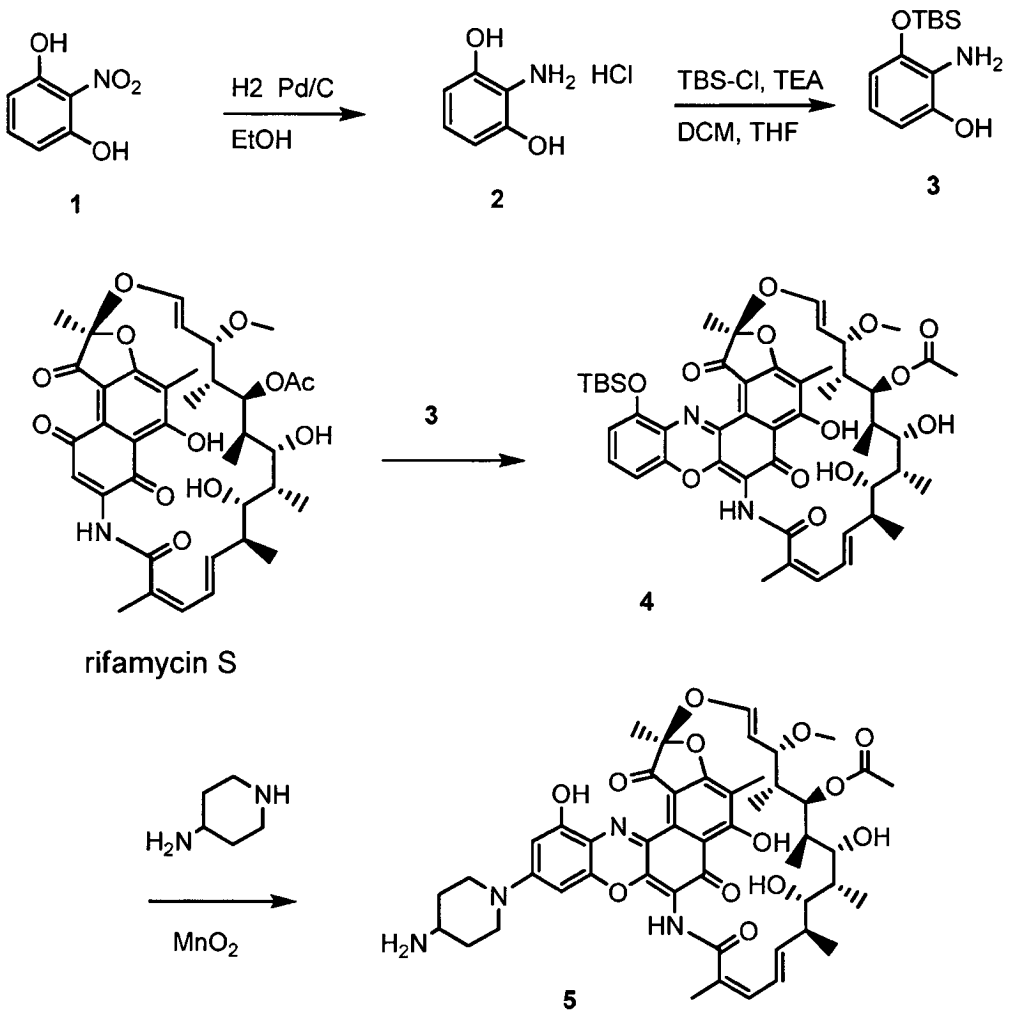
i. Generating stability variants

The rF1 Abs were engineered to improve certain properties (to avoid deamidation, aspartic acid isomerization, oxidation or N-linked glycosylation) and tested for retention of antigen binding as well as chemical stability after amino acid replacements . The amino acid alterations made were as described in US8,617,556.

iii. Generating Cys engineered mutants (ThioMabs)

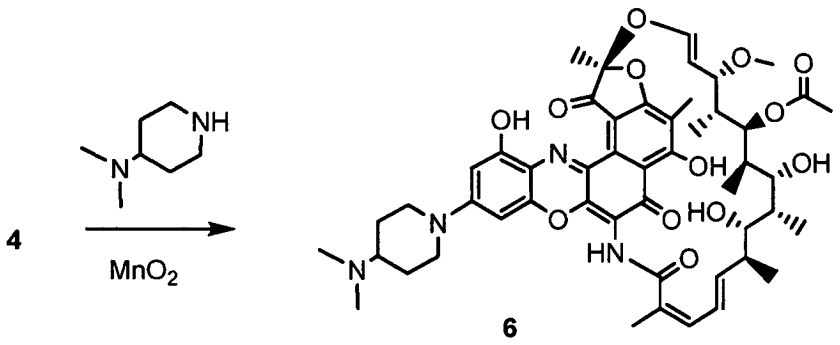
Full length ThioMabs were produced by introducing a Cysteine into the H chain (in CH1) or the L chain (Cκ) at a predetermined position as previously taught, e.g., at V205 in the kappa Constant region of the L chain and position A118 in the human Gamma 1 H chain (amino acid position numbers according to Eu convention) to allow conjugation of the antibody to a linker-antibiotic intermediate. H and L chains are then cloned into separate plasmids and the H and L encoding plasmids co-transfected into 293 cells where they are expressed and assembled into intact Abs. Both H and L chains can also be cloned into the same expression plasmid. IgG1 having 2 engineered Cys, one in each of H chains; or 2 engineered Cys, one in each of the L chains; or a combination of an engineered Cys in each of the H and L chains (HC LC Cys) leading to 4 engineered Cys per antibody tetramer, were generated by expressing the desired combination of cys mutant chains and wild type chains.

Example 7 Piperidyl benzoxazino rifamycin (pipBOR) **5**

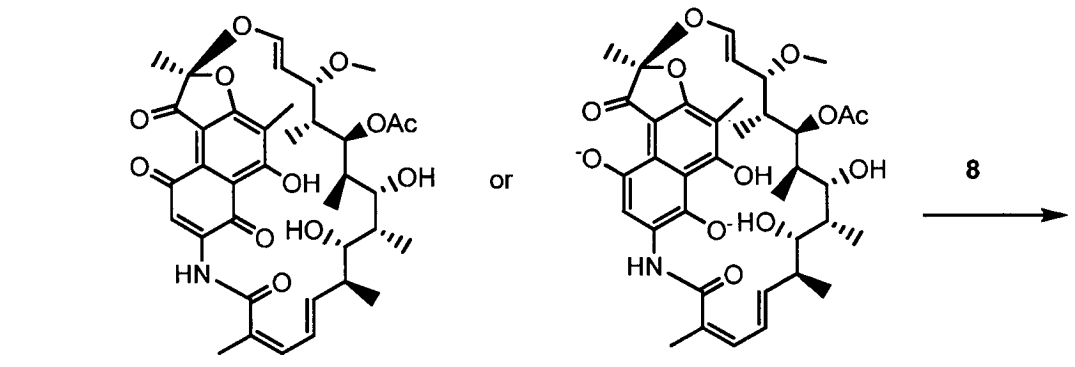


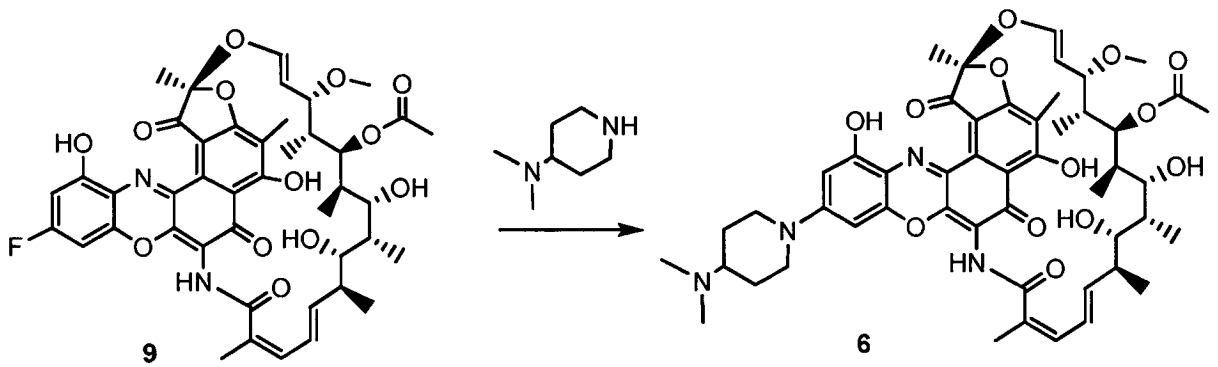
2-Nitrobenzene-1,3-diol **1** was hydrogenated under hydrogen gas with palladium/carbon catalyst in ethanol solvent to give 2-aminobenzene-1,3-diol **2**, isolated as the hydrochloride salt. Mono-protection of **2** with tert-butyldimethylsilyl chloride and triethylamine in dichloromethane/tetrahydrofuran gave 2-amino-3-(tert-butyldimethylsilyloxy)phenol **3**. Rifamycin S (ChemShuttle Inc., Fremont, CA, US 7342011; US 7271165; US 7547692) was reacted with **3** by oxidative condensation with manganese oxide or oxygen gas in toluene at room temperature to give TBS-protected benzoxazino rifamycin **4**. LCMS (ESI): $M+H^+$ = 915.41. Reaction of **4** with piperidin-4-amine and manganese oxide gave piperidyl benzoxazino rifamycin (pipBOR) **5**. LCMS (ESI): $M+H^+$ = 899.40

Example 8 DimethylpipBOR **6**



Reaction of N,N-dimethylpiperidin-4-amine with TBS-protected benzoxazino rifamycin **4** gave dimethylpiperidyl benzoxazino rifamycin (dimethylpipBOR) **6**



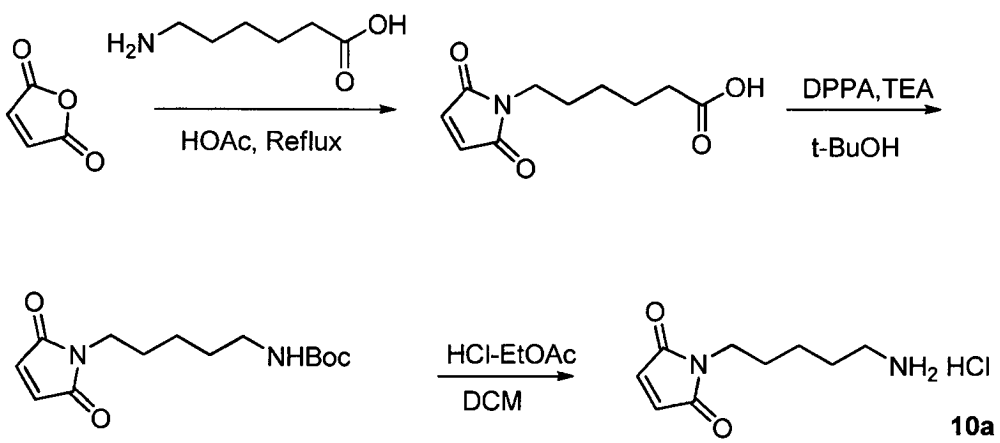


Alternatively, (5-fluoro-2-nitro-1,3-phenylene)bis(oxy)bis(methylene)dibenzene **7** was hydrogenated under hydrogen gas with palladium/carbon catalyst in tetrahydrofuran/methanol solvent to remove the benzyl groups to give 2-amino-5-fluorobenzene-1,3-diol **8**. LCMS (ESI): $M+H^+ = 144.04$. Commercially available Rifamycin S or Rifamycin SV sodium salt

(ChemShuttle Inc., Fremont, CA) was reacted with 2-amino-5-fluorobenzene-1,3-diol **8** by oxidative condensation in air or potassium ferric cyanide in ethyl acetate at 60 °C to give fluorobenzoxazino rifamycin **9**. Displacement of fluoride with N,N-dimethylpiperidin-4-amine gave dimethylpipBOR **6**. LCMS (ESI): $M+H^+ = 927.43$

Example 9 (S)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-N-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)cyclobutane-1,1-dicarboxamide **10**

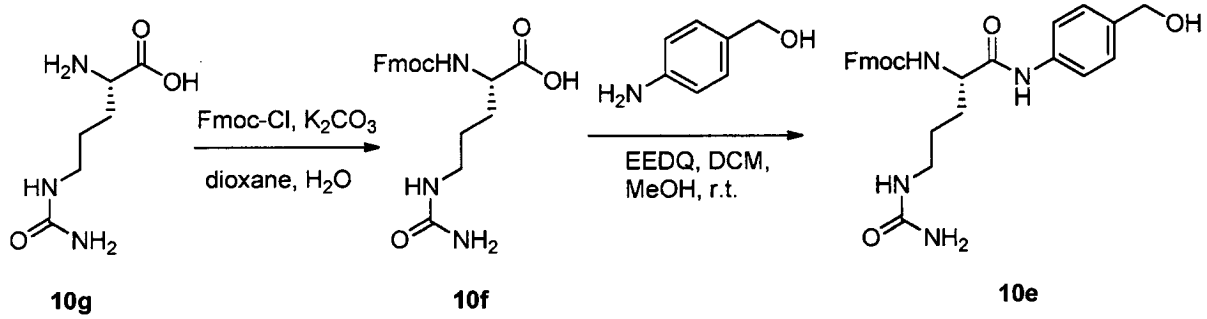
Step 1: Preparation of 1-(5-aminopentyl)-1H-pyrrole-2,5-dione hydrochloride **10a**



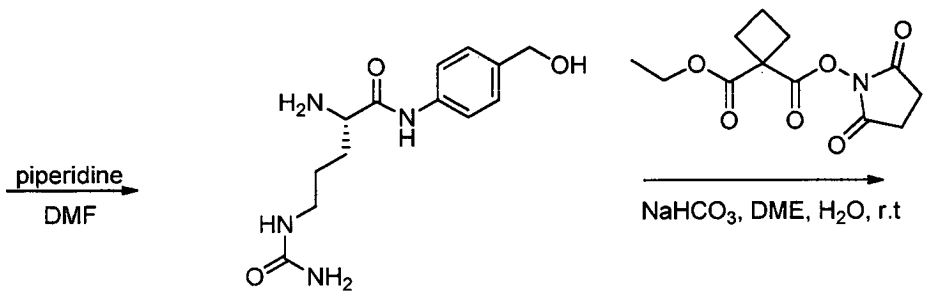
10a

Maleic anhydride, furan-2,5-dione (150 g, 1.53 mol) was added to a stirred solution of 6-aminohexanoic acid (201 g, 1.53 mol) in HOAc (1000 mL). After the mixture was stirred at r.t. for 2 h, it was heated at reflux for 8 h. The organic solvents were removed under reduced pressure and the residue was extracted with EtOAc (500 mL × 3), washed with H_2O. The combined organic layers was dried over Na_2SO_4 and concentrated to give the crude product. It was washed with petroleum ether to give 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoic acid as white solid (250 g, 77.4 %). DPPA (130 g, 473 mmol) and TEA (47.9 g, 473 mmol) was added to a solution of 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoic acid (100 g, 473 mmol) in t-BuOH (200 mL). The mixture was heated at reflux for 8 h under N_2. The mixture was concentrated, and the residue was purified by column chromatography on silica gel (PE:EtOAc= 3:1) to give tert-butyl 5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylcarbamate (13 g, 10 %). To a solution of tert-butyl 5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylcarbamate (28 g, 992 mmol) in anhydrous EtOAc (30 mL) was added HCl/EtOAc (50 mL) dropwise. After the mixture was stirred at r.t. for 5 h, it was filtered and the solid was dried to give 1-(5-aminopentyl)-1H-pyrrole-2,5-dione hydrochloride **10a** (16 g, 73.7 %). ^1H NMR (400 MHz, DMSO-d_6): δ 8.02 (s, 2H), 6.99 (s, 2H), 3.37-3.34 (m, 2H), 2.71-2.64 (m, 2H), 1.56-1.43 (m, 4H), 1.23-1.20 (m, 2H).

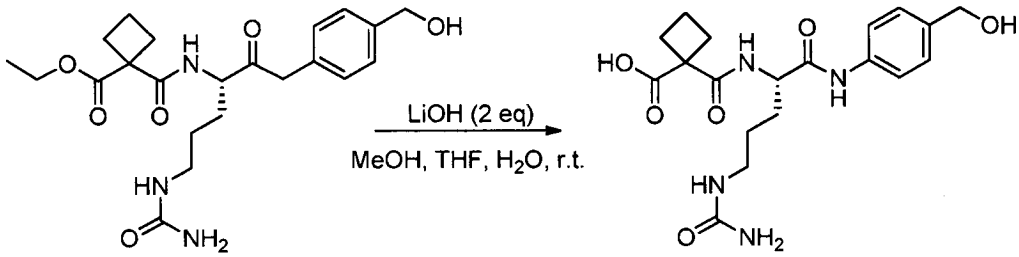
Step 2: Preparation of (S)-1-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-ylcarbamoyl)cyclobutanecarboxylic acid **10b**



10g **10f** **10e**



10d



10c **10b**

To a mixture of (S)-2-amino-5-ureidopentanoic acid **10g** (17.50 g, 0.10 mol) in a mixture of dioxane and H_2O (50 mL / 75 mL) was added K_2CO_3 (34.55 g, 0.25 mol). Fmoc-Cl (30.96 g, 0.12 mol) was added slowly at 0 °C. The reaction mixture was warmed to r.t. over 2 h. Organic solvent was removed under reduced pressure, and the water slurry was adjusted to pH = 3 with 6 M HCl solution, and extracted with EtOAc (100 mL × 3). The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-ureidopentanoic acid **10f** (38.0 g, 95.6 %). **10f** is commercially available.

To a solution of **10f** (4 g, 10 mmol) in a mixture of DCM and MeOH (100 mL / 50 mL) were added (4-aminophenyl)methanol (1.6 g, 13 mmol, 1.3 eq) and 2-Ethoxy-1-ethoxycarbonyl-

1,2-dihydroquinoline, EEDQ, Sigma-Aldrich CAS Reg. No. 16357-59-8 (3.2 g, 13 mmol, 1.3 eq). After the mixture was stirred at r.t. for 16 h under N_2, it was concentrated to give a brown solid. MTBE (200 mL) was added and it was stirred at 15°C for 2 h. The solid was collected by filtration, washed with MTBE (50 mL × 2) to give (S)-(9H-fluoren-9-yl)methyl (1-((4-(hydroxymethyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)carbamate **10e** as an orange solid (4.2 g, 84%). LCMS (ESI): m/z 503.0 [M+1].

To a stirred solution of **10e** (4.2 g, 8.3 mmol) in dry DMF (20 ml) was added piperidine (1.65 mL, 17 mmol, 2 eq) dropwise at r.t. The mixture was stirred at r.t. for 30 min, and solid precipitate formed. Dry DCM (50 mL) was added, and the mixture became transparent immediately. The mixture was stirred at r.t. for another 30 min, and LCMS showed **10e** was consumed. It was concentrated to dryness under reduced pressure (make sure no piperidine remained), and the residue was partitioned between EtOAc and H_2O (50 mL / 20 mL). Aqueous phase was washed with EtOAc (50 mL × 2) and concentrated to give (S)-2-amino-N-(4-(hydroxymethyl)phenyl)-5-ureidopentanamide **10d** as an oily residual (2.2 g, 94%) (contained small amount of DMF).

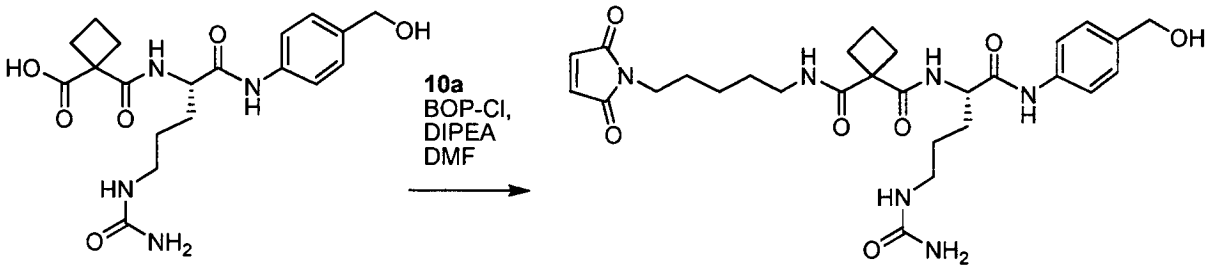
Commercially available 1,1-cyclobutanedicarboxylic acid, 1,1-diethyl ester (CAS Reg. No. 3779-29-1) was converted by limited saponification with aqueous base to the half acid/ester 1,1-cyclobutanedicarboxylic acid, 1-ethyl ester (CAS Reg No. 54450-84-9) and activation with a coupling reagent such as TBTU (*O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate, also called: *N,N,N',N'*-Tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate, CAS No. 125700-67-6, Sigma-Aldrich B-2903), and N-hydroxysuccinimide to the NHS ester, 1-(2,5-dioxopyrrolidin-1-yl) 1-ethyl cyclobutane-1,1-dicarboxylate.

To a solution of 1-(2,5-dioxopyrrolidin-1-yl) 1-ethyl cyclobutane-1,1-dicarboxylate (8 g, 29.7 mmol) in DME (50 mL) was added a solution of **10d** (6.0 g, 21.4 mmol) and $NaHCO_3$ (7.48 g, 89.0 mmol) in water (30 mL). After the mixture was stirred at r.t. for 16 h, it was concentrated to dryness under reduced pressure and the residue was purified by column chromatography (DCM:MeOH = 10:1) to give (S)-ethyl 1-((1-(4-(hydroxymethyl)phenyl)-2-oxo-6-ureidohexan-3-yl)carbamoyl)cyclobutanecarboxylate **10c** as white solid (6.4 g, 68.7%). LCMS (ESI): m/z 435.0 [M+1]

To a stirred solution of **10c** (6.4 g, 14.7 mmol) in a mixture of THF and MeOH (20 mL / 10 mL) was added a solution of LiOH • H_2O (1.2 g, 28.6 mmol) in H_2O (20 mL) at r.t. After the reaction mixture was stirred at r.t. for 16 h, solvent was removed under reduced pressure, the residue obtained was purified by prep-HPLC to give (S)-1-(1-(4-(hydroxymethyl)phenylamino)-

1-oxo-5-ureidopentan-2-ylcarbamoyl)cyclobutanecarboxylic acid **10b** (3.5 g, yield: 58.5%). LCMS (ESI): m/z 406.9 [M+1]. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.86 (d, J = 8.4 Hz, 2 H), 8.51 (d, J = 8.4 Hz, 2 H), 5.88 - 5.85 (m, 1 H), 5.78 (s, 2 H), 4.54 - 4.49 (m, 3 H), 4.38 - 4.32 (m, 1 H), 3.86 - 3.75 (m, 1 H), 3.84 - 3.80 (m, 2 H), 3.28 - 3.21 (m, 1 H), 3.30 - 3.24 (m, 1 H), 3.00 - 2.80 (m, 1 H), 2.37 - 2.28 (m, 2 H).

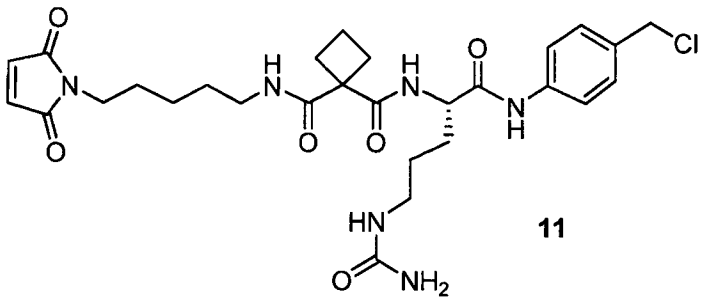
Step 3: Preparation of S)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-N-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)cyclobutane-1,1-dicarboxamide **10**



10b **10**

Diisopropylethylamine, DIPEA (1.59 g, 12.3 mmol) and bis(2-oxo-3-oxazolidinyl)phosphinic chloride, BOP-Cl (CAS Reg. No. 68641-49-6, Sigma-Aldrich, 692 mg, 2.71 mmol) was added to a solution of (S)-1-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-ylcarbamoyl)cyclobutanecarboxylic acid **10b** (1 g, 2.46 mmol) in DMF (10 mL) at 0 °C, followed by 1-(5-aminopentyl)-1H-pyrrole-2,5-dione hydrochloride 10a (592 mg, 2.71 mmol). The mixture was stirred at 0 °C for 0.5h. The reaction mixture was quenched with citric acid solution (10 mL), extracted with DCM/MeOH (10:1). The organic layer was dried and concentrated, and the residue was purified by column chromatography on silica gel (DCM:MeOH = 10:1) to give to give S)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-N-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)cyclobutane-1,1-dicarboxamide 10 (1.0 g, 71 %), also referred to as MC-CBDK-cit-PAB-OH. LCMS (ESI): M+H$^+$ = 571.28. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.00 (s, 1H), 7.82-7.77 (m, 2H), 7.53 (d, J = 8.4 Hz, 2 H), 7.19 (d, J = 8.4 Hz, 2 H), 6.96 (s, 2H), 5.95 (t, J = 6.4 Hz, 1H), 5.39 (s, 2H), 5.08 (t, J = 5.6 Hz, 1H), 4.40-4.35 (m, 3H), 4.09 (d, J = 4.8 Hz, 1 H), 3.01 (d, J = 3.2 Hz, 2 H), 3.05-2.72 (m, 4H), 2.68-2.58 (m, 3H), 2.40-2.36 (m, 4H), 1.72-1.70 (m, 3H), 1.44-1.42 (m, 1H), 1.40-1.23 (m, 6H), 1.21-1.16 (m, 4H).

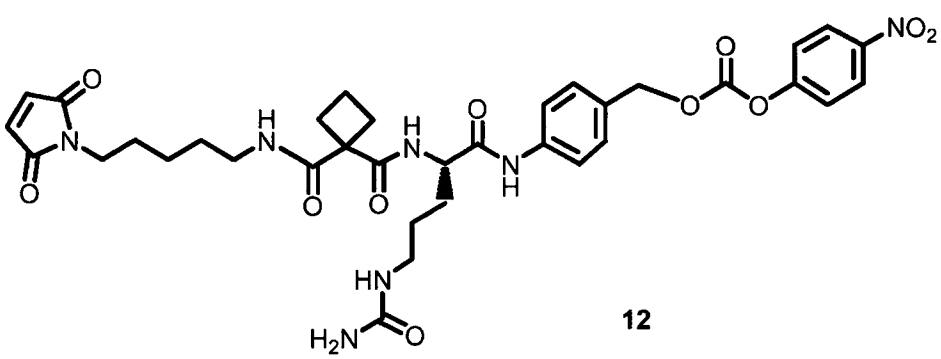
Example 10 (S)-N-(1-(4-(chloromethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)cyclobutane-1,1-dicarboxamide **11**



11

A solution of (S)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-N-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)cyclobutane-1,1-dicarboxamide **10** (2.0 g, 3.5 mmol) in N,N-dimethylformamide, DMF or N-methylpyrrolidone, NMP (50 mL) was treated with thionyl chloride, $SOCl_2$ (1.25 g, 10.5 mmol) in portions dropwise at 0 °C. The reaction remained yellow. The reaction was monitored by LC/MS indicating >90% conversion. After the reaction mixture was stirred at 20 °C for 30 min or several hours, it was diluted with water (50 mL) and extracted with EtOAc (50 mL x 3). The organic layer was dried, concentrated and purified by flash column (DCM : MeOH = 20 : 1) to form **11**, also referred to as MC-CBDK-cit-PAB-Cl as a gray solid. LCMS: (5-95, AB, 1.5 min), 0.696 min, *m/z* = 589.0 $[M+1]^+$.

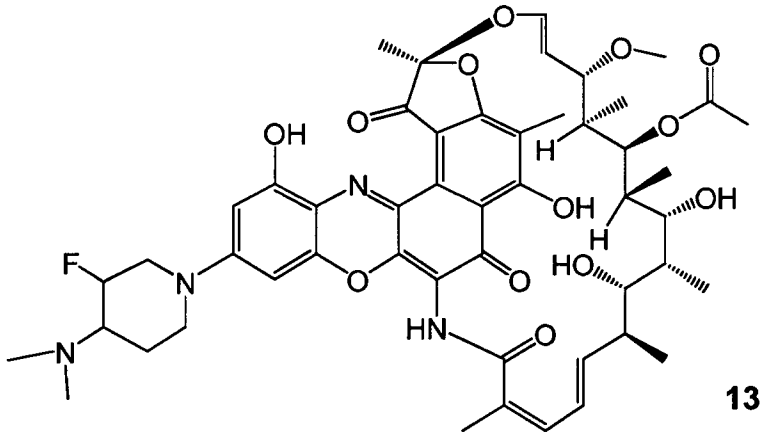
Example 11 (S)-4-(2-(1-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylcarbamoyl)cyclobutanecarboxamido)-5-ureidopentanamido)benzyl 4-nitrophenyl carbonate **12**



12

To a solution of (S)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)-N-(1-(4-(hydroxymethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)cyclobutane-1,1-dicarboxamide **10** in anhydrous DMF was added diisopropylethylamine (DIEA), followed by PNP carbonate (bis(4-nitrophenyl) carbonate). The reaction solution was stirred at room temperature (r.t.) for 4 hours and the mixture was purified by prep-HPLC to afford **12**. LCMS (ESI): $M+H^+$ = 736.29.

Example 12 Preparation of MC-(CBDK-cit)-PAB-(dimethyl, fluoropipBOR) - PLA-1



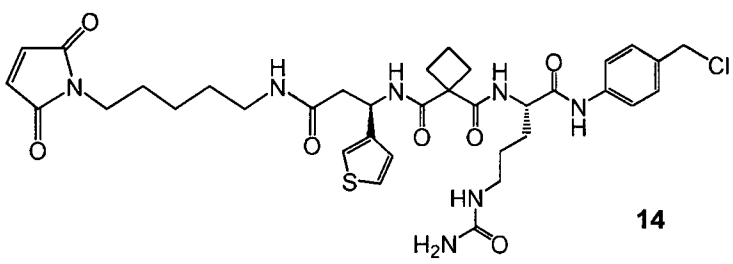
13

Following the procedure for PLA-2, (S)-N-(1-(4-(chloromethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)cyclobutane-1,1-dicarboxamide **11** and the fluorinated rifamycin-derivative, dimethylfluoropipBOR **13** (LCMS (ESI): $M+H^+$ = 945.43) were reacted to form MC-(CBDK-cit)-PAB-(dimethyl, fluoropipBOR) - PLA-1, Table 2. LCMS (ESI): $M+H^+$ = 1499.7

Example 13 Preparation of MC-(CBDK-cit)-PAB-(dimethylpipBOR) - PLA-2

(S)-N-(1-(4-(chloromethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)-N-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentyl)cyclobutane-1,1-dicarboxamide **11** (0.035 mmol) in DMF was cooled to 0 °C and dimethylpipBOR **6**, (10 mg, 0.011 mmol) was added. The mixture was diluted with another 0.5 mL of DMF. Stirred open to air for 30 minutes. N,N-diisopropylethylamine (DIEA, 10 µL, 0.05 mmol) was added and the reaction stirred overnight open to air. By LC/MS, 50% of desired product was observed. An additional 0.2 eq N,N-diisopropylethylamine base was added while the reaction stirred open to air for another 6 hours until the reaction appeared to stop progressing. The reaction mixture was diluted with DMF and purified on HPLC (20-60% ACN/HCOOH in H_2O) to give MC-(CBDK-cit)-PAB-(dimethylpipBOR) - PLA-2, Table 2. LCMS (ESI): $M+H^+$ = 1481.8, yield 31%.

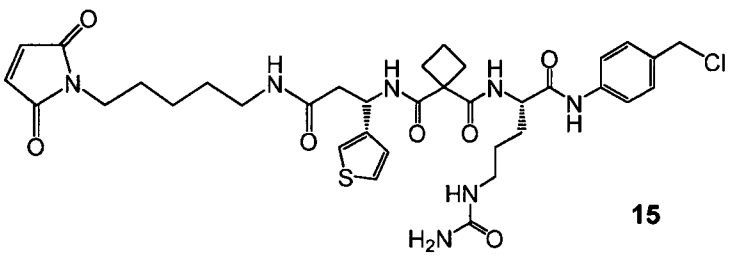
Example 14 Preparation of MC-((*R*)-thiophen-3-yl-CBDK-cit)-PAB-(dimethylpipBOR) (PLA-3)



14

Following the procedure for PLA-2, (N-((S)-1-(4-(chloromethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)-N-((R)-3-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylamino)-3-oxo-1-(thiophen-3-yl)propyl)cyclobutane-1,1-dicarboxamide **14** (LCMS (ESI): $M+H^+$ = 742.3) and dimethylpipBOR **6** were reacted to give MC-((*R*)-thiophen-3-yl-CBDK-cit)-PAB-(dimethylpipBOR) (PLA-3, Table 2). LCMS (ESI): $M+H^+$ = 1633.9

Example 15 Preparation of MC-((*S*)-thiophen-3-yl-CBDK-cit)-PAB-(dimethylpipBOR) (PLA-4)



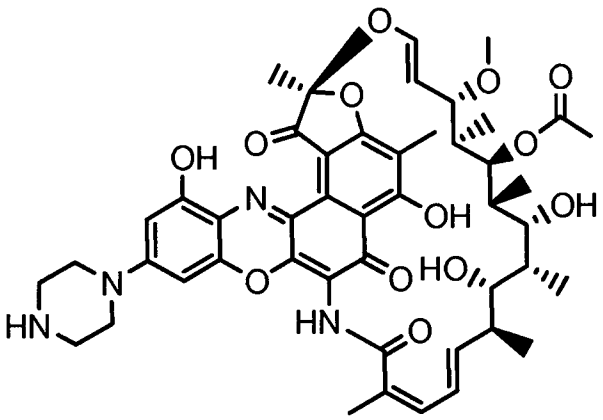
15

Following the procedure for PLA-2, (N-((R)-1-(4-(chloromethyl)phenylamino)-1-oxo-5-ureidopentan-2-yl)-N-((R)-3-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylamino)-3-oxo-1-(thiophen-3-yl)propyl)cyclobutane-1,1-dicarboxamide **15** (LCMS (ESI): $M+H^+$ = 742.3) and dimethylpipBOR **6** were reacted to give MC-((*R*)-thiophen-3-yl-CBDK-cit)-PAB-(dimethylpipBOR) (PLA-4, Table 2). LCMS (ESI): $M+H^+$ = 1633.9

Example 16 Preparation of MC-(CBDK-cit)-PABC-(pipBOR) (PLA-5)

Piperidyl benzoxazino rifamycin (pipBOR) **5** (15 mg, 0.0167 mmol), and then (S)-4-(2-(1-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylcarbamoyl)cyclobutanecarboxamido)-5-ureidopentanamido)benzyl 4-nitrophenyl carbonate **12** (12 mg, 0.0167 mmol) were weighed into a vial. Dimethylformamide, DMF (0.3 mL) was added, followed by diisopropylethylamine, DIEA (0.006 mL, 0.0334 mmol), and the reaction was allowed to stir at room temperature for 2 h. The reaction solution was directly purified by HPLC (30 to 70% MeCN/water + 1% formic acid) to give MC-(CBDK-cit)-PABC-(pipBOR) (PLA-5, Table 2). LCMS (ESI): $M+H^+$ = 1496.5

Example 17 Preparation of MC-(CBDK-cit)-PABC-(piperazBTR) (PLA-6)



16

Following the procedures for PLA-5,the piperidine rifamycin derivative, piperazBOR **16** (LCMS (ESI): $M+H^+$ = 885.4) and (S)-4-(2-(1-(5-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)pentylcarbamoyl)cyclobutanecarboxamido)-5-ureidopentanamido)benzyl 4-nitrophenyl carbonate **12** were reacted to give MC-(CBDK-cit)-PABC-(piperazBTR) (PLA-6. Table 2). LCMS (ESI): $M+H^+$ = 1482.5

Example 18 Preparation of rF1 Antibody-Antibiotic Conjugates

Antibody-antibiotic conjugates (AAC) Table 3 were prepared by conjugating an rF1 antibody to a PML Linker-Antibiotic intermediate, including those from Table 2. Prior to conjugation, the rF1 antibodies were partially reduced with TCEP using standard methods in accordance with the methodology described in WO 2004/010957, the teachings of which are incorporated by reference for this purpose. The partially reduced antibodies were conjugated to the linker-antibiotic intermediate using standard methods in accordance with the methodology described, e.g., in Doronina et al. (2003) *Nat. Biotechnol.* 21:778-784 and US 2005/0238649 A1. Briefly, the partially reduced antibodies were combined with the linker-antibiotic intermediate to allow conjugation of the linker-antibiotic intermediate to reduced cysteine residues of the antibody. The conjugation reactions were quenched, and the AAC were purified. The antibiotic load (average number of antibiotic moieties per antibody) for each AAC was determined and was between about 1 to about 2 for the rF1 antibodies engineered with a single cysteine mutant site.

Reduction/Oxidation of ThioMabs for Conjugation: Full length, cysteine engineered monoclonal antibodies (ThioMabs - Junutula, et al., 2008b Nature Biotech., 26(8):925-932; Dornan et al (2009) Blood 114(13):2721-2729; US 7521541; US 7723485; WO2009/052249, Shen et al (2012) Nature Biotech., 30(2):184-191; Junutula et al (2008) Jour of Immun. Methods 332:41-52) expressed in CHO cells were reduced with about a 20-40 fold excess of TCEP

(tris(2-carboxyethyl)phosphine hydrochloride or DTT (dithiothreitol) in 50 mM Tris pH 7.5 with 2 mM EDTA for 3 hrs at 37 °C or overnight at room temperature.(Getz *et al* (1999) *Anal. Biochem.* Vol 273:73-80; Soltec Ventures, Beverly, MA). The reduced ThioMab was diluted and loaded onto a HiTrap S column in 10 mM sodium acetate, pH 5, and eluted with PBS containing 0.3M sodium chloride. Alternatively, the antibody was acidified by addition of 1/20th volume of 10 % acetic acid, diluted with 10 mM succinate pH 5, loaded onto the column and then washed with 10 column volumes of succinate buffer. The column was eluted with 50 mM Tris pH7.5, 2 mM EDTA.

The eluted reduced ThioMab was treated with 15 fold molar excess of DHAA (dehydroascorbic acid) or 200 nM aqueous copper sulfate ($CuSO_4$). Oxidation of the interchain disulfide bonds was complete in about three hours or more. Ambient air oxidation was also effective. The re-oxidized antibody was dialyzed into 20 mM sodium succinate pH 5, 150 mM NaCl, 2 mM EDTA and stored frozen at -20 °C.

Conjugation of ThioMabs with linker-antibiotic intermediates: The deblocked, reoxidized, thio-antibodies (ThioMab) were reacted with 6-8 fold molar excess of the linker-antibiotic intermediate of Table 2 (from a DMSO stock at a concentration of 20 mM) in 50 mM Tris, pH 8, until the reaction was complete (16-24 hours) as determined by LC-MS analysis of the reaction mixture.

The crude antibody-antibiotic conjugates (AAC) were then applied to a cation exchange column after dilution with 20 mM sodium succinate, pH 5. The column was washed with at least 10 column volumes of 20 mM sodium succinate, pH 5, and the antibody was eluted with PBS. The AAC were formulated into 20 mM His/acetate, pH 5, with 240 mM sucrose using gel filtration columns. AAC were characterized by UV spectroscopy to determine protein concentration, analytical SEC (size-exclusion chromatography) for aggregation analysis and LC-MS before and after treatment with Lysine C endopeptidase.

Size exclusion chromatography was performed using a Shodex KW802.5 column in 0.2M potassium phosphate pH 6.2 with 0.25 mM potassium chloride and 15% IPA at a flow rate of 0.75 ml/min. Aggregation state of AAC was determined by integration of eluted peak area absorbance at 280 nm.

LC-MS analysis was performed using an Agilent QTOF 6520 ESI instrument. As an example, an AAC generated using this chemistry was treated with 1:500 w/w Endoproteinase Lys C (Promega) in Tris, pH 7.5, for 30 min at 37 °C. The resulting cleavage fragments were loaded onto a 1000A, 8 um PLRP-S column heated to 80°C and eluted with a gradient of 30% B to 40% B in 5 minutes. Mobile phase A: H_2O with 0.05% TFA. Mobile phase B: acetonitrile

with 0.04% TFA. Flow rate: 0.5ml/min. Protein elution was monitored by UV absorbance detection at 280 nm prior to electrospray ionization and MS analysis. Chromatographic resolution of the unconjugated Fc fragment, residual unconjugated Fab and antibiotic-Fab was usually achieved. The obtained m/z spectra were deconvoluted using Mass Hunter™ software (Agilent Technologies) to calculate the mass of the antibody fragments.

The AAC, **103** (AAR = 1.9) thio-rF1-HC-121C, LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR) was made using the rF1 L chain of SEQ ID NO. 9 containing the engineered Cys 205, and the rF1 H chain comprising SEQ ID NO. 10. The AAC **102** (AAR = 3.9) thio-rF1-HC-121C, LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR) was made using the rF1 L chain of SEQ ID NO. 9 in the preceding containing the engineered Cys 205, and the rF1 H chain comprising SEQ ID NO. 12 which contains the engineered Cys 114 (114 Kabat numbering is the same as 118 Eu numbering and 121 sequential numbering). The Cys engineered L and/or H chain was conjugated to the PML linker and rifamycin-type antibiotic as shown in Table 2.

Example 19 *In vitro* efficacy of rF1-AACs

S. aureus (USA300 NRS384 strain) was incubated with various doses (100 ug/mL, 10 ug/mL, 1 ug/mL or 0.1 ug/mL) of an anti-S. aureus unconjugated antibody, **103** AAC loaded with 1.9 average antibiotic molecules per antibody (AAR2) or with **102** AAC loaded with 3.9 average antibiotic molecules per antibody (AAR4) for 1 hour to permit binding of the antibody to the bacteria. The resulting opsonized bacteria were fed to murine macrophages and incubated at 37°C to permit phagocytosis *(in vitro* macrophage assay). After 2 hours, the infection mix was removed and replaced with normal growth media supplemented with 50 ug/mL of gentamycin to kill any remaining extracellular bacteria. The total number of surviving intracellular bacteria was determined 2 days later by plating serial dilutions of the macrophage lysates on Tryptic Soy Agar plates.

The results are shown in Figure 10. Both of the AACs tested (AAR2 vs. AAR4) showed a similar dose response and yielded maximal killing at a dose of 10 ug/mL or above with partial to no killing at 1 ug/mL and below, suggesting that the dose response for the AAC is limited by the number of antibody binding sites on the bacterium. By loading 4 antibiotic molecules per antibody, bacterial killing by AACs and overall killing of bacteria was superior with the AAR4 AAC at all doses tested. At the highest dose tested, the 2DAR AAC reduced bacterial loads by 350-fold, whereas the 4AAR AAC reduced bacterial loads by more than 4,000-fold. (dashed line indicates the limit of detection for the assays shown).

This example demonstrates that rF1-AAC, **102** (AAR = 3.9) and **103** (AAR = 1.9) thio-rF1-HC-121C, LC-V205C-MC-(CBDK-cit)-PAB-(dimethylpipBOR) from Table 3 killed intracellular MRSA in a macrophage assay in vitro. The results are shown in Figure 10.

Example 20 *In vivo* efficacy of rF1-AACs

This example demonstrates that the rF1-AACs were effective in greatly reducing or eradicating intracellular S. aureus infections, in a murine intravenous infection model.

Peritonitis Model. 7 week old female A/J mice (Jackson Laboratories) are infected by peritoneal injection with $5x10^7$ CFU of USA300. Mice are sacrificed 2 days post infection and the peritoneum is flushed with 5 mL of cold phosphate buffered saline solution (PBS). Kidneys are homogenized in 5 mL of PBS as described below for the intravenous infection model. Peritoneal washes are centrifuged for 5 minutes at 1,000 rpm at 4°C in a table top centrifuge. The supernatant is collected as the extracellular bacteria and the cell pellet containing peritoneal cells is collected as the intracellular fraction. The cells are treated with 50 μg/mL of lysostaphin for 20 minutes at 37°C to kill contaminating extracellular bacteria. Peritoneal cells are washed 3x in ice cold PBS to remove the lysostaphin prior to analysis. To count the number of intracellular CFUs, peritoneal cells are lysed in HB (Hanks Balanced Salt Solution supplemented with 10 mM HEPES and .1% Bovine Serum Albumin) with 0.1% Triton-X, and serial dilutions of the lysate are made in PBS with 0.05% tween-20.

Murine intravenous infection model. For studies involving competing human IgG (SCID IVIG model), CB17.SCID mice (Charles River Laboratories, Hollister, CA) were reconstituted with GammaGard S/D IGIV Immune Globulin (ASD Healthcare, Brooks KY) using a dosing regimen optimized to achieve constant serum levels of at least 10 mg/mL of human IgG in serum. IGIV was administered with an initial intravenous dose of 30 mg per mouse followed by a second dose of 15 mg/mouse by intraperitoneal injection after 6 hours, and subsequent daily dosing of 15 mg per mouse by intraperitoneal injection for 3 consecutive days.

Mice (n=8 for each of antibody or AAC) were infected 4 hours after the first dose of IGIV with $1x10^7$ CFU of MRSA (USA300 NRS384 strain) diluted in phosphate buffered saline by intravenous injection. Infected mice were treated with 50 mg/kg of rF1 naked antibody, **103** AAC DAR2 or **102** AAC DAR4. Mice were given a single dose of AAC 26h post infection by intravenous injection, sacrificed on day 4 post infection, and kidneys and hearts were harvested in 5 mL of phosphate buffered saline. The tissue samples were homogenized using a GentleMACS Dissociator™ (Miltenyi Biotec, Auburn, CA). The total number of bacteria

recovered per organ was determined by plating serial dilutions of the tissue homogenate in PBS .05% Tween on Tryptic Soy Agar with 5% defibrinated sheep blood.

Figure 11A shows the results of in vivo treatment with AACs on the bacterial load in the kidneys of the infected mice. Treatment with AAC containing 2 antibiotic molecules per antibody (DAR2) reduced bacterial load by approximately 30-fold and treatment with the AAC containing 4 antibiotic molecules per antibody (AAR4) reduced bacterial burdens by more than 30,000-fold.

Figure 11B shows the results of in vivo treatment with AACs on the bacterial count in the heart. Treatment with AAC AAR2 reduced bacterial burdens by approximately 70-fold with 6 out of 8 mice having undetectable level of bacteria in hearts; treatment with the AAC DAR4 completely eradicated infection in hearts resulting in 8 out of 8 mice having undetectable levels of bacteria.

Although the foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the invention. All patents, patent applications, and references cited throughout the specification are expressly incorporated by reference.

CLAIMS

We claim:

1. An antibody-antibiotic conjugate compound comprising an anti-serine-aspartate repeat (SDR) antibody, covalently attached by a protease-cleavable, non-peptide linker to a rifamycin-type antibiotic.

2. The antibody-antibiotic conjugate compound of claim 1 having the formula:

$$Ab–(PML–abx)_p$$

wherein:

Ab is the rF1 antibody;

PML is the protease-cleavable, non-peptide linker having the formula:

$$–Str–PM–Y–$$

where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit;

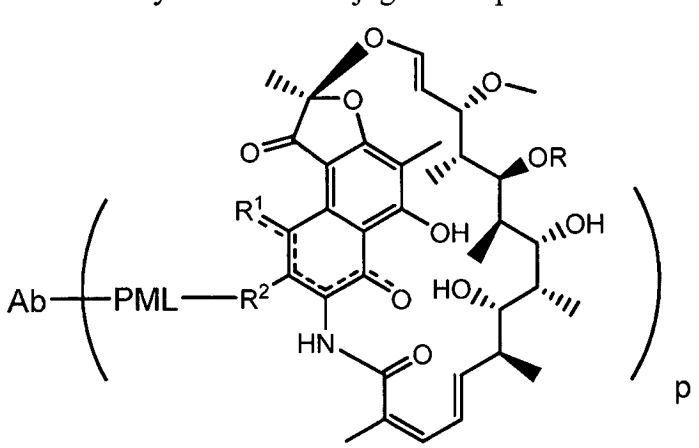
abx is the rifamycin-type antibiotic; and

p is an integer from 1 to 8.

3. The antibody-antibiotic conjugate compound of claim 2 wherein the rifamycin-type antibiotic is a rifalazil-type antibiotic.

4. The antibody-antibiotic conjugate compound of claim 2 wherein the rifamycin-type antibiotic comprises a quaternary amine attached to the protease-cleavable, non-peptide linker.

5. The antibody-antibiotic conjugate compound of claim 2 having Formula I:



I

wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

R^1 is OH;

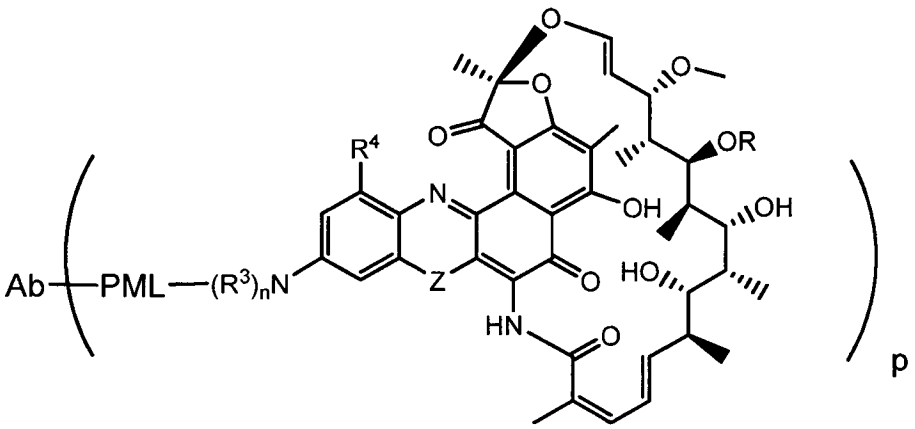
R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH;

PML is the protease-cleavable, non-peptide linker attached to R^2 or the fused heteroaryl or heterocyclyl formed by R^1 and R^2; and

Ab is the rF1 antibody.

6. The antibody-antibiotic conjugate compound of claim 5 having the formula:



wherein

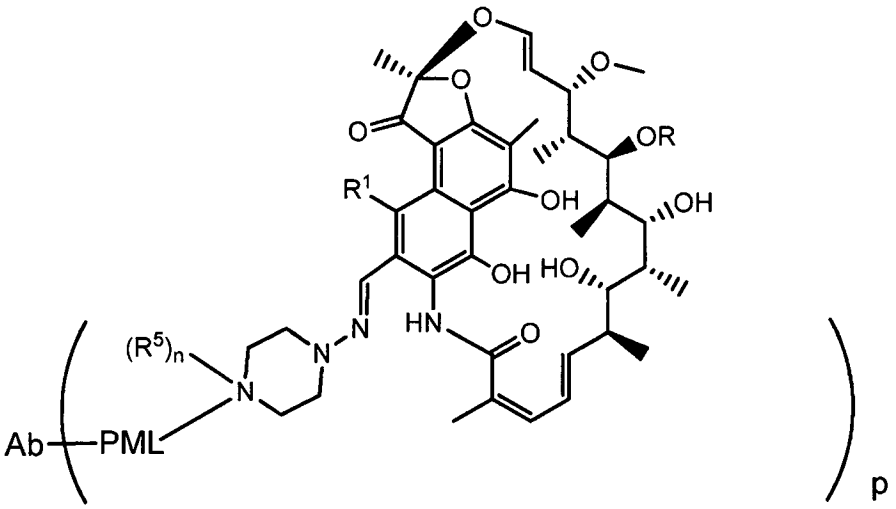
R^3 is independently selected from H and C_1–C_{12} alkyl;

n is 1 or 2;

R^4 is selected from H, F, Cl, Br, I, C_1–C_{12} alkyl, and OH; and

Z is selected from NH, $N(C_1$–C_{12} alkyl), O and S.

7. The antibody-antibiotic conjugate compound of claim 2 having the formula:

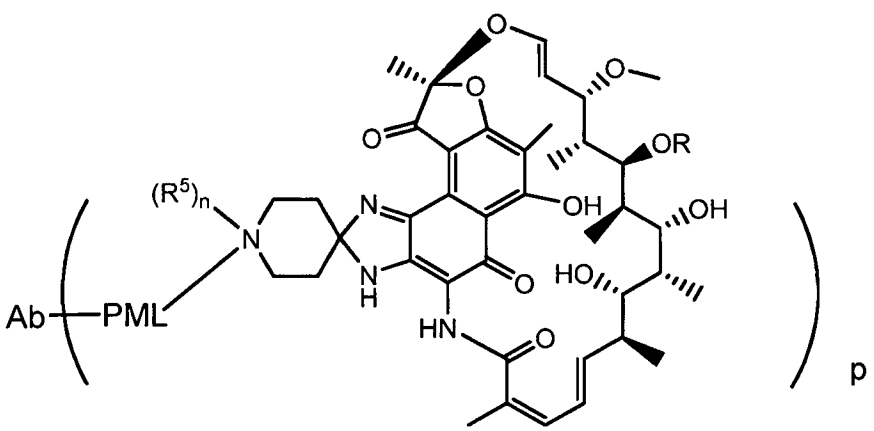


wherein

R^5 is selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

8. The antibody-antibiotic conjugate compound of claim 2 having the formula:

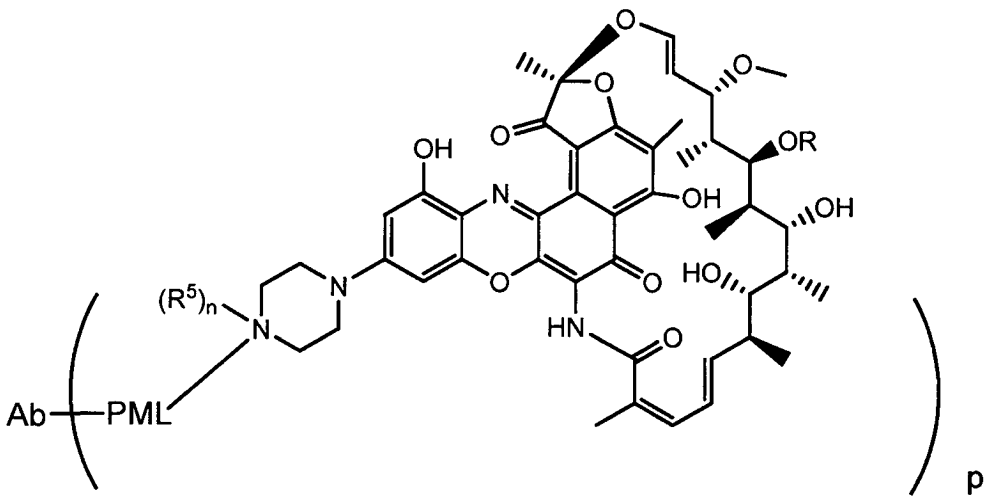


wherein

R^5 is selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

9. The antibody-antibiotic conjugate compound of claim 2 having the formula:

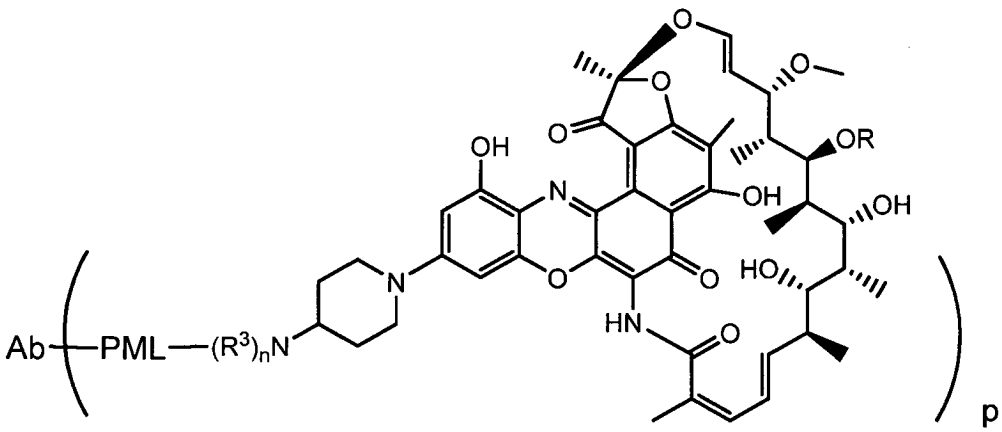


wherein

R^5 is independently selected from H and C_1–C_{12} alkyl; and

n is 0 or 1.

10. The antibody-antibiotic conjugate compound of claim 2 having the formula:

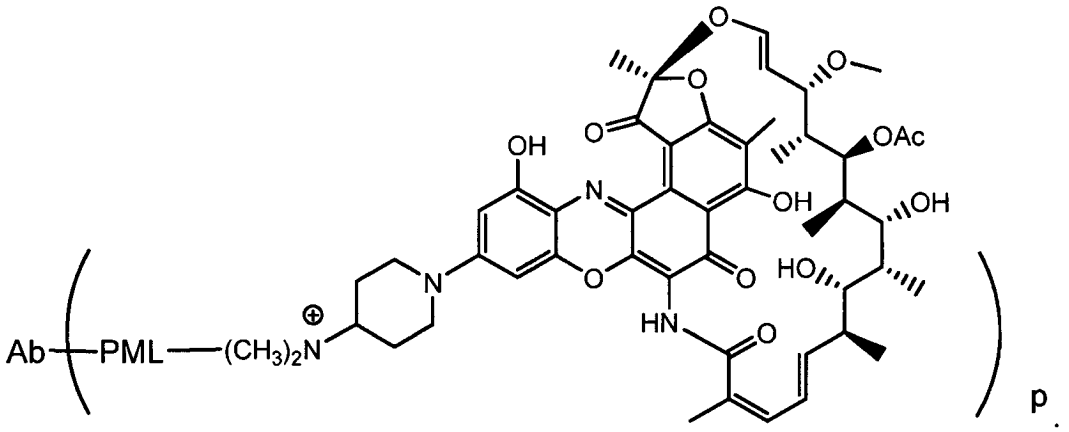


wherein

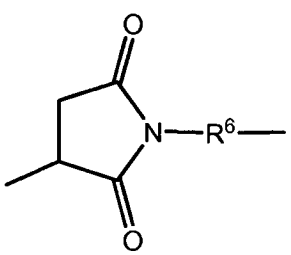
R^3 is independently selected from H and C_1–C_{12} alkyl; and

n is 1 or 2.

11. The antibody-antibiotic conjugate compound of claim 10 having the formula:



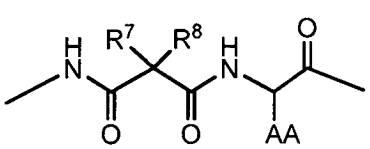
12. The antibody-antibiotic conjugate compound of claim 2 wherein Str has the formula:



wherein R^6 is selected from the group consisting of C_1-C_{12} alkylene, C_1-C_{12} alkylene-C(=O), C_1-C_{12} alkylene-NH, $(CH_2CH_2O)_r$, $(CH_2CH_2O)_r$-C(=O), $(CH_2CH_2O)_r$-CH_2, and C_1-C_{12} alkylene-NHC(=O)CH_2CH(thiophen-3-yl), where r is an integer ranging from 1 to 10.

13. The antibody-antibiotic conjugate compound of claim 12 wherein R^6 is $(CH_2)_5$.

14. The antibody-antibiotic conjugate compound of claim 2 wherein PM has the formula:

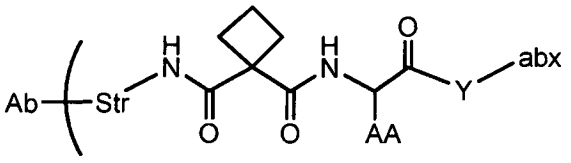


where R^7 and R^8 together form a C_3-C_7 cycloalkyl ring, and

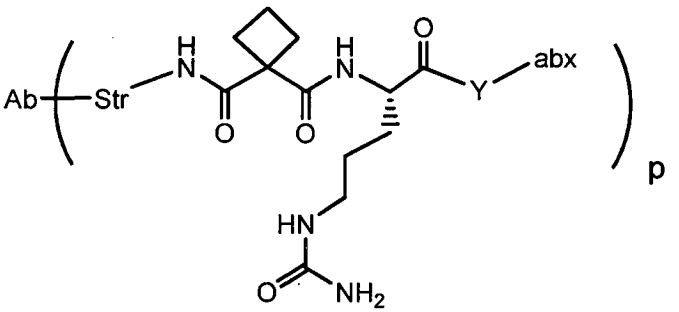
AA is an amino acid side chain selected from H, $-CH_3$, $-CH_2(C_6H_5)$, $-CH_2CH_2CH_2CH_2NH_2$, $-CH_2CH_2CH_2NHC(NH)NH_2$, $-CHCH(CH_3)CH_3$, and $-CH_2CH_2CH_2NHC(O)NH_2$.

15. The antibody-antibiotic conjugate compound of claim 2 wherein Y comprises para-aminobenzyl or para-aminobenzyloxycarbonyl.

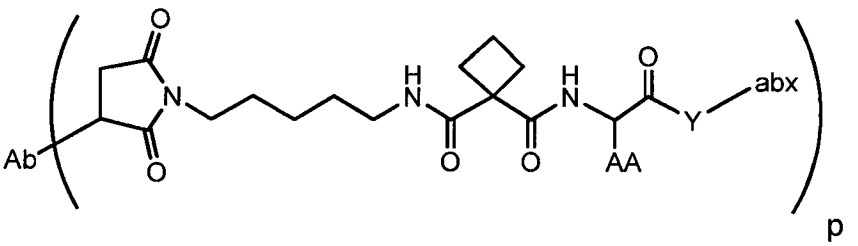
16. The antibody-antibiotic conjugate compound of claim 2 having the formula:



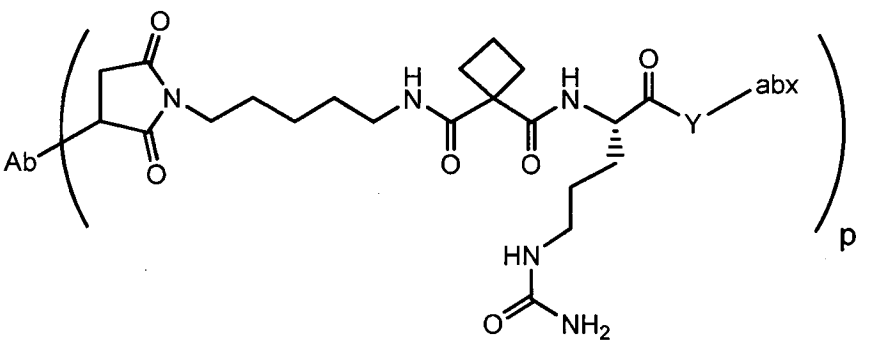
17. The antibody-antibiotic conjugate compound of claim 16 having the formula:



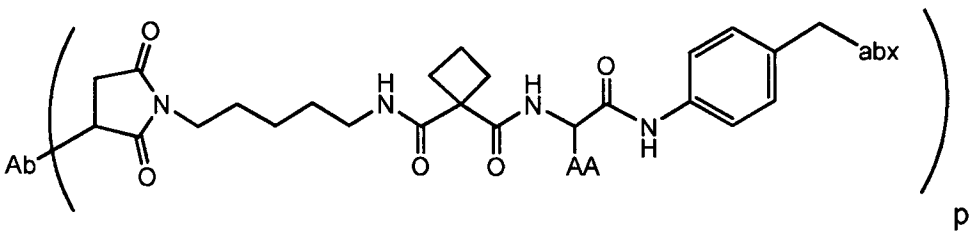
18. The antibody-antibiotic conjugate compound of claim 15 having the formula:



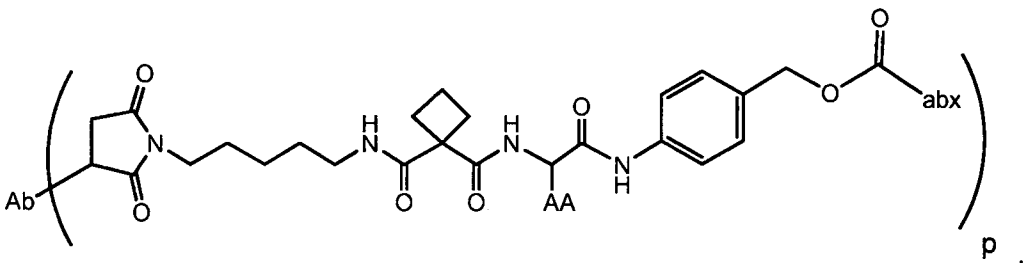
19. The antibody-antibiotic conjugate compound of claim 18 having the formula:



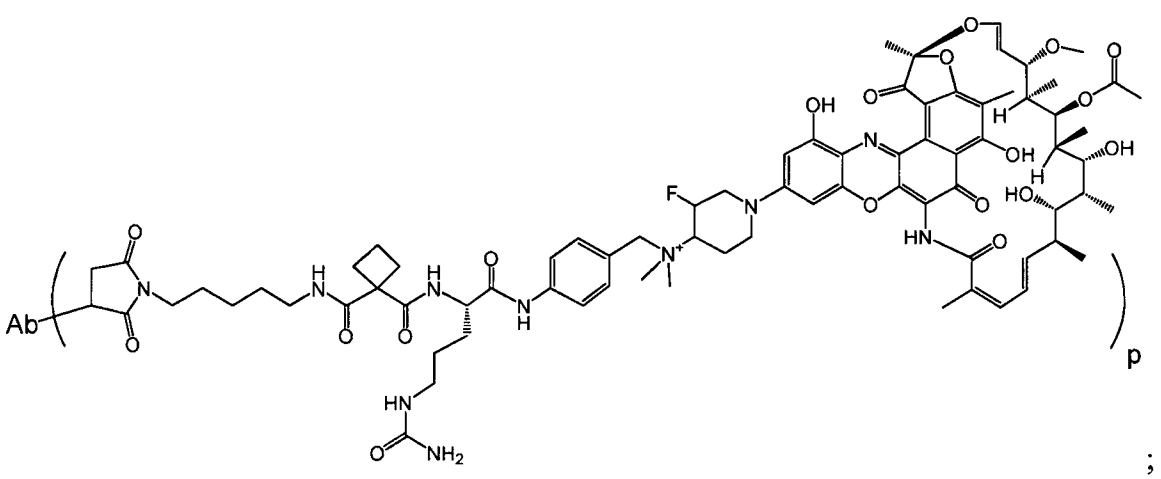
20. The antibody-antibiotic conjugate compound of claim 15 selected from the formulas:



and



21. The antibody-antibiotic conjugate compound of claim 16 selected from the formulas:



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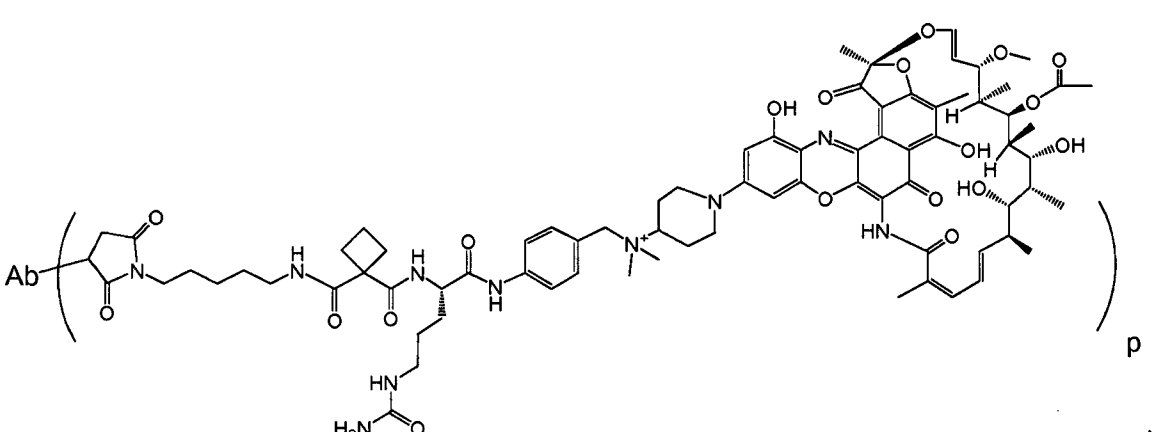

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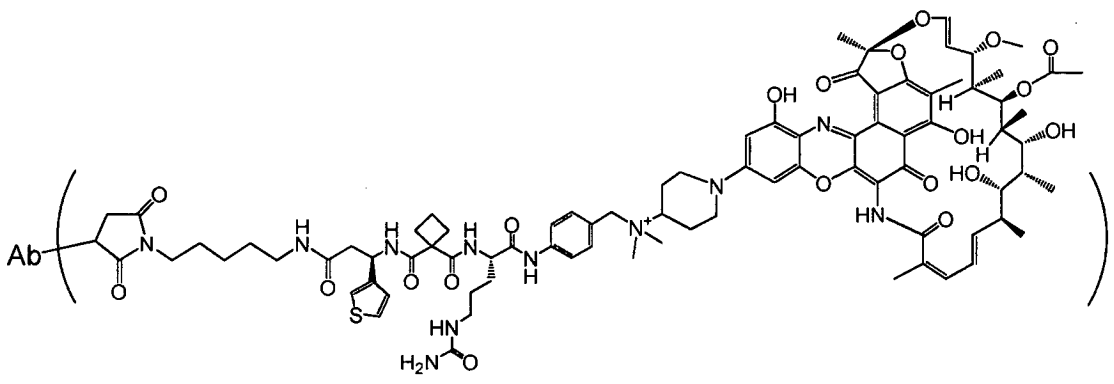
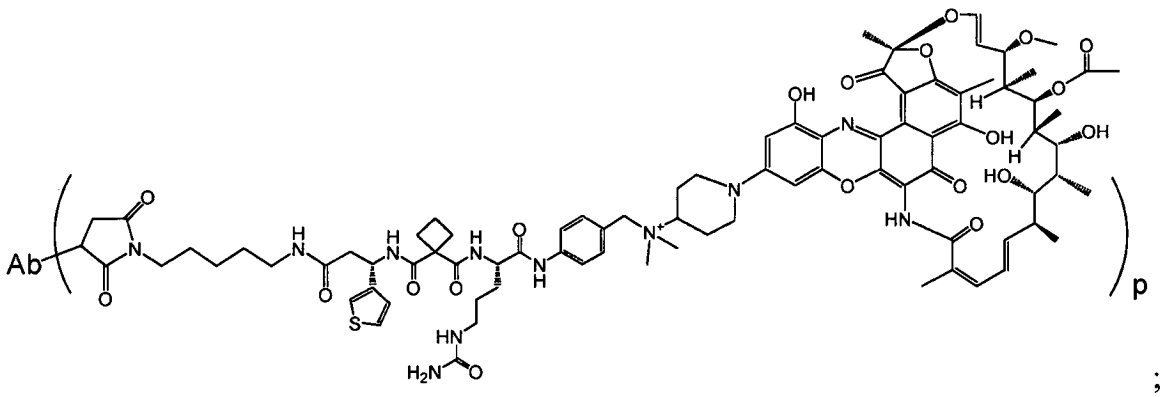
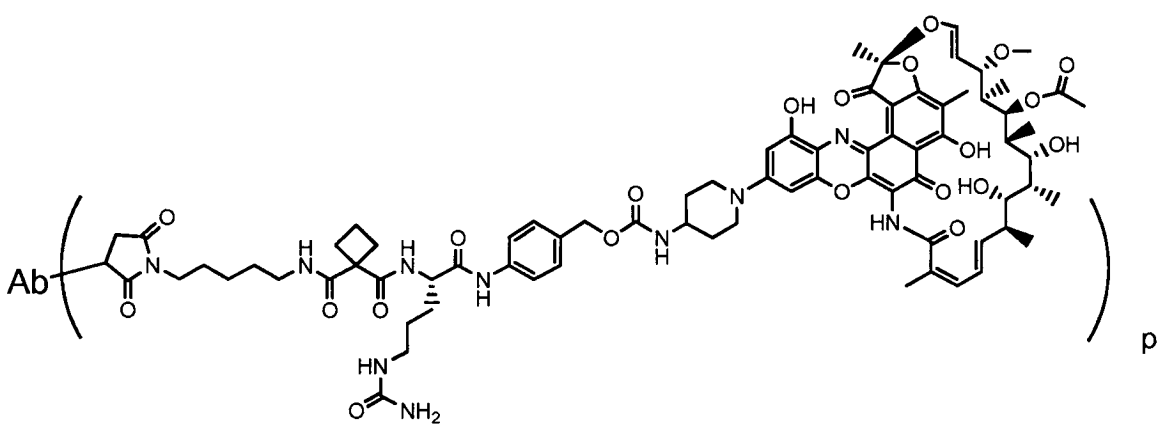


;

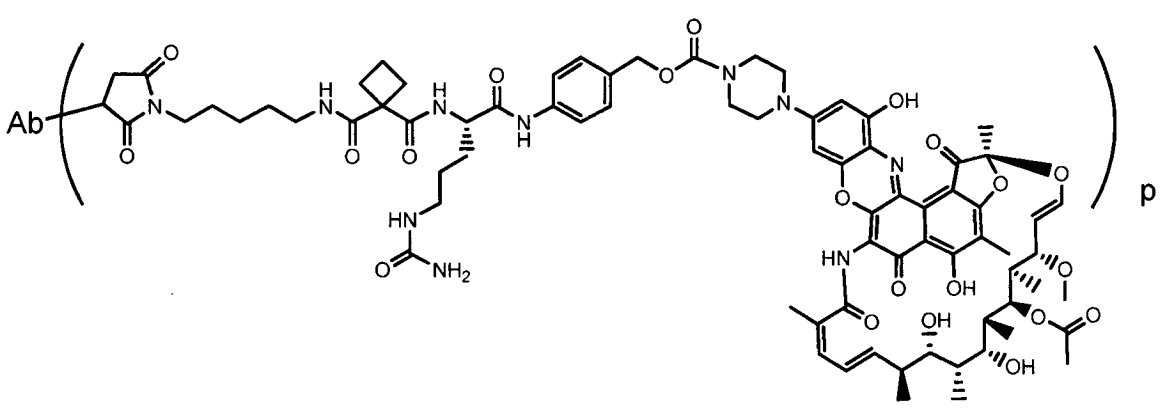


;



;

and



.

22. The antibody-antibiotic conjugate compound of claim 1, wherein the anti-SDR antibody is a rF1 antibody.

23. The antibody-antibiotic conjugate of claim 22, wherein the rF1 antibody comprises a light (L) chain and a heavy (H) chain, the L chain comprising CDR L1, CDR L2, and CDR L3 and the H chain comprising CDR H1, CDR H2 and CDR H3, wherein the CDR L1, CDR L2, and CDR L3 and CDR H1, CDR H2 and CDR H3 comprise the amino acid sequences of the CDRs of each of Abs F1, rF1, rF1.v1 and rF1.v6 (SEQ ID NO.1-8), respectively, as shown in Table 4A and Table 4B .

24. The antibody-antibiotic conjugate of claim 22 wherein the rF1 antibody comprises a heavy chain variable region (VH), wherein the VH comprises at least 95% sequence identity over the length of the VH region of SEQ ID NO.13.

25. The antibody-antibiotic conjugate compound of claim 24, wherein the VL comprises at least 95% sequence identity over the length of the VL region of SEQ ID NO. 14 or SEQ ID NO.15.

26. The antibody-antibiotic conjugate compound of any one of claim 1 or claim 22 wherein the anti-SDR antibody binds to *Staphylococcus aureus* and/or *Staphylococcus epidermidis* in vivo.

27. The antibody-antibiotic conjugate compound of any of the preceding claims, wherein the antibody is a F(ab) or a $F(ab')_2$.

28. A pharmaceutical composition comprising the antibody-antibiotic conjugate compound of claim 1, and a pharmaceutically acceptable carrier, glidant, diluent, or excipient.

29. A method of treating a Staphyloccocal bacterial infection in a patient comprising administering to the patient a therapeutically-effective amount of the antibody-antibiotic conjugate compound of claim 1.

30. The method of claim 29 wherein the patient is infected with Staphylococcus aureus.

31. The method of claim 30 wherein the patient is infected with Staphylococcus epidermidis.

32. The method of claim 29 wherein the antibody-antibiotic conjugate compound is administered to the patient at a dose in the range of about 50mg/kg to 100mg/kg.

33. The method of claim 29 wherein the patient is administered the antibody-antibiotic conjugate compound in conjunction with treatment with a second antibiotic.

34. A method of killing intracellular Staph aureus in the cells of a staph aureus infected patient without killing the host cells by administering an antibody-antibiotic conjugate compound of claim 1.

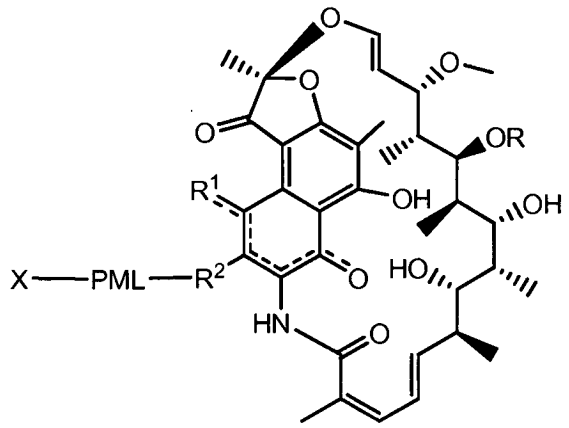
35. A process for making the antibody-antibiotic conjugate compound of claim 1 comprising conjugating a rifamycin-type antibiotic to an rF1 antibody.

36. A kit for treating a bacterial infection, comprising:

a) the pharmaceutical composition of claim 23; and

b) instructions for use.

37. An antibiotic-linker intermediate having Formula II:



II

wherein:

the dashed lines indicate an optional bond;

R is H, C_1–C_{12} alkyl, or $C(O)CH_3$;

R^1 is OH;

R^2 is CH=N–(heterocyclyl), wherein the heterocyclyl is optionally substituted with one or more groups independently selected from $C(O)CH_3$, C_1–C_{12} alkyl, C_1–C_{12} heteroaryl, C_2–C_{20} heterocyclyl, C_6–C_{20} aryl, and C_3–C_{12} carbocyclyl;

or R^1 and R^2 form a five- or six-membered fused heteroaryl or heterocyclyl, and optionally forming a spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring, wherein the spiro or fused six-membered heteroaryl, heterocyclyl, aryl, or carbocyclyl ring is optionally substituted H, F, Cl, Br, I, C_1–C_{12} alkyl, or OH;

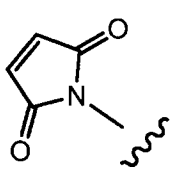
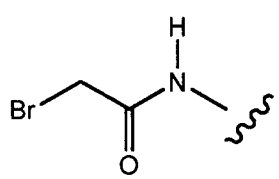
PML is a protease-cleavable, non-peptide linker attached to R^2 or the fused heteroaryl or heterocyclyl formed by R^1 and R^2, and having the formula:

–Str–PM–Y–

where Str is a stretcher unit; PM is a peptidomimetic unit, and Y is a spacer unit; and

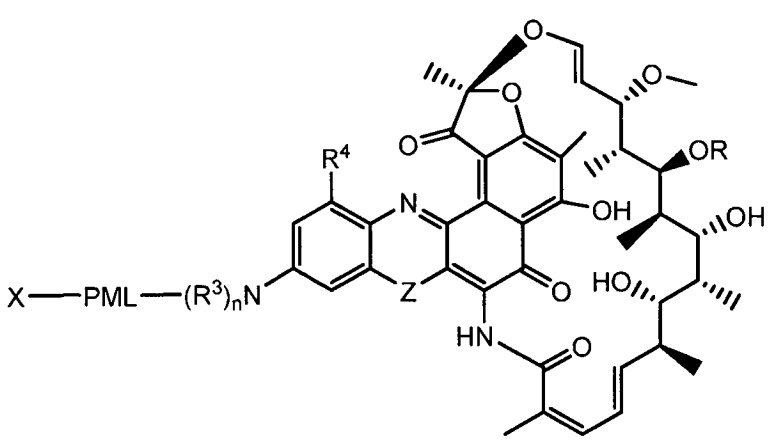
X is a reactive functional group selected from maleimide, thiol, amino, bromide, bromoacetamido, iodoacetamido, p-toluenesulfonate, iodide, hydroxyl, carboxyl, pyridyl disulfide, and N-hydroxysuccinimide.

38. The antibiotic-linker intermediate of claim 37 wherein X is

or

39. The antibiotic-linker intermediate of claim 37 having the formula:



wherein

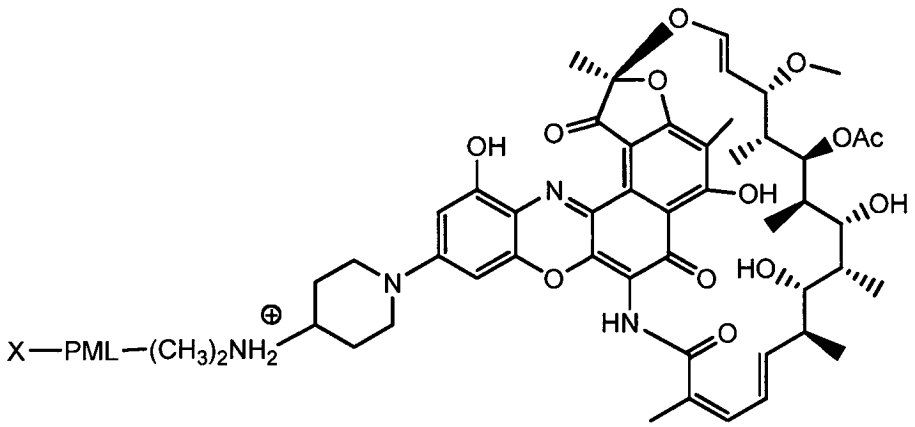
R^3 is independently selected from H and C_1–C_{12} alkyl;

n is 1 or 2;

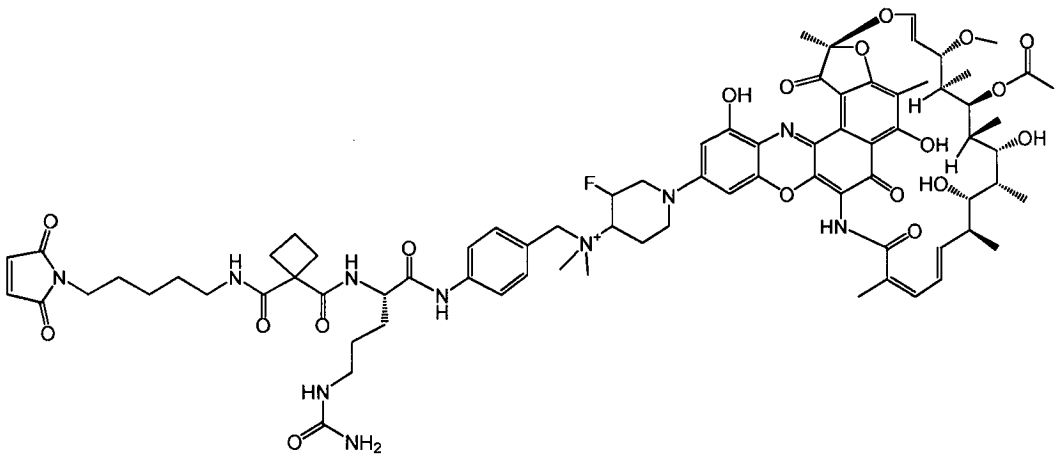
R^4 is selected from H, F, Cl, Br, I, C_1–C_{12} alkyl, and OH; and

Z is selected from NH, N(C_1–C_{12} alkyl), O and S.

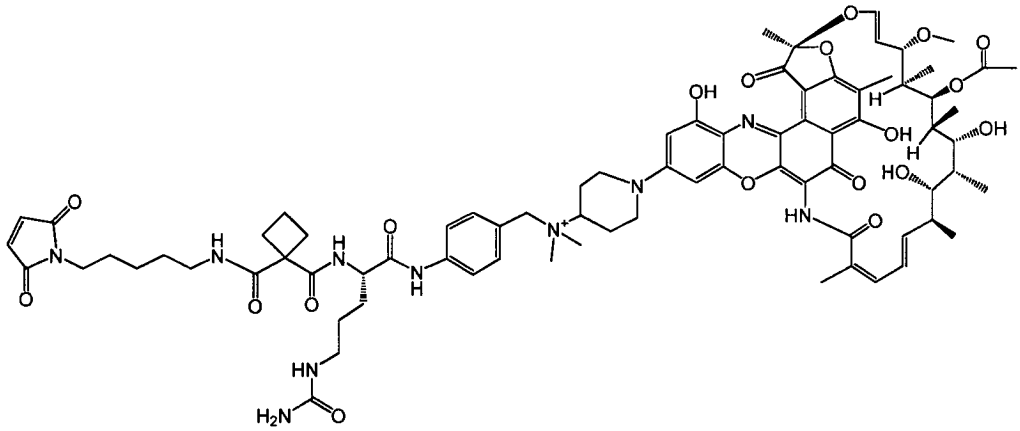
40. The antibiotic-linker intermediate of claim 37 having the formula:



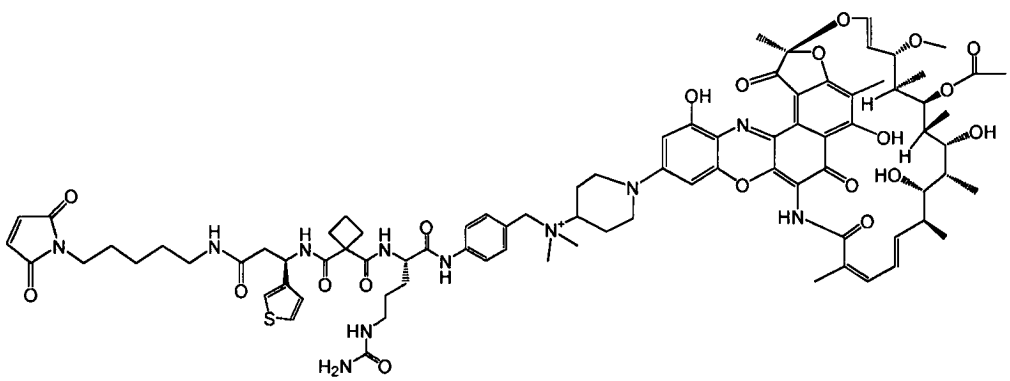
41. The antibiotic-linker intermediate of claim 37 selected from the formulas:



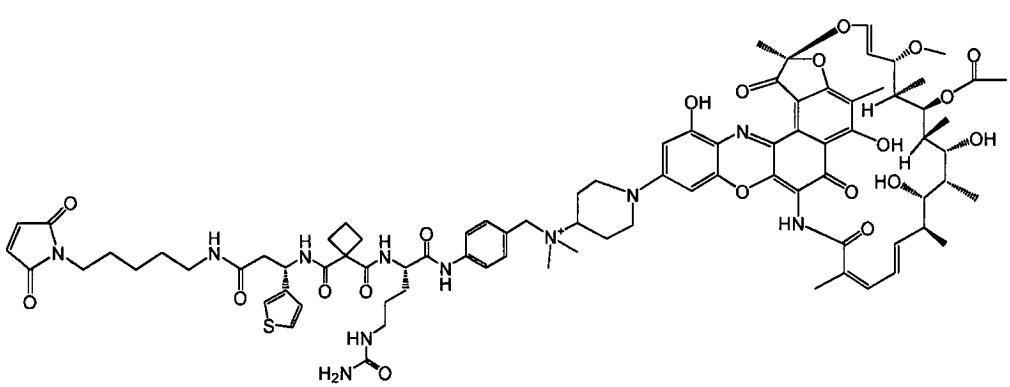
;



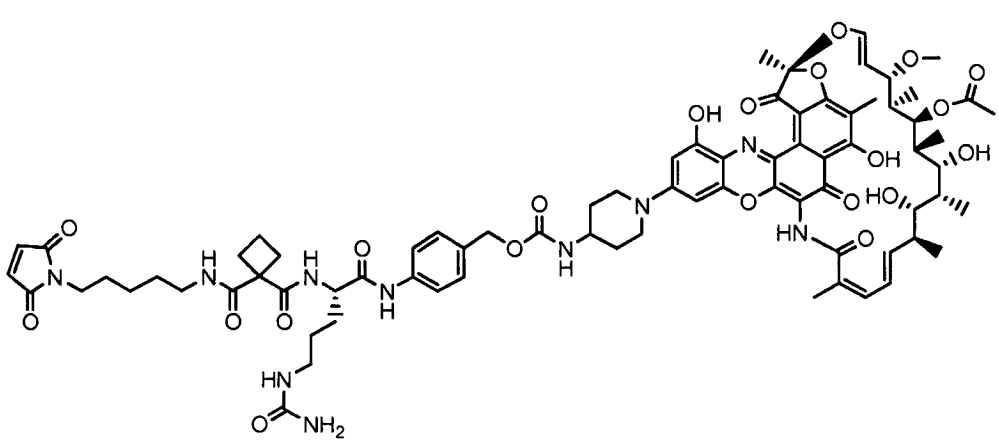
;



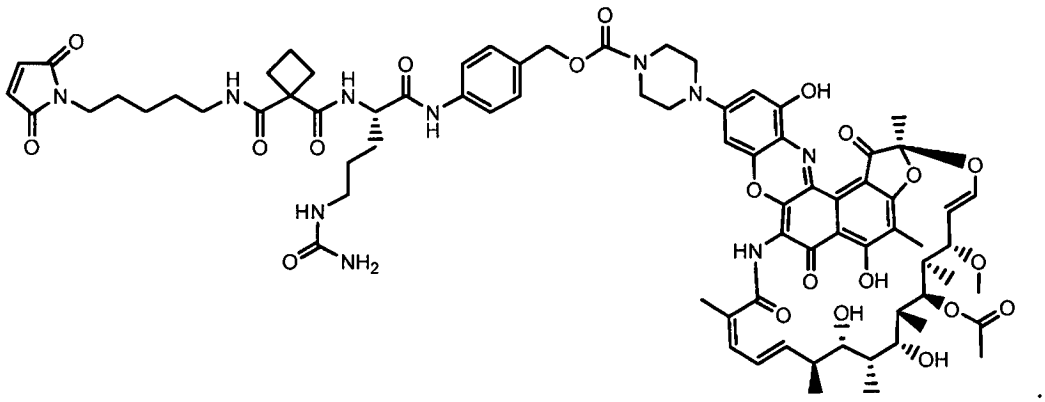
;



;



; and



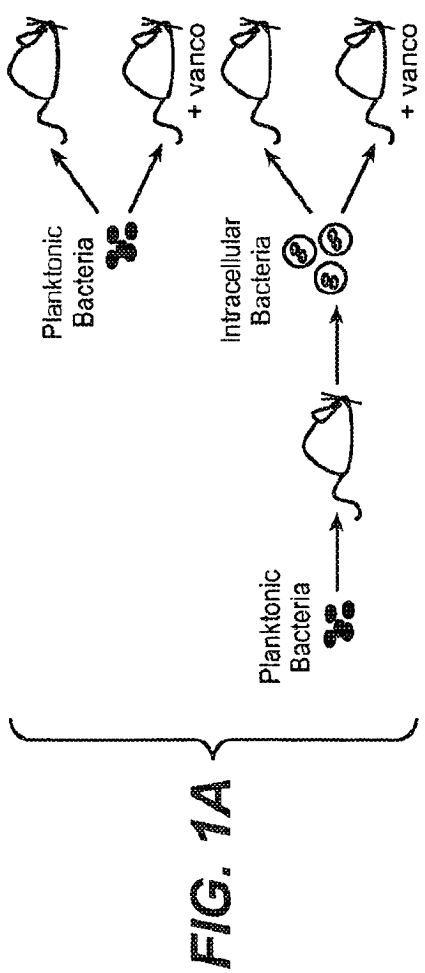


FIG. 1A

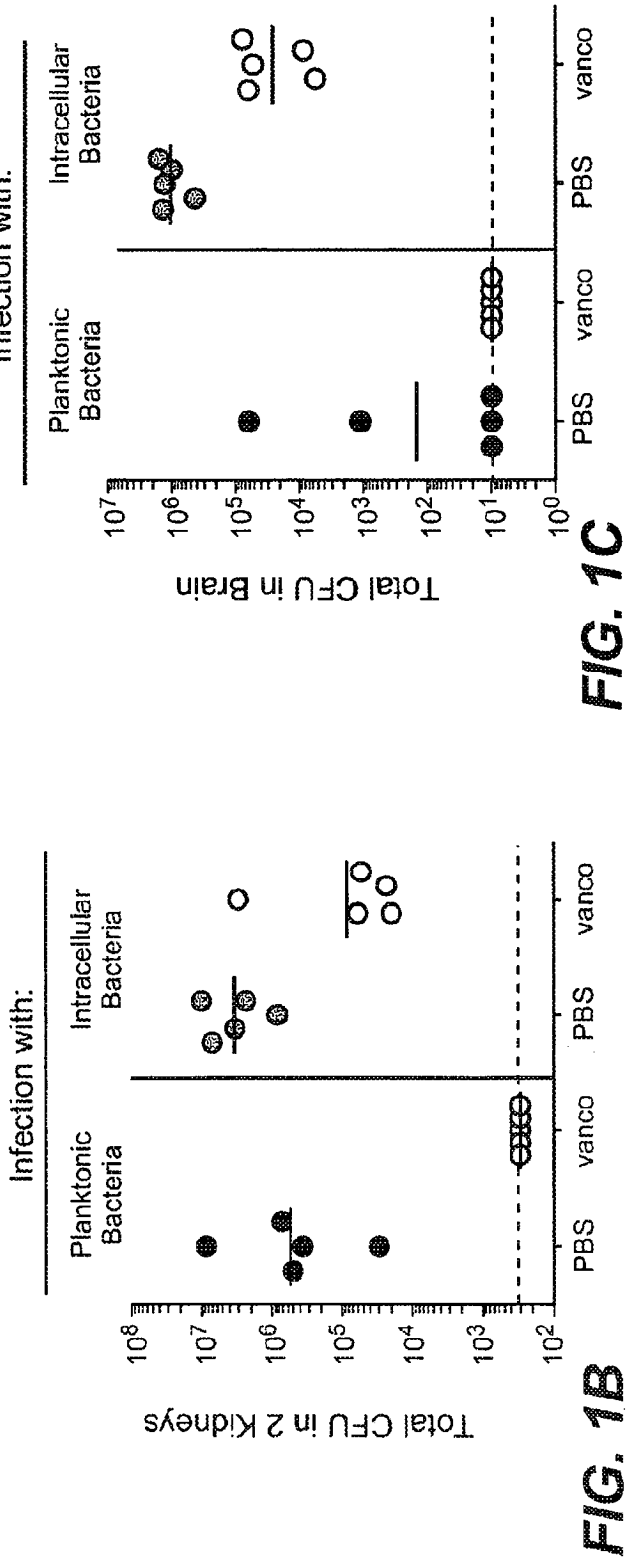


FIG. 1B

FIG. 1C

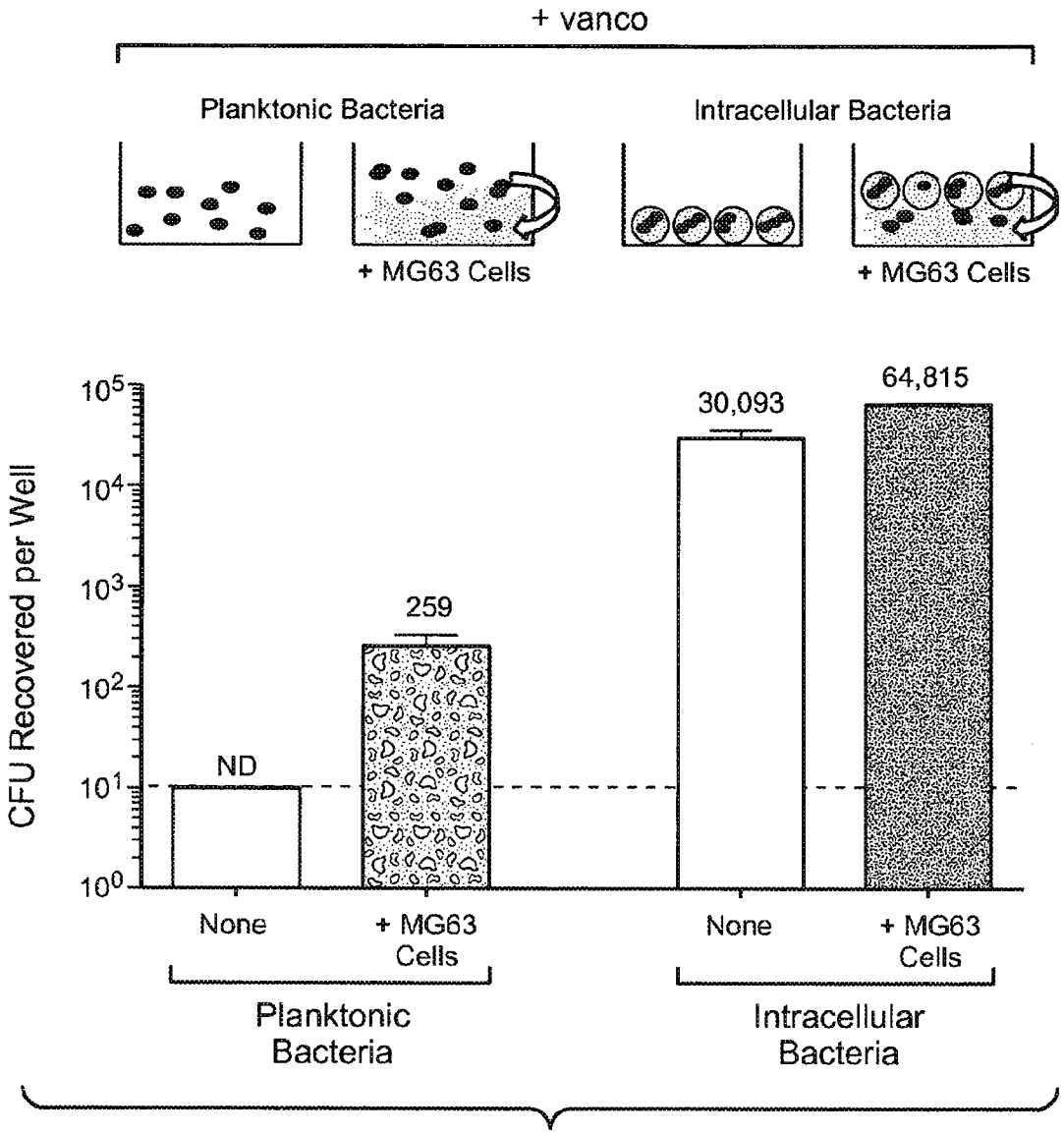

+ vanco
Planktonic Bacteria
Intracellular Bacteria
+ MG63 Cells
+ MG63 Cells
CFU Recovered per Well
10^5
10^4
10^3
10^2
10^1
10^0
ND
259
30,093
64,815
None
+ MG63 Cells
None
+ MG63 Cells
Planktonic Bacteria
Intracellular Bacteria

FIG. 1D

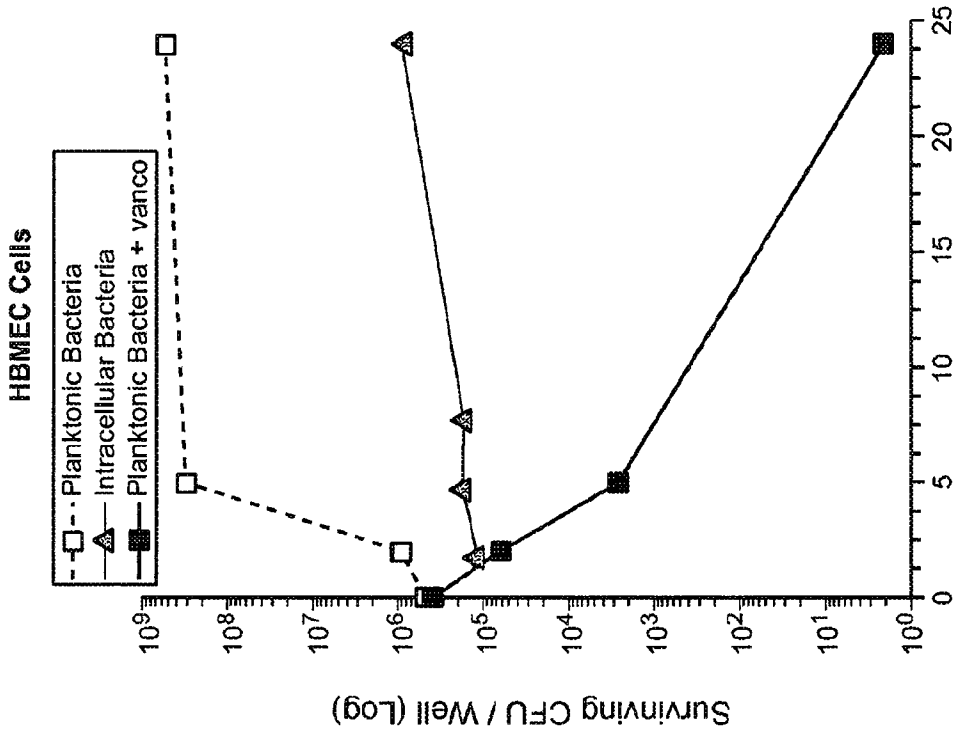

HBMEC Cells
Planktonic Bacteria
Intracellular Bacteria
Planktonic Bacteria + vanco
Surviving CFU / Well (Log)
Time (Hrs)

FIG. 1F

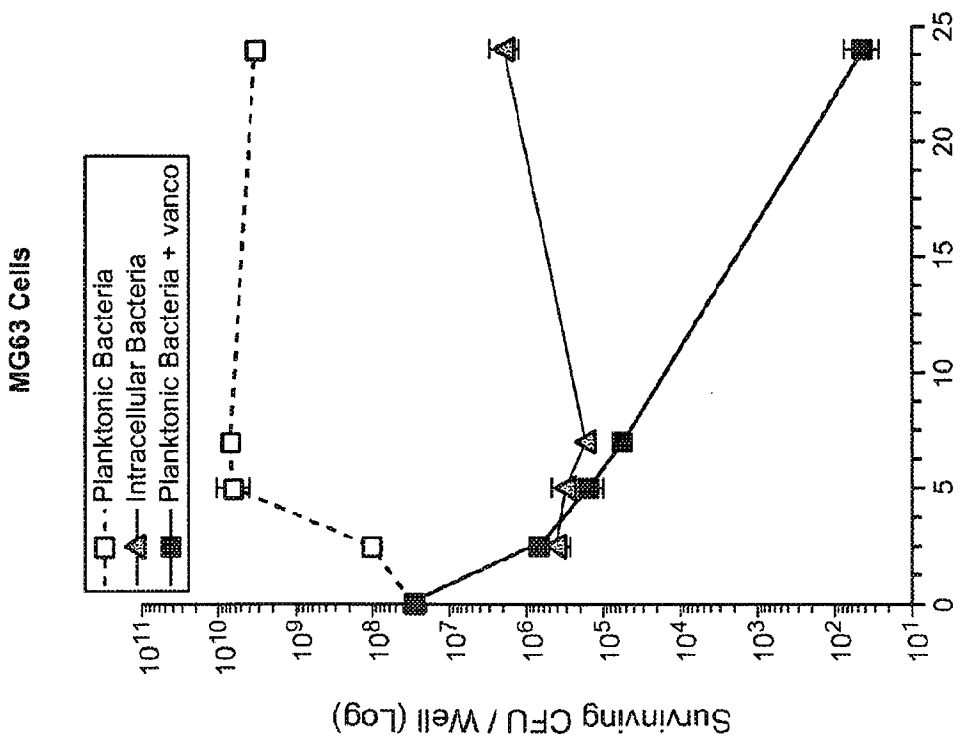
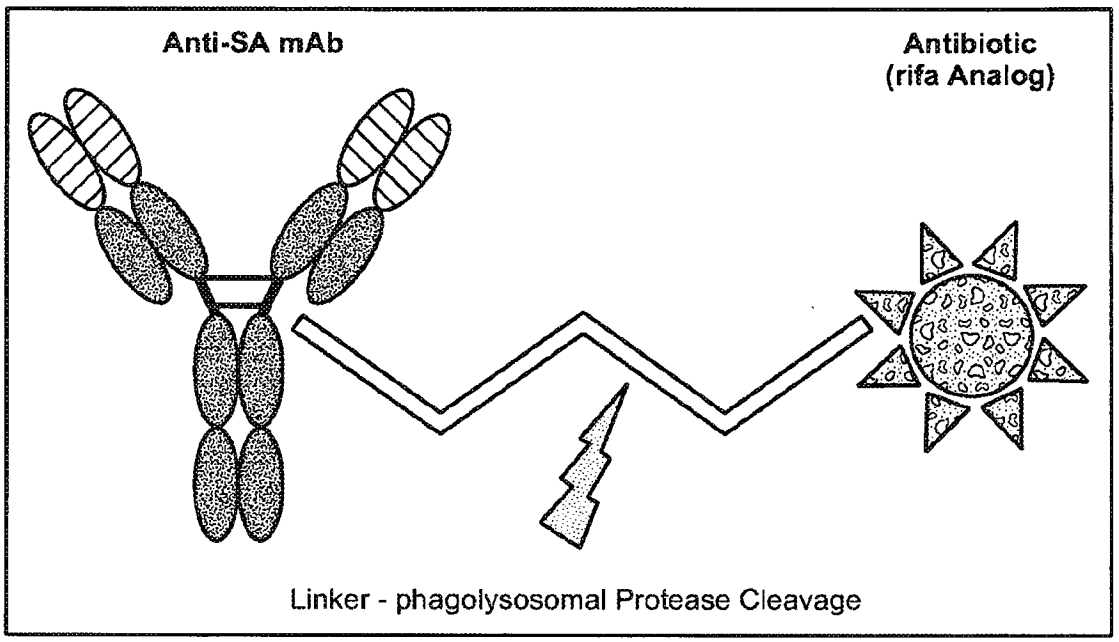

MG63 Cells
Planktonic Bacteria
Intracellular Bacteria
Planktonic Bacteria + vanco
Surviving CFU / Well (Log)
Time (Hrs)

FIG. 1E



• **Concept of TAC:**
Antibiotic is released from TAC by
Phagolysosomal Proteases

FIG. 2

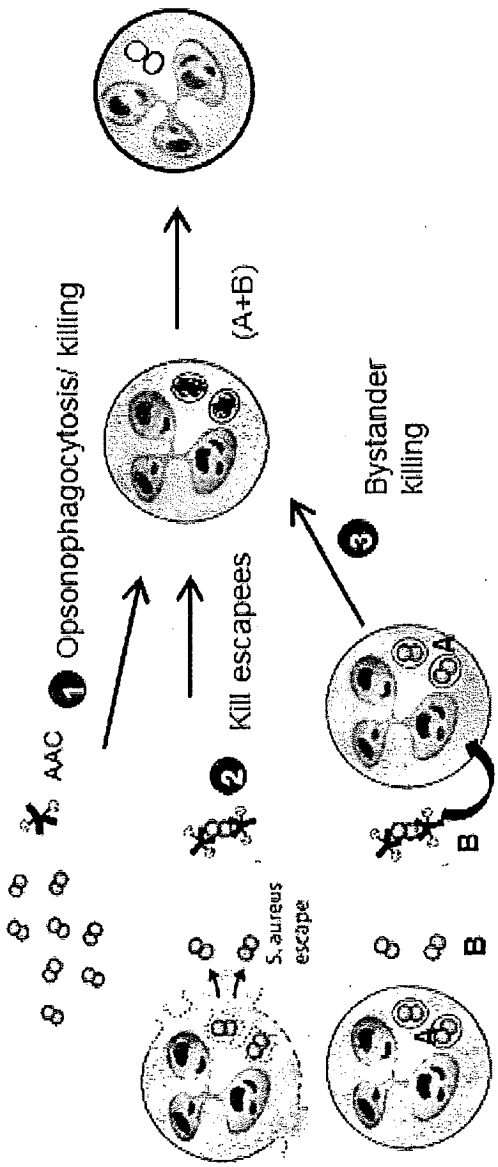

Opsonophagocytosis/ killing
(A+B)
Bystander killing
AAC
Kill escapees
S. aureus escape
A
B

FIG. 3

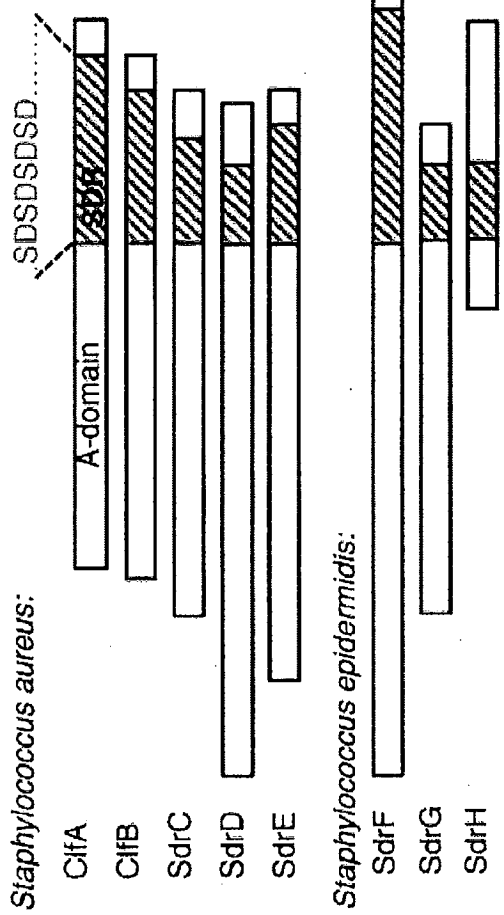

Staphylococcus aureus:
ClfA
ClfB
SdrC
SdrD
SdrE
A-domain
SDSDSDSD
Staphylococcus epidermidis:
SdrF
SdrG
SdrH

FIG. 4A

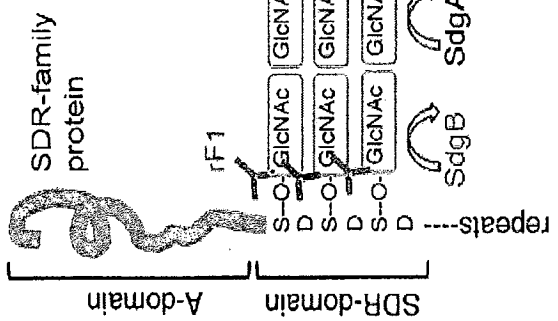

SDR-family protein
rF1
GlcNAc
SdgB
SdgA
repeats
A-domain
SDR-domain

FIG. 4B

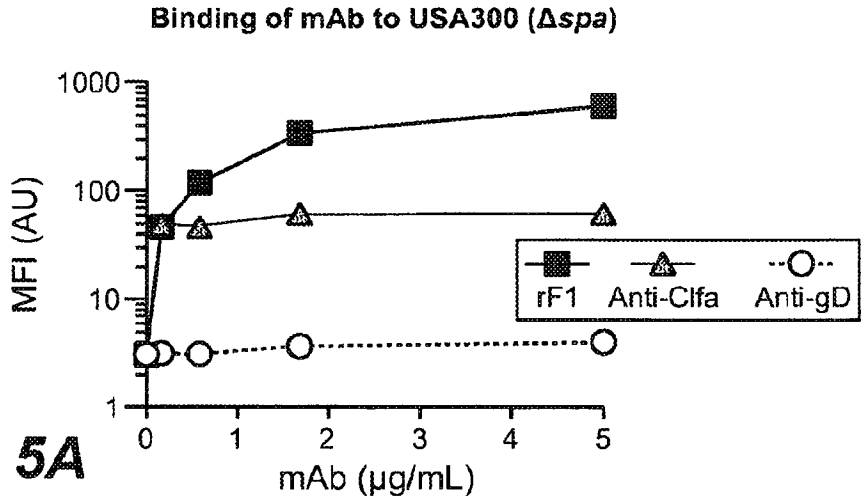


FIG. 5A

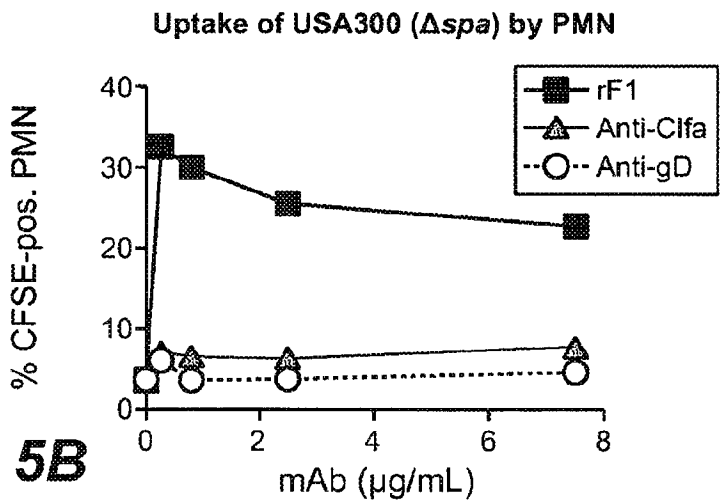


FIG. 5B

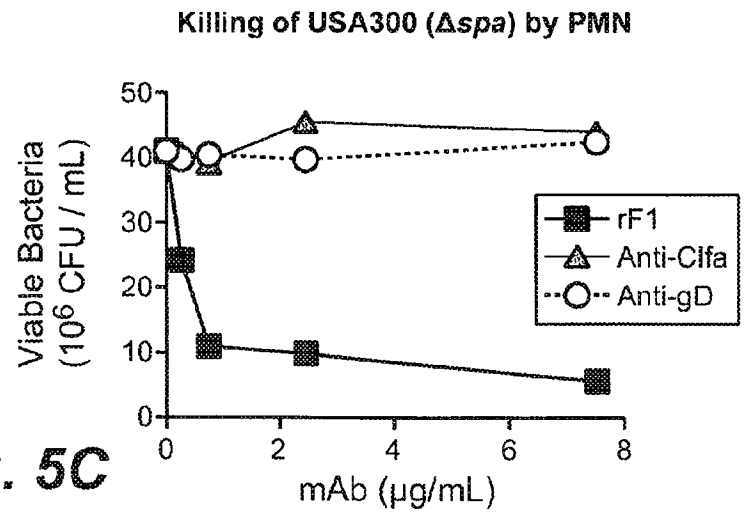


FIG. 5C

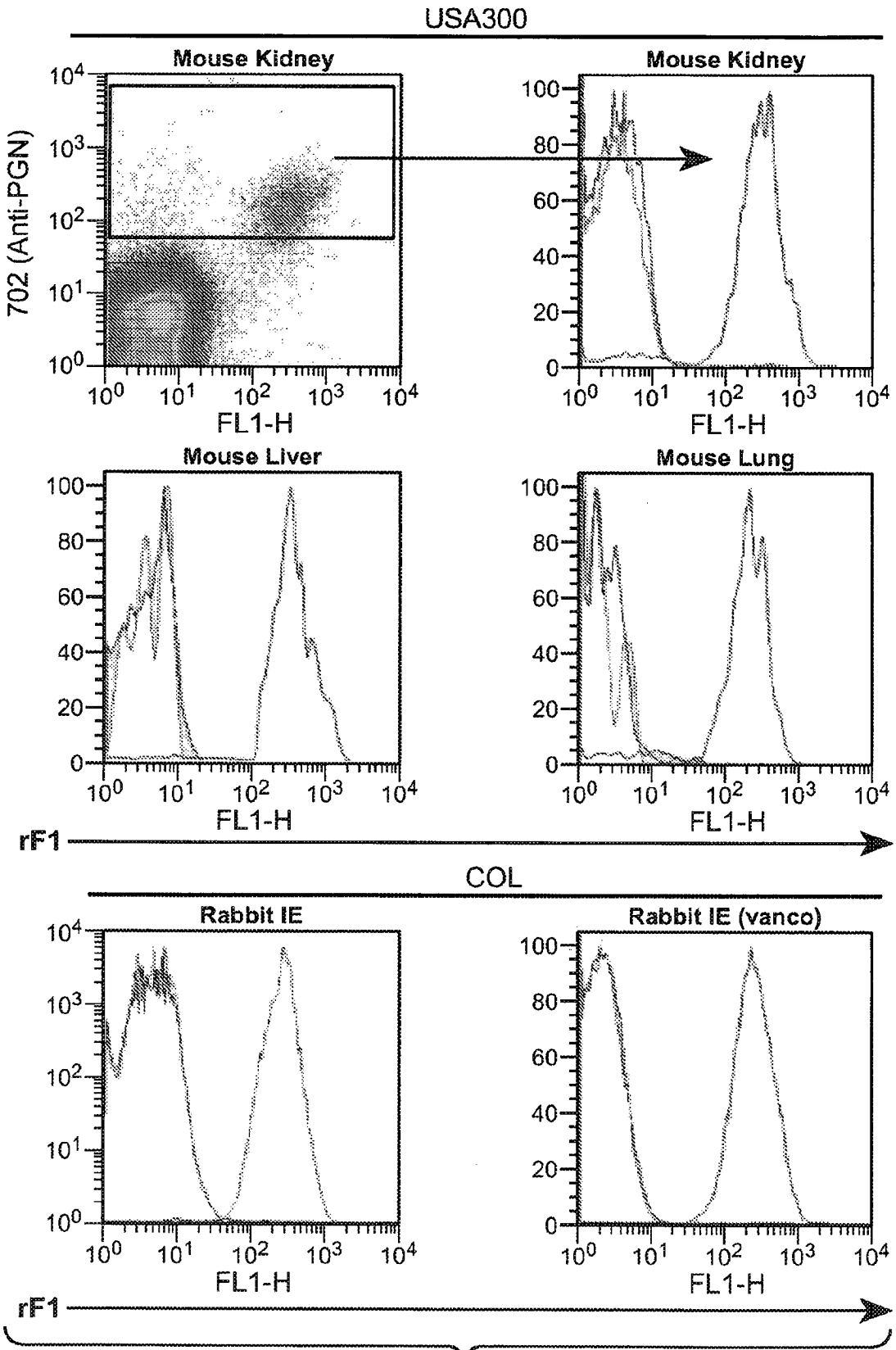

USA300
Mouse Kidney
Mouse Kidney
702 (Anti-PGN)
FL1-H
FL1-H
Mouse Liver
Mouse Lung
FL1-H
FL1-H
rF1
COL
Rabbit IE
Rabbit IE (vanco)
FL1-H
FL1-H
rF1

FIG. 6

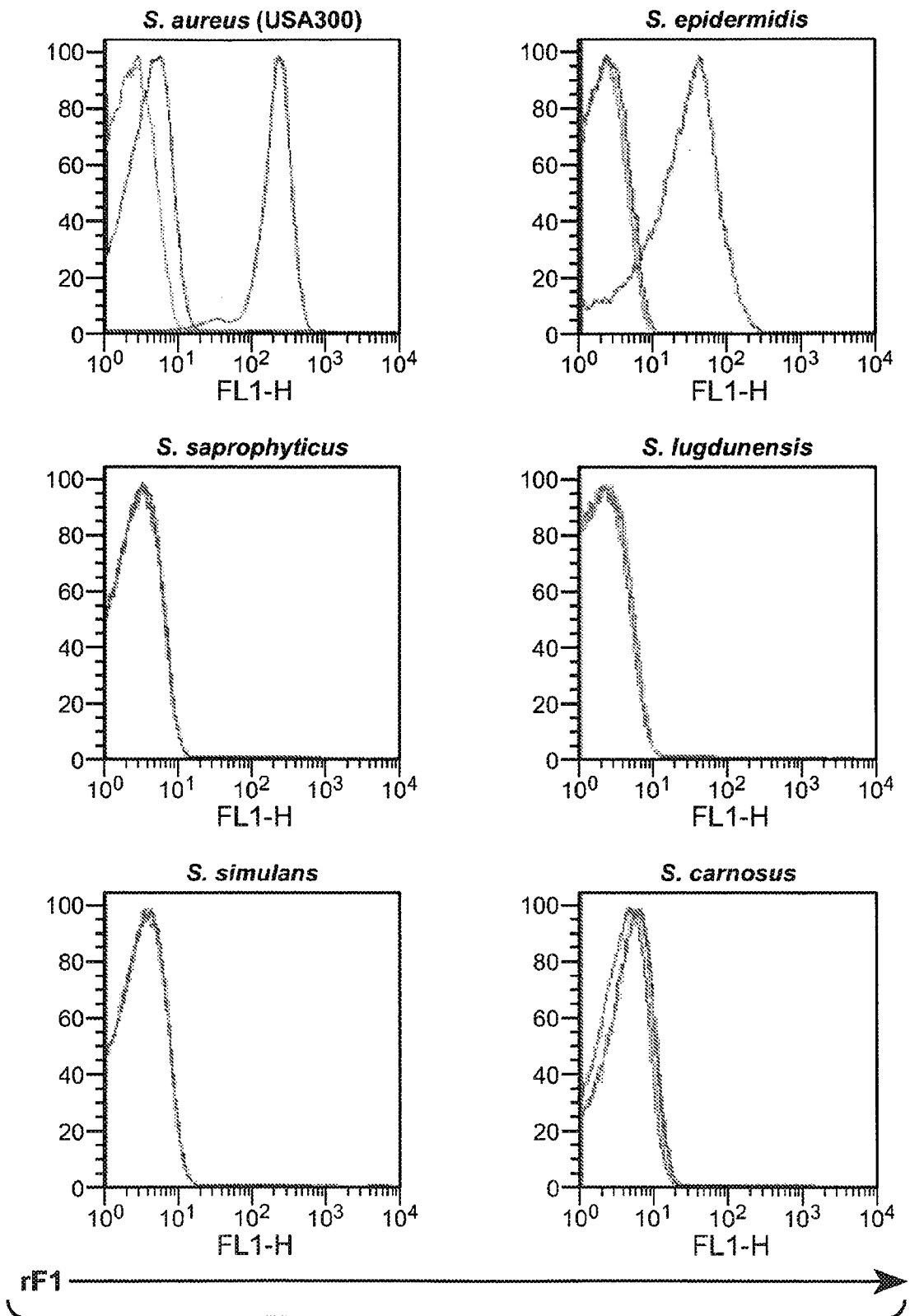

S. aureus (USA300)
S. epidermidis
S. saprophyticus
S. lugdunensis
S. simulans
S. carnosus
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4
FL1-H
rF1

FIG. 7A

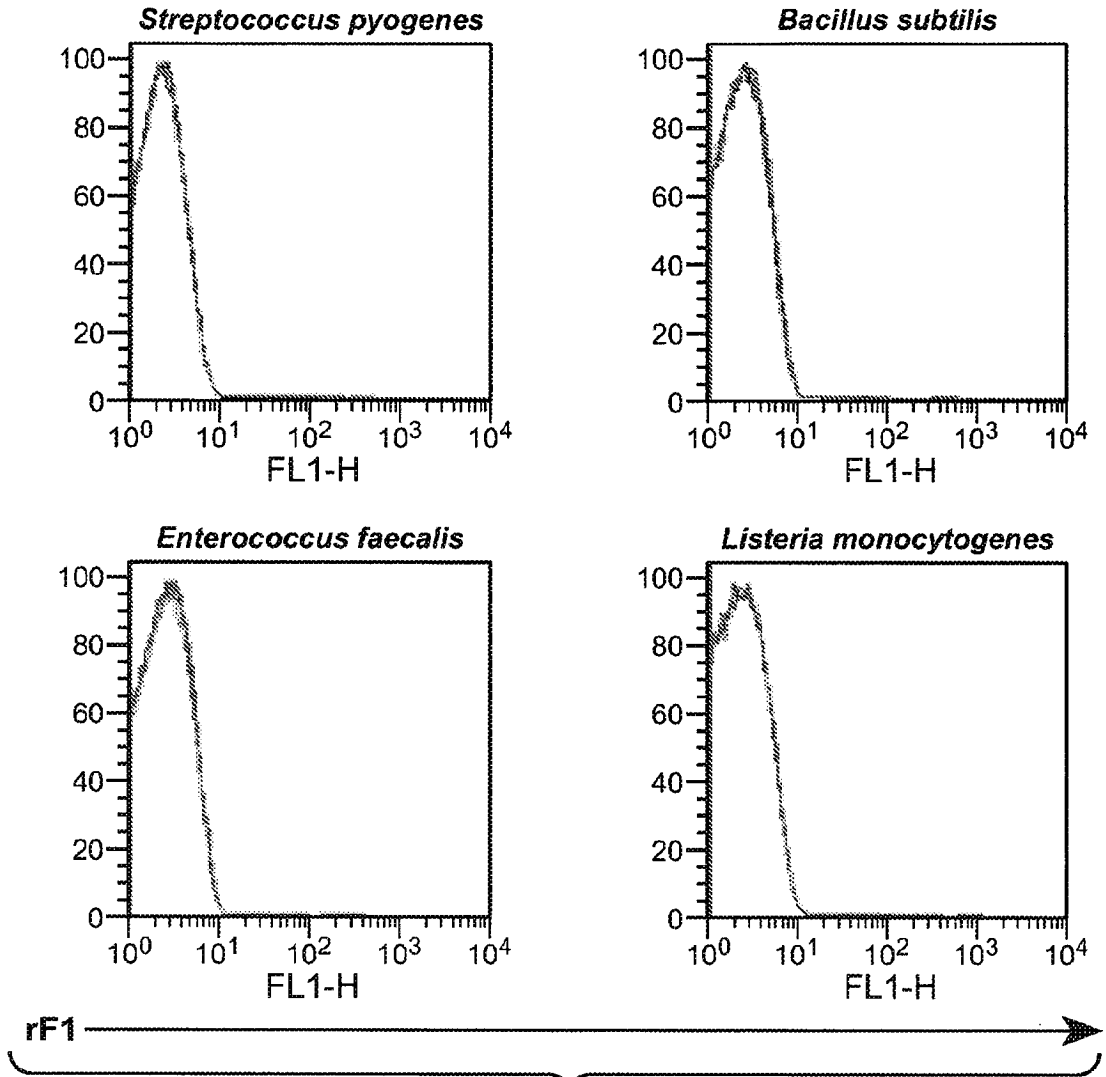

Streptococcus pyogenes
Bacillus subtilis
Enterococcus faecalis
Listeria monocytogenes
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4
FL1-H
rF1

FIG. 7B

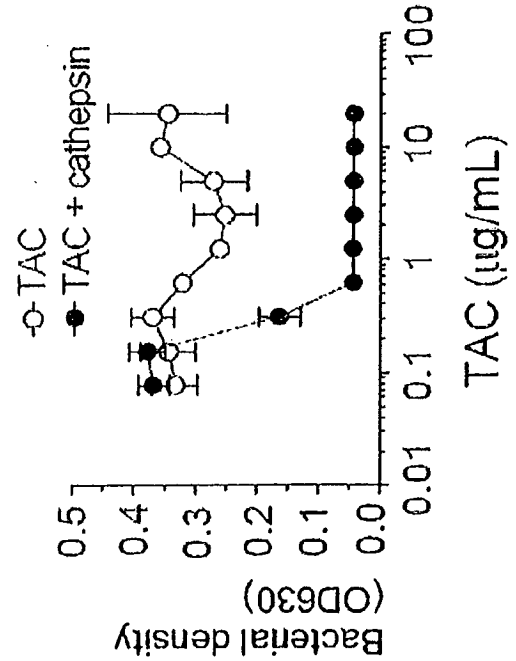

TAC
TAC + cathepsin
Bacterial density (OD630)
0.5
0.4
0.3
0.2
0.1
0.0
0.01
0.1
1
10
100
TAC (µg/mL)

FIG. 9

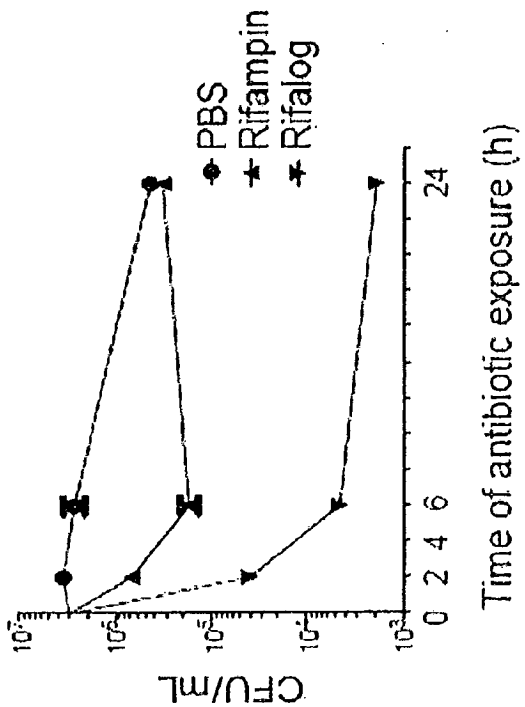

PBS
Rifampin
Rifalog
CFU/mL
0 2 4 6
24
Time of antibiotic exposure (h)

FIG. 8

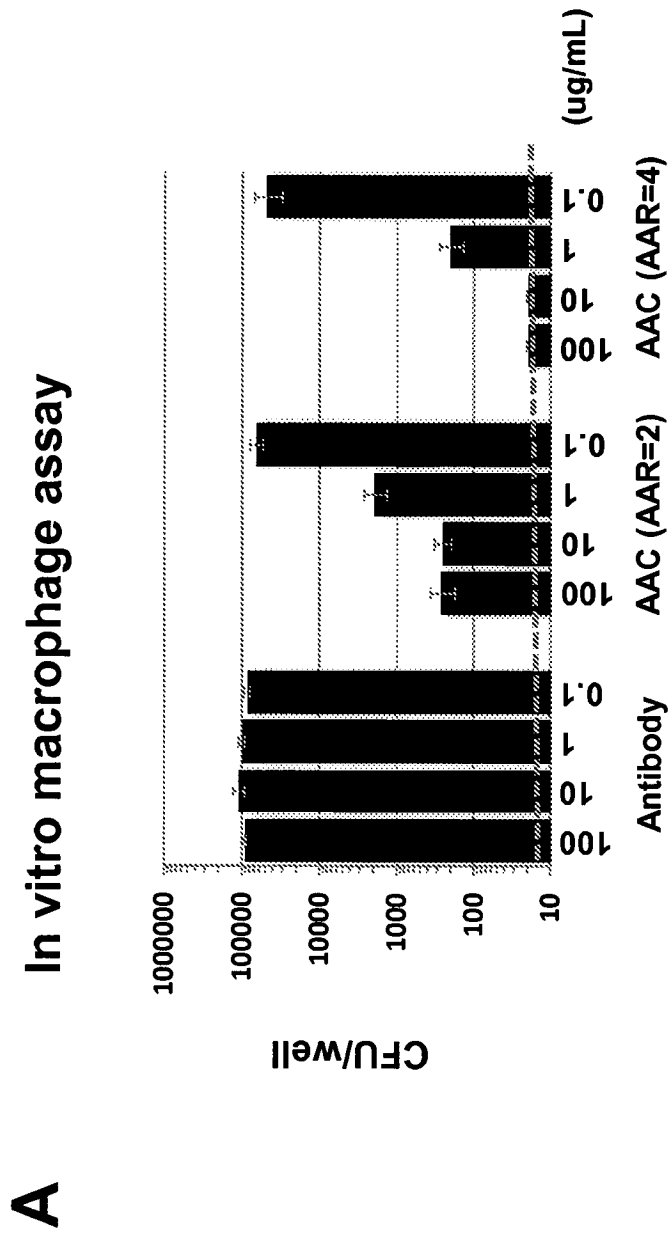

A
In vitro macrophage assay
CFU/well
1000000
100000
10000
1000
100
10
Antibody
100
10
1
0.1
AAC (AAR=2)
100
10
1
0.1
AAC (AAR=4)
100
10
1
0.1
(ug/mL)

FIG. 10

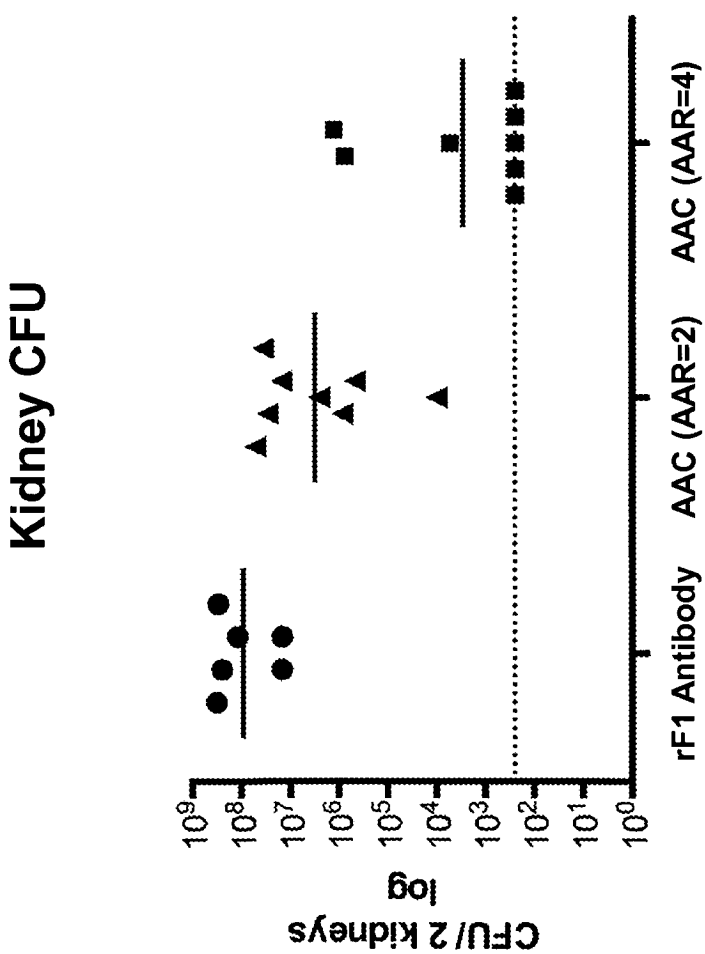


Figure 11A

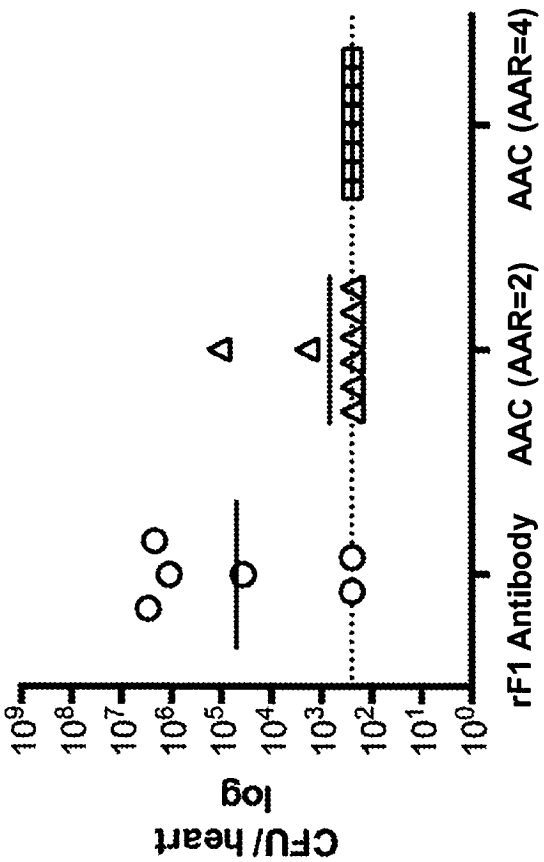


Figure 11B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/063515

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2011/008092 A2 (AIMM THERAPEUTICS BV; GENENTECH INC) 20 January 2011 (2011-01-20) pages 19-20; claims 1-7,14-16,25; example 5; sequences 1-10;27-30 pages 2-11	1,22-36 1-13, 15-21, 37-41

X A	WO 2014/080251 A1 (HANGZHOU DAC BIOTECH CO LTD) 30 May 2014 (2014-05-30) pages 35-36; claims 14; 11,12; figures 18,19 pages 43-44	12,13, 15,37,38 1-3,14, 16-20, 22, 26-30, 32,34,35

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 February 2016	12/02/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Kanbier, Titia

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2015/063515

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/020885 A2 (UNIV CALIFORNIA) 13 March 2003 (2003-03-13)	1,3, 28-30, 34,35
A	pages 44,47-48, paragraphs 10,25,33-35,86,90-94; claims 33-35,37-39,41	6-11, 16-22, 26,27, 32,33, 37-41

Y	WO 2005/081711 A2 (SEATTLE GENETICS INC) 9 September 2005 (2005-09-09) cited in the application claims 4,12-13,19-21,52,54,5829-30	1-13, 15-21, 37-41

Y	WO 2005/082023 A2 (GENENTECH INC) 9 September 2005 (2005-09-09) pages 14-15; claims 5,6,12,19	1-13, 15-21, 37-41

Y	WO 2008/141044 A2 (GENENTECH INC) 20 November 2008 (2008-11-20) page 68, paragraph 269 pages 59-67,90	1-13, 15-21, 37-41

X	WO 2007/096703 A2 (TARGANTA THERAPEUTICS INC) 30 August 2007 (2007-08-30) page 66, lines 9-11; example 2 page 1, line 34 - page 2, line 9; claims 1-20,22,29-36 page 41, lines 11-14 page 42, line 28 - page 47, line 13 page 66, lines 15-34 page 69, lines 13-18	5-11,21, 37-41

A	WO 03/045319 A2 (ACTIVBIOTICS INC) 5 June 2003 (2003-06-05) pages 2-13; figures 1,3; examples 2-4 pages 16-19 pages 35-42; claims 1-10,14-19	5-11,21, 37-41

X,P	WO 2014/194247 A1 (GENENTECH INC) 4 December 2014 (2014-12-04) pages 18-26 pages 69-81; claims	1-41

X,P	WO 2014/193722 A1 (GENENTECH INC) 4 December 2014 (2014-12-04) pages 58-80; claims; examples 4,14,17	1-41

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/063515

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011008092 A2	20-01-2011	AR 077756 A1	21-09-2011
		AU 2010271582 A1	01-03-2012
		CA 2768204 A1	20-01-2011
		CN 102666583 A	12-09-2012
		EA 201390967 A1	30-01-2014
		EP 2454284 A2	23-05-2012
		IL 217497 A	30-11-2015
		JP 2012533293 A	27-12-2012
		KR 20120065326 A	20-06-2012
		NZ 598063 A	25-07-2014
		NZ 616921 A	26-06-2015
		SG 177653 A1	28-02-2012
		TW 201107470 A	01-03-2011
		US 2011020323 A1	27-01-2011
		US 2012157665 A1	21-06-2012
		US 2013253175 A1	26-09-2013
		US 2013261293 A1	03-10-2013
		US 2014235828 A1	21-08-2014
		US 2015376266 A1	31-12-2015
		WO 2011008092 A2	20-01-2011
WO 2014080251 A1	30-05-2014	AU 2012395148 A1	21-05-2015
		CA 2891280 A1	30-05-2014
		EP 2922818 A1	30-09-2015
		US 2015250896 A1	10-09-2015
		WO 2014080251 A1	30-05-2014
WO 03020885 A2	13-03-2003	AU 2002326691 A1	18-03-2003
		CA 2455435 A1	13-03-2003
		EP 1416955 A2	12-05-2004
		US 2003153020 A1	14-08-2003
		WO 03020885 A2	13-03-2003
WO 2005081711 A2	09-09-2005	AU 2004316290 A1	09-09-2005
		BR PI0416028 A	02-01-2007
		CA 2543888 A1	09-09-2005
		CA 2841741 A1	09-09-2005
		CN 1938046 A	28-03-2007
		CN 103394083 A	20-11-2013
		CN 104998273 A	28-10-2015
		DK 1725249 T3	17-03-2014
		DK 2489364 T3	02-03-2015
		EP 1725249 A2	29-11-2006
		EP 2260858 A2	15-12-2010
		EP 2478912 A1	25-07-2012
		EP 2486933 A1	15-08-2012
		EP 2489364 A1	22-08-2012
		ES 2456325 T3	22-04-2014
		ES 2534185 T3	20-04-2015
		ES 2557389 T3	25-01-2016
		HK 1098071 A1	06-06-2014
		HK 1173684 A1	11-09-2015
		IL 175247 A	30-04-2013
		JP 5020636 B2	05-09-2012
		JP 5470288 B2	16-04-2014
		JP 5551661 B2	16-07-2014
		JP 2007514652 A	07-06-2007
		JP 2011121969 A	23-06-2011

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/063515

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		JP 2011231131 A	17-11-2011
		JP 2013151567 A	08-08-2013
		KR 20060111473 A	27-10-2006
		KR 20130119004 A	30-10-2013
		KR 20140015148 A	06-02-2014
		NZ 547633 A	27-08-2010
		NZ 583292 A	30-03-2012
		PT 1725249 E	10-04-2014
		PT 2489364 E	16-04-2015
		SG 149815 A1	27-02-2009
		SG 195524 A1	30-12-2013
		SI 1725249 T1	30-04-2014
		SI 2489364 T1	30-04-2015
		US 2005238649 A1	27-10-2005
		US 2008226657 A1	18-09-2008
		US 2008248051 A1	09-10-2008
		US 2008248053 A1	09-10-2008
		US 2009047296 A1	19-02-2009
		US 2012003247 A1	05-01-2012
		US 2012003248 A1	05-01-2012
		US 2012027783 A1	02-02-2012
		US 2012027784 A1	02-02-2012
		US 2012034246 A1	09-02-2012
		US 2012034247 A1	09-02-2012
		US 2012141508 A1	07-06-2012
		US 2012141509 A1	07-06-2012
		US 2012141510 A1	07-06-2012
		US 2012148608 A1	14-06-2012
		US 2012148610 A1	14-06-2012
		US 2014220047 A1	07-08-2014
		WO 2005081711 A2	09-09-2005
		ZA 200603619 A	29-10-2008
WO 2005082023 A2	09-09-2005	AU 2005216251 A1	09-09-2005
		CA 2556752 A1	09-09-2005
		EP 1718667 A2	08-11-2006
		JP 5064037 B2	31-10-2012
		JP 2007527421 A	27-09-2007
		US 2005256030 A1	17-11-2005
		US 2009041791 A1	12-02-2009
		US 2010273843 A1	28-10-2010
		WO 2005082023 A2	09-09-2005
WO 2008141044 A2	20-11-2008	AR 066476 A1	19-08-2009
		AU 2008251608 A1	20-11-2008
		CA 2683568 A1	20-11-2008
		CL 2008001334 A1	22-09-2008
		CN 101687037 A	31-03-2010
		EP 2144628 A2	20-01-2010
		JP 5290276 B2	18-09-2013
		JP 2010526821 A	05-08-2010
		PE 02452009 A1	17-03-2009
		TW 200902554 A	16-01-2009
		US 2008311134 A1	18-12-2008
		WO 2008141044 A2	20-11-2008
WO 2007096703 A2	30-08-2007	AU 2006339058 A1	30-08-2007
		BR PI0614758 A2	12-04-2011

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2015/063515

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
		CA	2618741 A1	30-08-2007
		CN	101282981 A	08-10-2008
		EA	200800570 A1	27-02-2009
		EP	1928890 A2	11-06-2008
		IL	189270 A	31-05-2015
		JP	5368092 B2	18-12-2013
		JP	2009505974 A	12-02-2009
		KR	20080035687 A	23-04-2008
		US	2011263534 A1	27-10-2011
		WO	2007096703 A2	30-08-2007
WO 03045319 A2	05-06-2003	AU	2002360416 A1	10-06-2003
		CA	2465846 A1	05-06-2003
		EP	1453837 A2	08-09-2004
		JP	2005510534 A	21-04-2005
		US	2004063718 A1	01-04-2004
		WO	03045319 A2	05-06-2003
WO 2014194247 A1	04-12-2014	AU	2014273939 A1	26-11-2015
		CA	2910029 A1	04-12-2014
		WO	2014194247 A1	04-12-2014
WO 2014193722 A1	04-12-2014	CA	2913011 A1	04-12-2014
		US	2015366985 A1	24-12-2015
		WO	2014193722 A1	04-12-2014

(19)中华人民共和国国家知识产权局

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WO2016/090040 EN 2016.06.09

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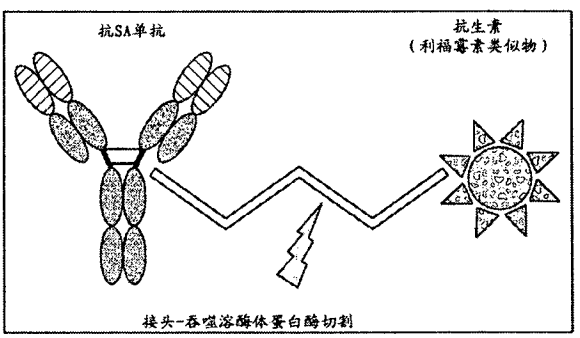
(54)发明名称

抗金黄色葡萄球菌抗体利福霉素缀合物及其用途

(57)摘要

本发明提供rF1抗体抗生素缀合物以及使用其的方法。



·TAC的概念：
抗生素通过吞噬溶酶体蛋白酶
从TAC释放

CN 107249642 A

1.一种抗体-抗生素缀合化合物，其包含抗丝氨酸-天冬氨酸重复序列(SDR)的抗体，所述抗体通过蛋白酶可切割的非肽类接头与利福霉素型抗生素共价连接。

2.权利要求1的抗体-抗生素缀合化合物，其具有下式：

Ab-(PML-abx)$_p$

其中：

Ab是rF1抗体；

PML是具有下式的蛋白酶可切割的非肽类接头：

-Str-PM-Y-

其中Str是延伸单元；PM是拟肽单元，和Y是间隔单元；

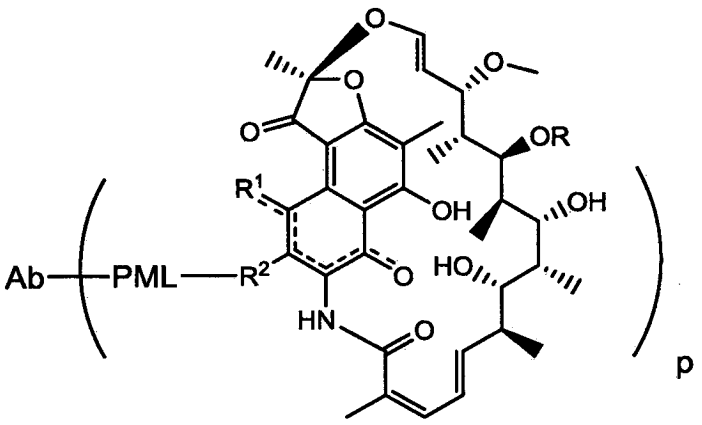
abx是利福霉素型抗生素；并且

p是从1到8的整数。

3.权利要求2的抗体-抗生素缀合化合物，其中利福霉素型抗生素是利福拉齐型抗生素。

4.权利要求2的抗体-抗生素缀合化合物，其中利福霉素型抗生素包含与蛋白酶可切割的非肽类接头连接的季胺。

5.权利要求2的抗体-抗生素缀合化合物，其具有式I：



I

其中：

虚线表示任选的键；

R是H、C_1-C_{12}烷基或C(O)CH_3；

R^1是OH；

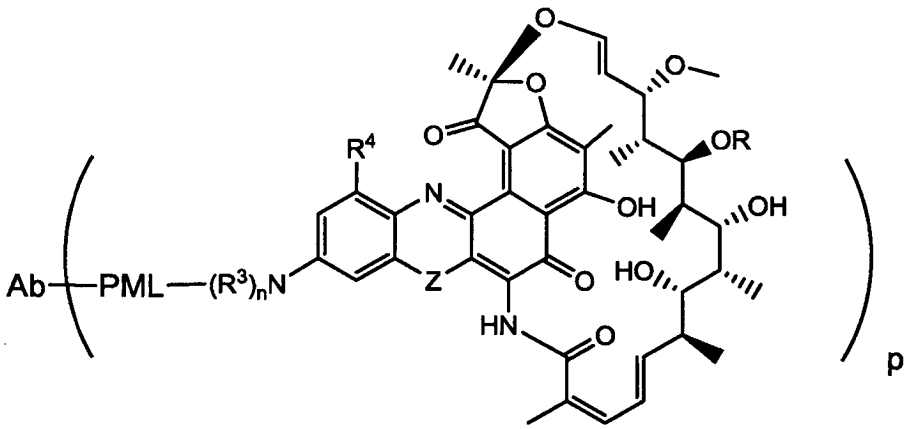
R^2是CH=N-(杂环基)，其中所述杂环基任选地被一个或多个独立地选自C(O)CH_3、C_1-C_{12}烷基、C_1-C_{12}杂芳基、C_2-C_{20}杂环基、C_6-C_{20}芳基和C_3-C_{12}碳环基的基团取代；

或R^1和R^2形成五元或六元稠合杂芳基或杂环基，并且任选地形成螺或稠合的六元杂芳基、杂环基、芳基或碳环基环，其中所述螺或稠合的六元杂芳基、杂环基、芳基或碳环基环任选地被H、F、Cl、Br、I、C_1-C_{12}烷基或OH取代；

PML是连接到R^2或由R^1和R^2形成的稠合杂芳基或杂环基的蛋白酶可切割的非肽类接头；并且

Ab是rF1抗体。

6.权利要求5的抗体-抗生素缀合化合物，其具有下式：



其中，

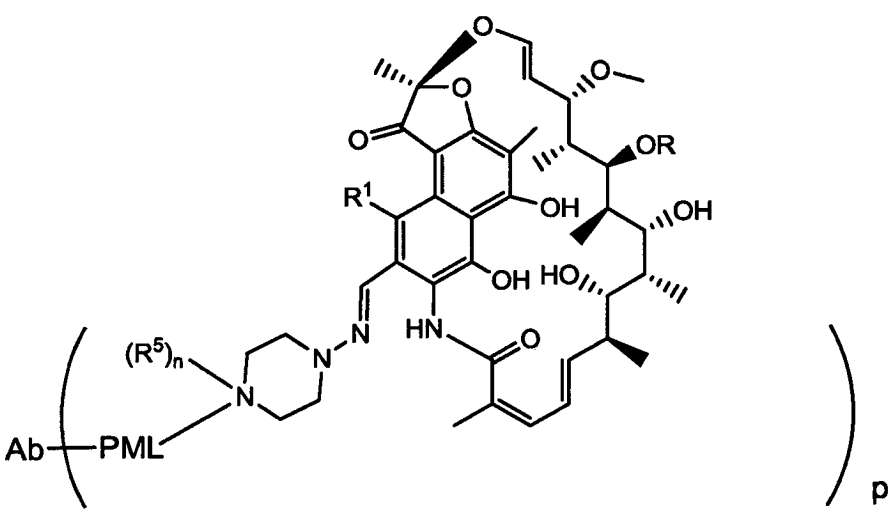
R^3独立地选自H和C_1-C_{12}烷基；

n为1或2；

R^4选自H、F、Cl、Br、I、C_1-C_{12}烷基和OH；并且

Z选自NH、N(C_1-C_{12}烷基)、O和S。

7.权利要求2的抗体-抗生素缀合化合物，其具有下式：

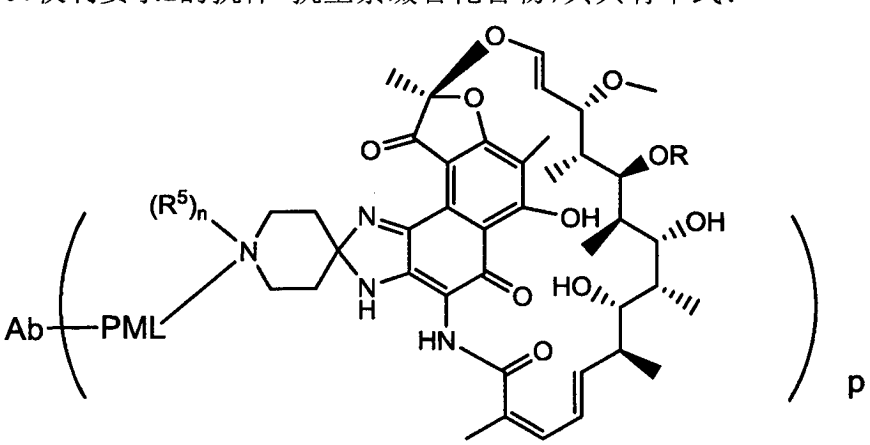


其中，

R^5选自H和C_1-C_{12}烷基；和

n为0或1。

8.权利要求2的抗体-抗生素缀合化合物，其具有下式：

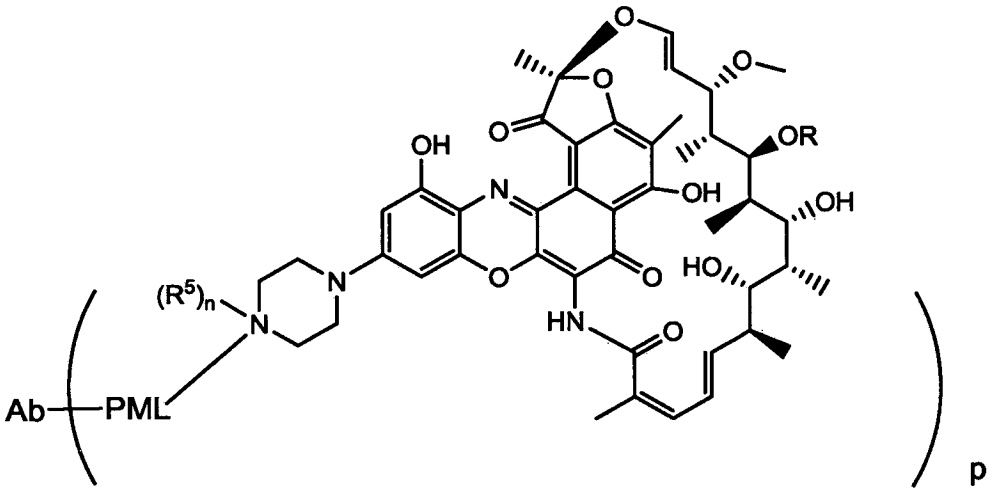


其中，

R^5选自H和C_1-C_{12}烷基；和

n为0或1。

9.权利要求2的抗体-抗生素缀合化合物，其具有下式：

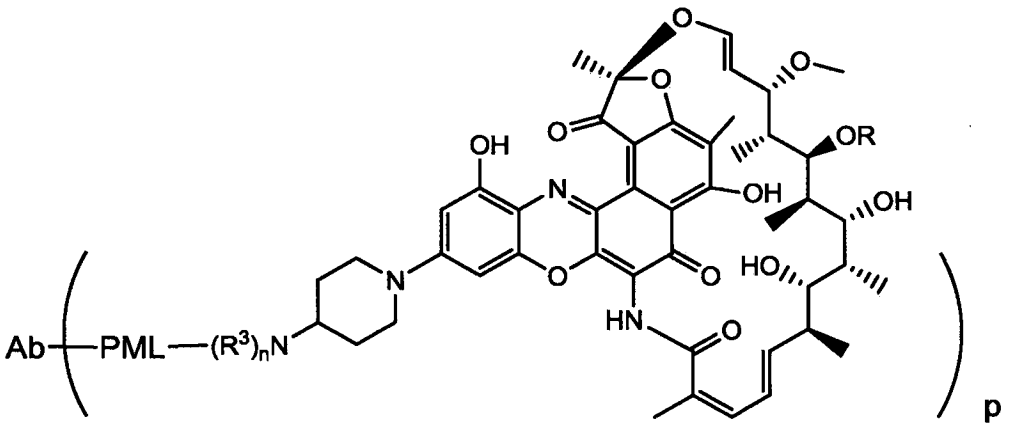


其中，

R^5独立地选自H和C_1-C_{12}烷基；和

n为0或1。

10.权利要求2的抗体-抗生素缀合化合物，其具有下式：

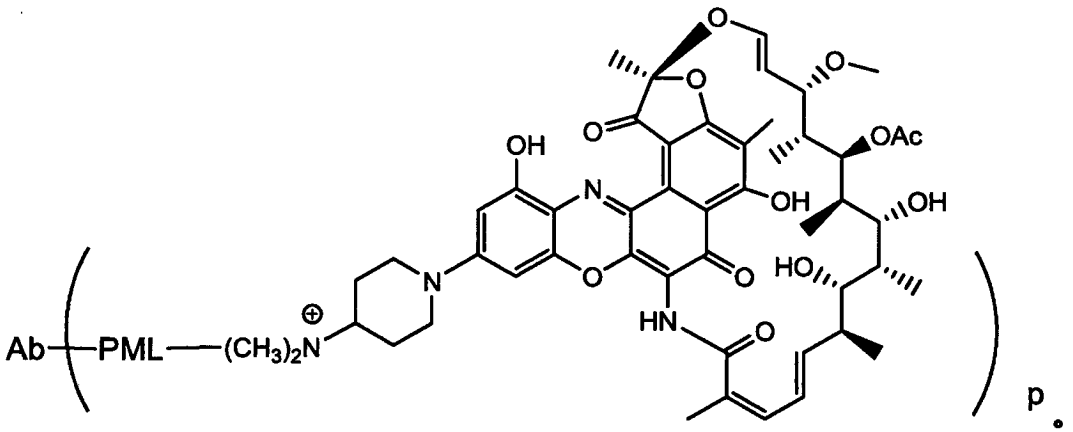


其中，

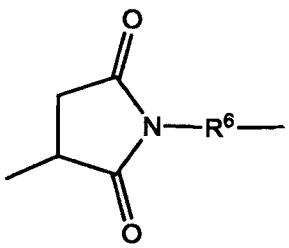
R^3独立地选自H和C_1-C_{12}烷基；和

n为1或2。

11.权利要求10的抗体-抗生素缀合化合物，其具有下式：



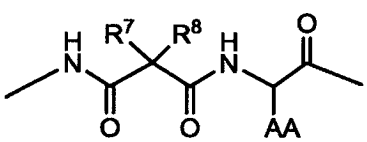
12.权利要求2的抗体-抗生素缀合化合物，其中Str具有下式：



其中R^6选自C_1-C_{12}亚烷基、C_1-C_{12}亚烷基-C(=O)、C_1-C_{12}亚烷基-NH、$(CH_2CH_2O)_r$、$(CH_2CH_2O)_r$-C(=O)、$(CH_2CH_2O)_r$-CH_2、和C_1-C_{12}亚烷基-NHC(=O)CH_2CH(噻吩-3-基)，其中r是1至10的整数。

13.权利要求12的抗体-抗生素缀合化合物，其中R^6是$(CH_2)_5$。

14.权利要求2的抗体-抗生素缀合化合物，其中PM具有下式：

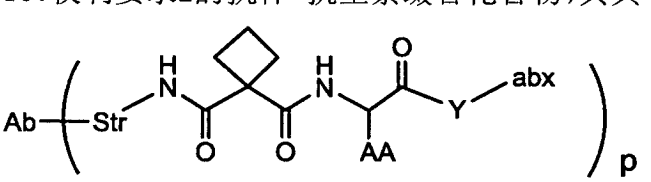


其中R^7和R^8一起形成C_3-C_7环烷基环，和

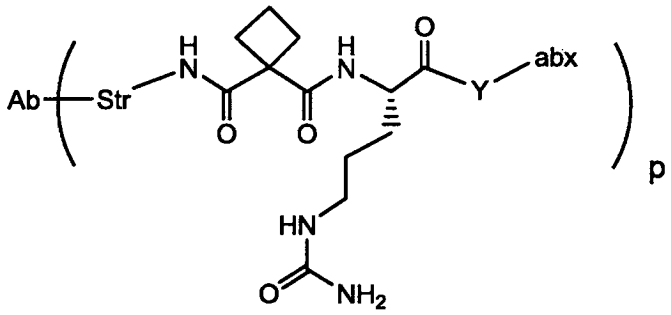
AA是选自H、$-CH_3$、$-CH_2(C_6H_5)$、$-CH_2CH_2CH_2CH_2NH_2$、$-CH_2CH_2CH_2NHC(NH)NH_2$、$-CHCH(CH_3)CH_3$、和$-CH_2CH_2CH_2NHC(O)NH_2$的氨基酸侧链。

15.权利要求2的抗体-抗生素缀合化合物，其中Y包括对氨基苄基或对氨基苄氧基羰基。

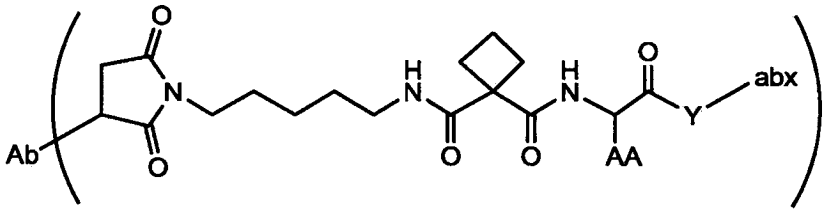
16.权利要求2的抗体-抗生素缀合化合物，其具有下式：



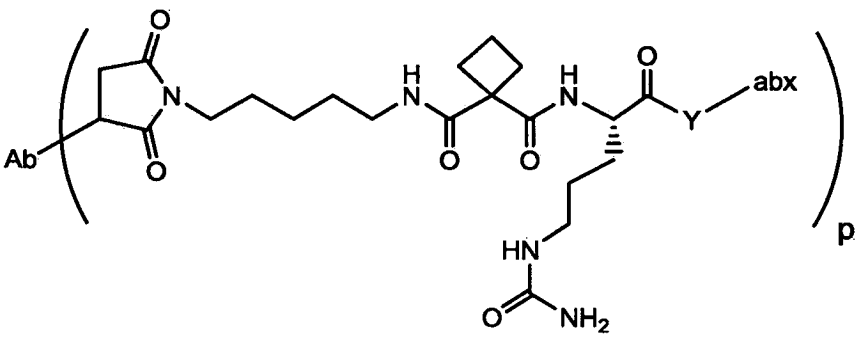
17.权利要求16的抗体-抗生素缀合化合物，其具有下式：



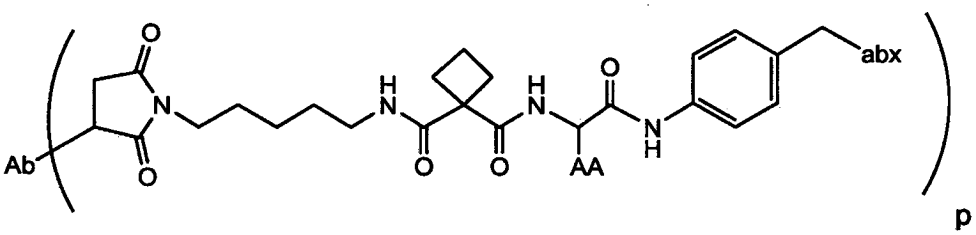
18.权利要求15的抗体-抗生素缀合化合物，其具有下式：



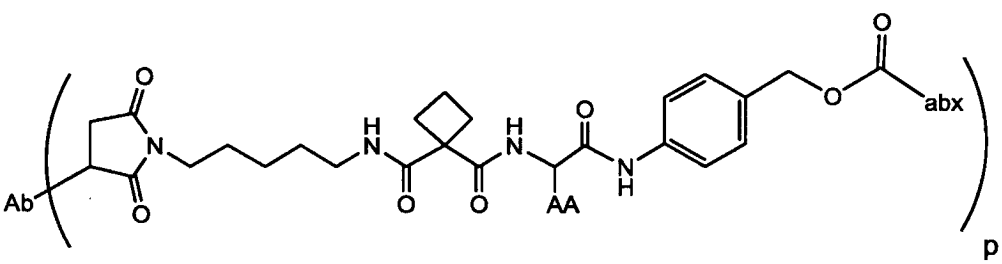
19.权利要求18的抗体-抗生素缀合化合物，其具有下式：



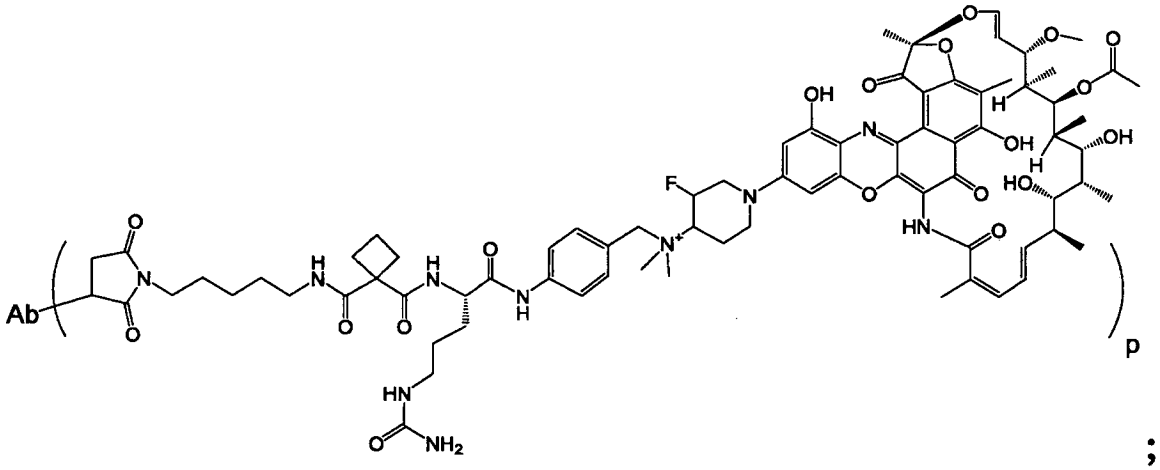
20.权利要求15的抗体-抗生素缀合化合物，其选自下式：



和



21.权利要求16的抗体-抗生素缀合化合物，其选自下式：


Ab
O
N
O
H
N
O
O
H
N
O
N
H
HN
O
NH2
F
N
N+
OH
N
O
OH
O
HN
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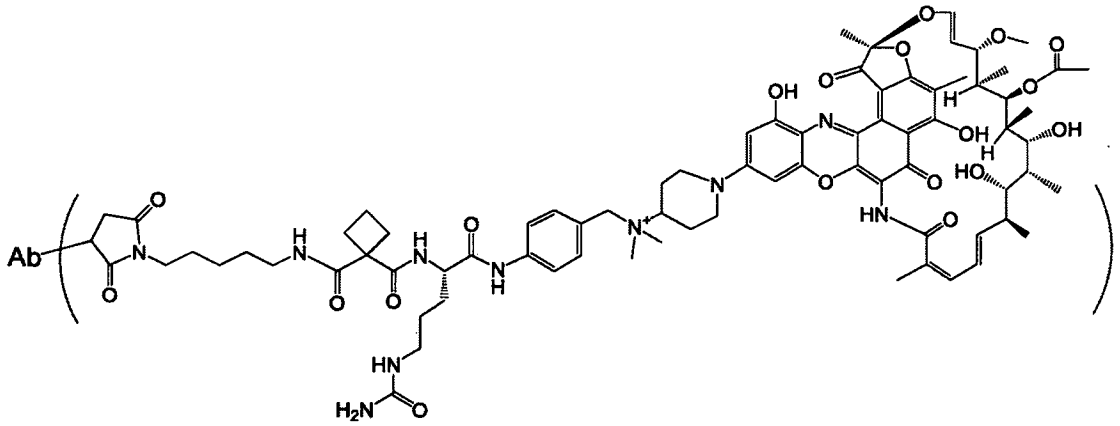
O
O
O
O
O
H
OH
H
HO
p

;



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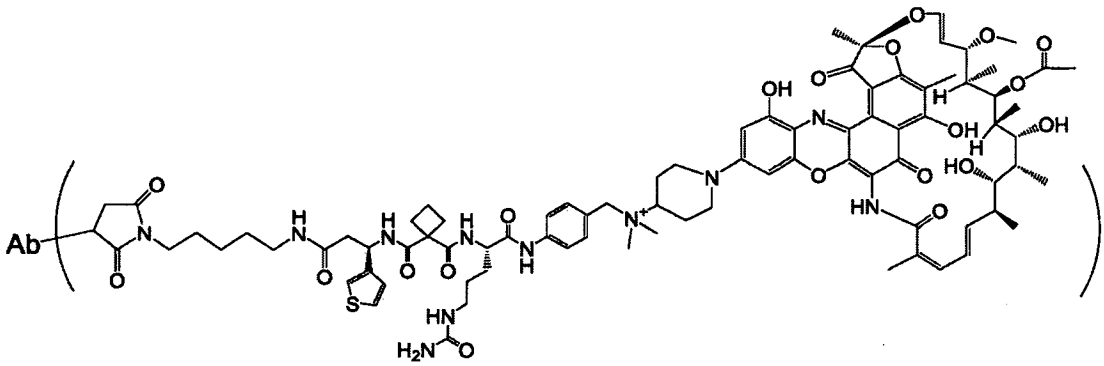

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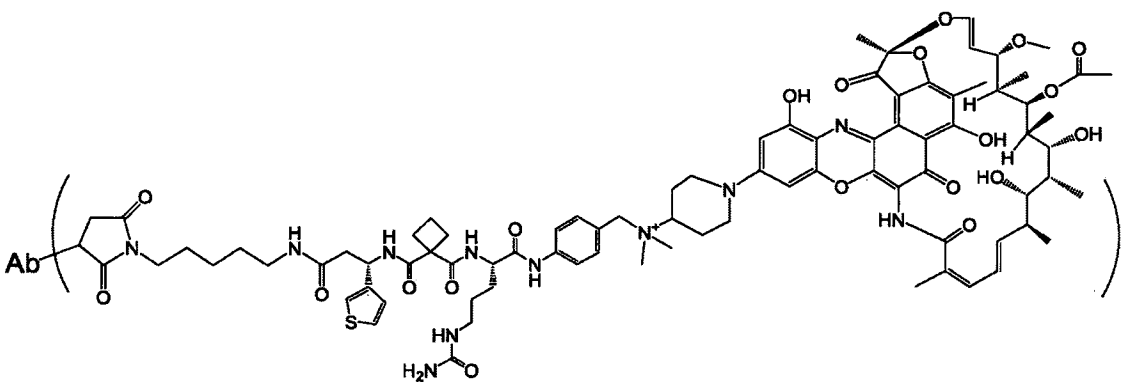

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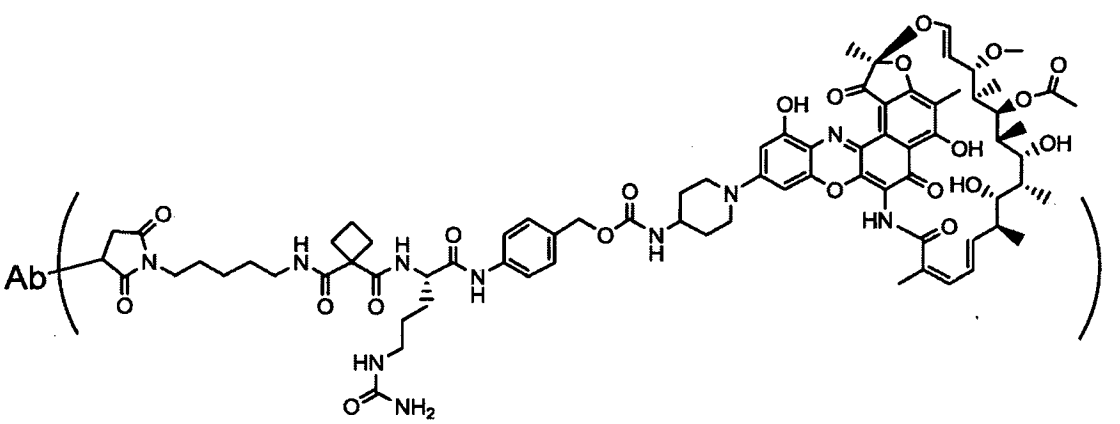

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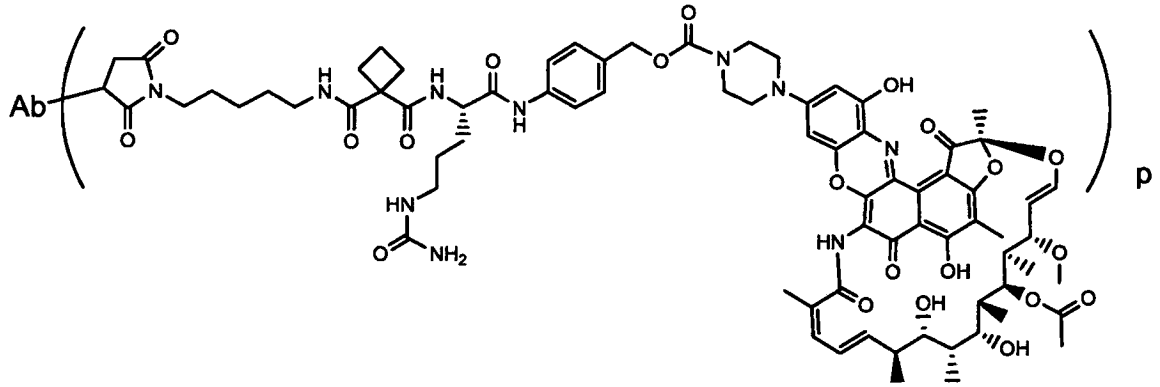
和



22.权利要求1的抗体-抗生素缀合化合物，其中抗SDR抗体是rF1抗体。

23.权利要求22的抗体-抗生素缀合物，其中rF1抗体包含轻链(L)链和重链(H)链，L链包含CDR L1、CDR L2和CDR L3，以及H链包含CDR H1、CDR H2和CDR H3，其中CDR L1、CDR L2和CDR L3与CDR H1、CDR H2和CDR H3分别包含每个抗体F1、rF1、rF1.v1和rF1.v6(SEQ ID NO.1-8)的CDR的氨基酸序列，如表4A和表4B所示。

24.权利要求22的抗体-抗生素缀合物，其中rF1抗体包含重链可变区(VH)，其中VH包含相对于SEQ ID NO.13的VH区的长度上至少95%的序列同一性。

25.权利要求24的抗体-抗生素缀合化合物，其中VL包含相对于SEQ ID NO.14或SEQ ID NO.15的VL区的长度上至少95%的序列同一性。

26.权利要求1或22中任一项的抗体-抗生素缀合化合物，其中抗SDR抗体在体内结合金黄色葡萄球菌和/或表皮葡萄球菌。

27.前述权利要求中任一项的抗体-抗生素缀合化合物，其中抗体是F(ab)或F(ab')2。

28.一种药物组合物，其包含权利要求1的抗体-抗生素缀合化合物和药学上可接受的载体、助流剂、稀释剂或赋形剂。

29.一种治疗患者的葡萄球菌细菌感染的方法，其包含向患者施用治疗有效量的权利要求1的抗体-抗生素缀合化合物。

30.权利要求29的方法，其中患者感染了金黄色葡萄球菌。

31.权利要求30的方法，其中患者感染了表皮葡萄球菌。

32.权利要求29的方法，其中抗体-抗生素缀合化合物以约50mg/kg至100mg/kg的剂量施用于患者。

33.权利要求29的方法，其中患者施用与第二抗生素治疗联合的抗体-抗生素缀合物。

34.一种通过施用权利要求1的抗体-抗生素缀合化合物在金黄色葡萄球菌感染的患者的细胞中杀死细胞内金黄色葡萄球菌、而不杀死宿主细胞的方法。

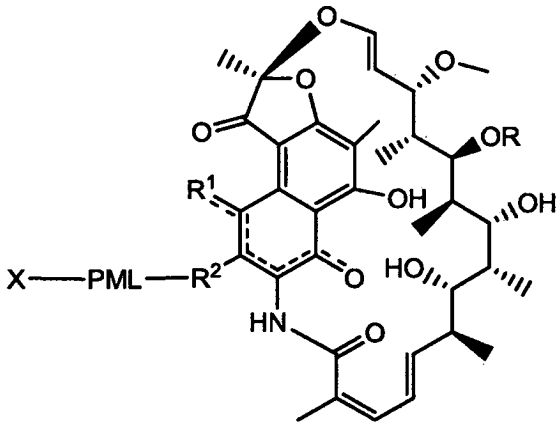
35.一种制备权利要求1的抗体-抗生素缀合化合物的方法，其包括将利福霉素型抗生素与rF1抗体缀合。

36.一种用于治疗细菌感染的药盒，其包含：

a)权利要求23的药物组合物；和

b)使用说明。

37.一种具有式II的抗生素-接头中间体：



II

其中：

虚线表示任选的键；

R是H、C_1-C_{12}烷基或C(O)CH_3；

R^1是OH；

R^2是CH=N-(杂环基)，其中所述杂环基任选地被一个或多个独立地选自C(O)CH_3、C_1-C_{12}烷基、C_1-C_{12}杂芳基、C_2-C_{20}杂环基、C_6-C_{20}芳基和C_3-C_{12}碳环基的基团取代；

或R^1和R^2形成五元或六元稠合杂芳基或杂环基，并且任选地形成螺或稠合的六元杂芳基、杂环基、芳基或碳环基环，其中所述螺或稠合的六元杂芳基、杂环基、芳基或碳环基环任选地被H、F、Cl、Br、I、C_1-C_{12}烷基或OH取代；

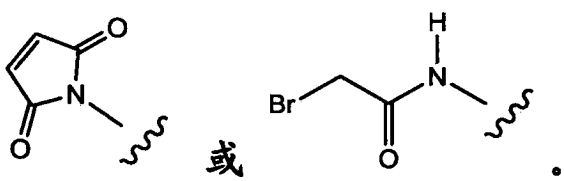
PML是连接到R^2或由R^1和R^2形成的稠合杂芳基或杂环基的蛋白酶可切割的非肽类接头；并具有下式：

-Str-PM-Y-

其中Str是延伸单元；PM是拟肽单元，和Y是间隔单元；并且

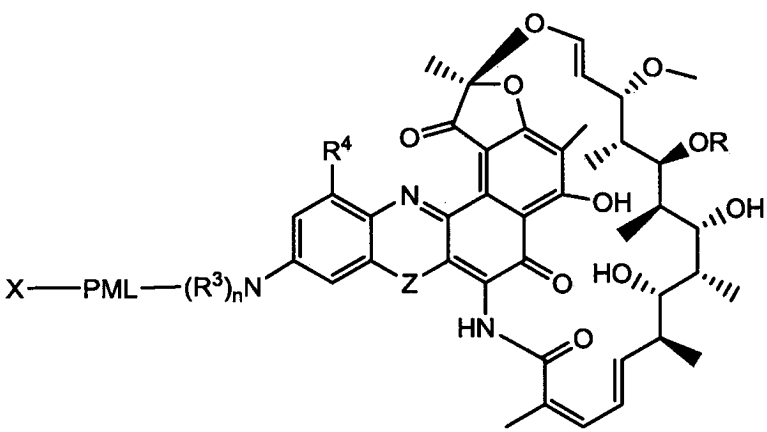
X是选自马来酰亚胺、硫醇、氨基、溴化物、溴乙酰氨基、碘乙酰氨基、对甲苯磺酸酯、碘化物、羟基、羧基、吡啶基二硫化物和N-羟基琥珀酰亚胺的反应性官能团。

38.权利要求37的抗生素-接头中间体，其中X为



或

39.权利要求37的抗生素-接头中间体，其具有下式：



其中，

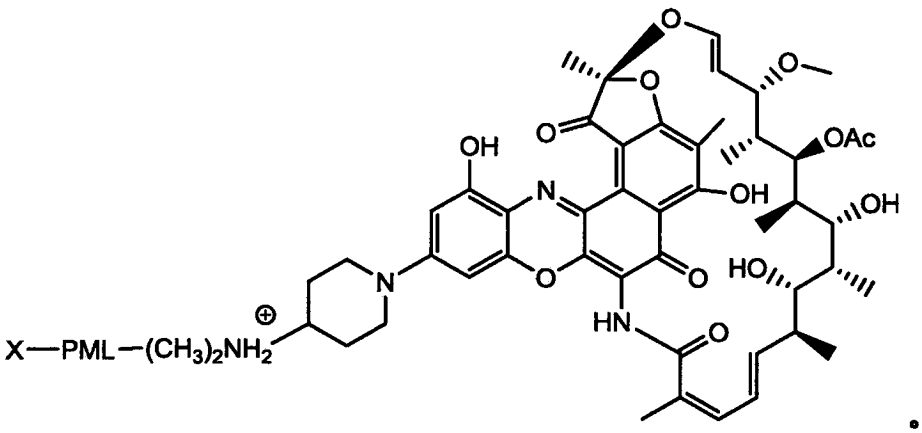
R^3独立地选自H和C_1-C_{12}烷基；

n为1或2；

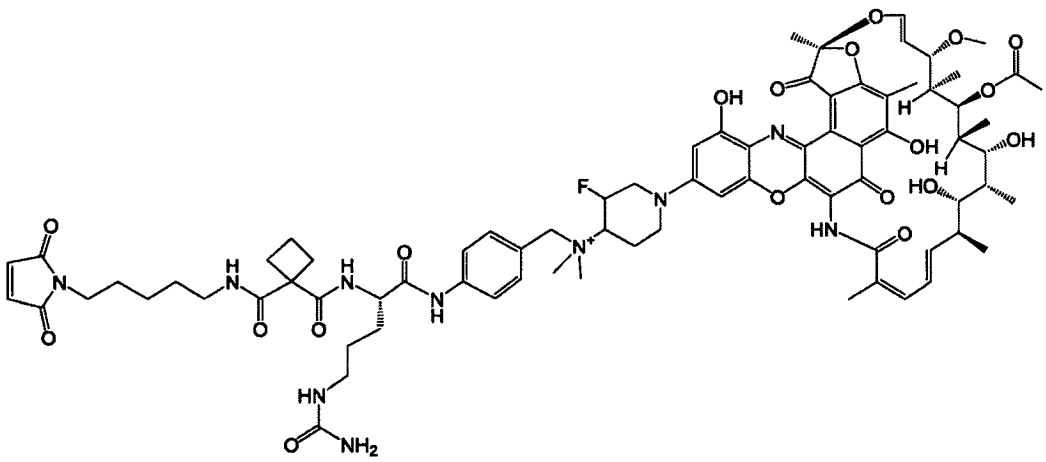
R^4选自H、F、Cl、Br、I、C_1-C_{12}烷基和OH；和

Z选自NH、N(C_1-C_{12}烷基)、O和S。

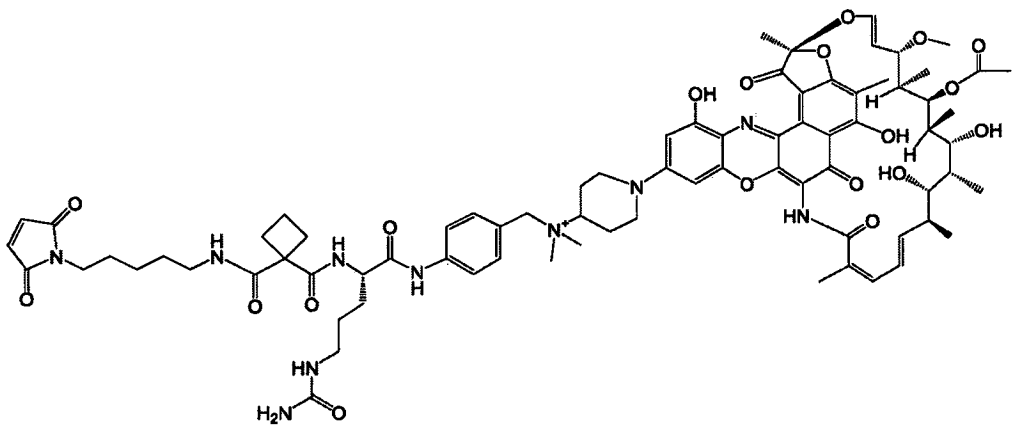
40.权利要求37的抗生素-接头中间体，其具有下式：



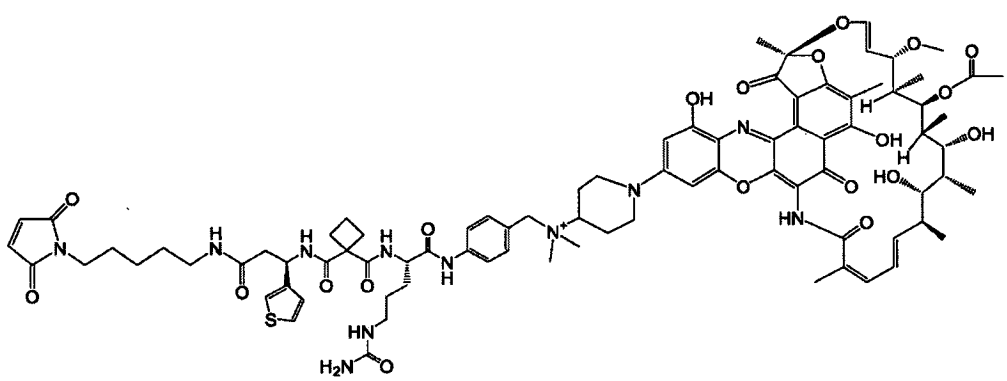
41.权利要求37的抗生素-接头中间体，其选自下式：



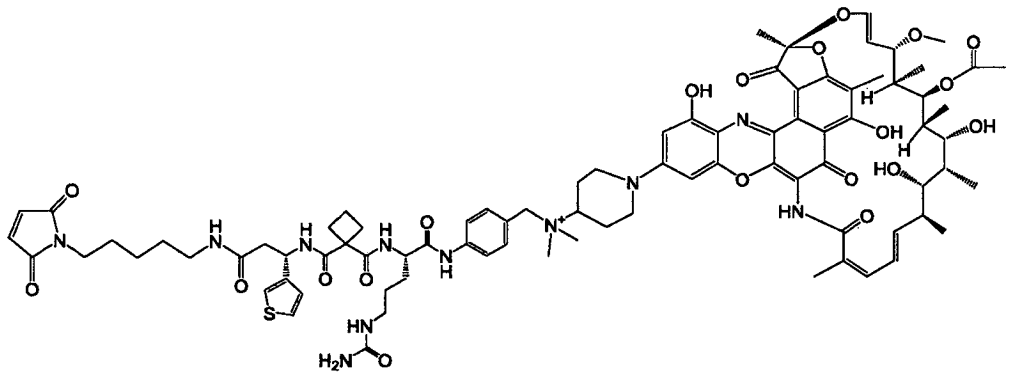
;



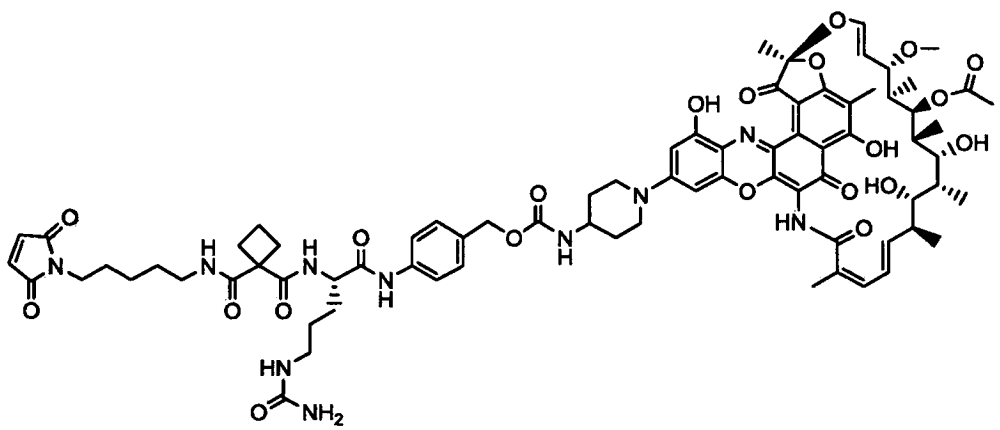
;



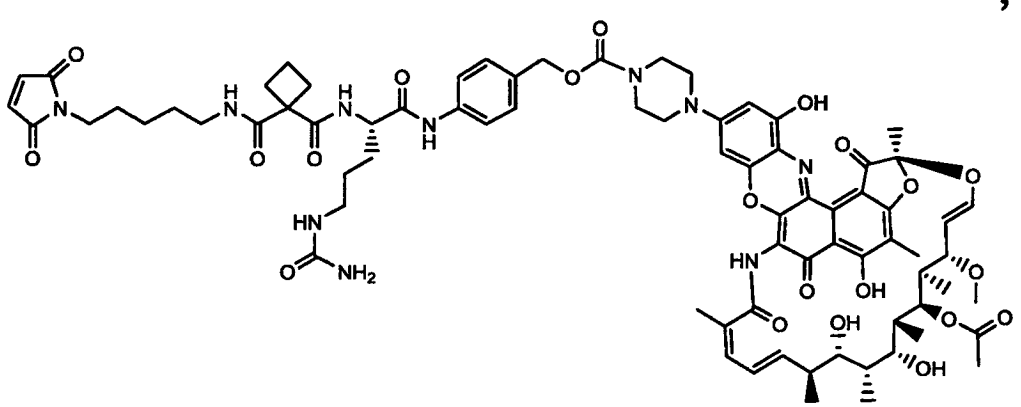
;



；



；和



。

抗金黄色葡萄球菌抗体利福霉素缀合物及其用途

[0001] 相关申请的交叉引用

[0002] 本申请要求2014年12月3日提交的美国临时申请号62/087,213的优先权，其全部内容通过引用并入本文，用于所有目的。

[0003] 序列表

[0004] 本申请包含已经以ASCII格式电子提交的序列表，其全部内容通过引用并入本文。所述ASCII副本，于2015年12月1日创建，名称为P32350WO_PCTSequenceListing.txt，且大小为26,747字节。

技术领域

[0005] 本发明涉及与利福霉素型抗生素缀合的抗葡萄球菌抗体，以及所得抗体-抗生素缀合物在治疗葡萄球菌感染中的用途。

[0006] 发明背景

[0007] 金黄色葡萄球菌和表皮葡萄球菌是成功的人类共生体，其主要定殖于鼻孔和皮肤。金黄色葡萄球菌(S.aureus;SA)也可侵袭各种组织，导致危及生命的感染；它是全球范围人类细菌感染的重要原因。最近出现的金黄色葡萄球菌菌株显示增加的毒性和在其他健康个体中增强的引起疾病的能力。在过去几十年中，由于耐甲氧西林金黄色葡萄球菌(MRSA)的出现和快速传播，金黄色葡萄球菌的感染变得越来越难以治疗，这种耐药菌对所有已知的β-内酰胺抗生素具有抗性(Boucher,H.W.等人，(2009)Clin Infect Dis 48,1-12)。目前，耐甲氧西林金黄色葡萄球菌(MRSA)的最普遍和最致命的临床菌株是USA300，其具有产生大量毒性因子并引起感染个体死亡的能力(Chambers,HF和Deleo FR(2009)自然微生物学综述(Nature Reviews Microbiology)7:629-641)。最严重的感染如心内膜炎、骨髓炎、坏死性肺炎和败血症在细菌传播到血液中之后发生(Lowy,F.D.(1998)N ENGL J MED 339,520-532)。与金黄色葡萄球菌密切相关的表皮葡萄球菌通常与医院获得性的感染相关，并且代表内置医疗器械最常见的感染源。

[0008] 对于葡萄球菌粘附和成功定殖宿主组织而言，重要的是细胞壁蛋白家族，其特征在于在葡萄球菌中存在的粘附性A-结构域附近的大量丝氨酸-天冬氨酸二肽(SDR)重复序列(Foster TJ,Hook M(1998)微生物学进展(Trends Microbiol)6:484-488)。对粘附重要的这些蛋白质包括团集因子(Clf)A和ClfB(Foster TJ,见上文)。除了ClfA和ClfB之外，金黄色葡萄球菌还表达三种SDR蛋白SdrC、SdrD和SdrE，它们在基因组中串联排列。这些蛋白质也被认为参与组织定殖，并且其中任一种的消除降低了细菌的毒性(Cheng AG等人(2009)FASEB Journal 23:3393-3404)。这个家族的另外三个成员SrdF、SdrG和SdrH存在于大多数表皮葡萄球菌菌株中(McCrea KW等人(2000)The serine-aspartate repeat(Sdr)protein family in Staphylococcus epidermidis.Microbiology 146(Pt 7):1535-1546)。在这些蛋白质的每一个中，含有25-275个SD-二肽重复序列的SDR区域(SEQ ID NO:24)位于N-末端配体结合A-结构域和C-末端LPXTG基序(SEQ ID NO:25)之间，其介导通过转肽酶，即分选酶A锚定到细胞壁上。SDR-结构域的功能仍然是未知的，但已经提出，其充当使

这些蛋白质的N末端配体结合位点暴露的细胞壁跨越结构域(Hartford O,等人(1997)Mol Microbiol 25:1065-1076)。

[0009] 发现金黄色葡萄球菌和表皮葡萄球菌的所有SDR蛋白的SDR结构域被两个新的糖基转移酶SdgA和SdgB高度糖基化,其以两个步骤负责糖基化(Hazenbos等人(2013)PLOS Pathogens 9(10):1-18)。这些糖基化事件阻止宿主蛋白酶降解这些蛋白质,从而保护细菌宿主组织相互作用。Hazenbos等人(2013)还表明,SdgB介导的糖基化在人体中产生高度调理素化抗体的免疫显性表位。这些抗体占总抗葡萄球菌IgG反应的很大比例。

[0010] 侵袭性MRSA感染难以治疗,死亡率约为20%,是美国传染性疾病死亡的主要原因。因此,万古霉素、利奈唑胺和达托霉素成为少数用于治疗侵袭性MRSA感染的抗生素(Boucher,H.,Miller,L.G.&Razonable,R.R.(2010)Clin Infect Dis 51Suppl 2,S183-197)。然而,在MRSA临床菌株中,已经报道了对万古霉素降低的敏感性和对利奈唑胺和达托霉素的交叉耐药性(Nannini,E.,Murray,B.E.&Arias,C.A.(2010)Curr Opin Pharmacol 10,516-521)。随着时间的推移,克服抗性所需的万古霉素剂量已经上升至发生肾毒性的水平。因此,尽管有这些抗生素,侵袭性MRSA感染的死亡率和发病率仍然很高。

[0011] 调查显示,金黄色葡萄球菌能够在哺乳动物细胞内侵袭和存活,包括负责细菌清除的吞噬细胞(Thwaites,G.E.&Gant,V.(2011)Nat Rev Microbiol 9,215-222);Rogers.D.E.,Tompsett,R.(1952)J.Exp.Med 95,209-230);Gresham,H.D.,等人(2000)J Immunol 164,3713-3722);Kapral,F.A.&Shayegani,M.G.(1959)J Exp Med 110,123-138;Anwar,S.,等人(2009)Clin Exp Immunol 157,216-224);Fraunholz,M.&Sinha,B.(2012)Front Cell Infect Microbiol 2,43);Garzoni,C.&Kelley,W.L.(2011)EMBO Mol Med 3,115-117)。在静脉内感染后几分钟内,金黄色葡萄球菌被宿主吞噬细胞(主要是嗜中性粒细胞和巨噬细胞)摄取(Rogers,D.E.(1956)JEM 103,713)。尽管大多数细菌被这些细胞有效地杀死,但血液中吞噬细胞内的金黄色葡萄球菌的不完全清除,可以使这些被感染的细胞作为"特洛伊木马"用于从初始感染部位传播细菌。实际上,正常的嗜中性粒细胞计数患者可能比那些减少的嗜中性粒细胞计数患者更容易传播疾病(Thwaites,G.E.&Gant,V.(2011)见上文)。一旦传递到组织,金黄色葡萄球菌可以侵袭各种非吞噬细胞类型,并且组织中的细胞内金黄色葡萄球菌与慢性或复发性感染相关。此外,细胞内细菌暴露于次优的抗生素浓度可能会促进抗生素耐药菌株的出现,从而使临床问题更加突出。与这些观察结果一致,用万古霉素或达托霉素治疗侵袭性MRSA感染的患者如菌血症或心内膜炎,与超过50%的失败率相关(Kullar,R.,Davis,S.L.,Levine,D.P.&Rybak,M.J.,Impact of vancomycin exposure on outcomes in patients with methicillin-resistant Staphylococcus aureus bacteremia:support for consensus guidelines suggested targets.Clinical infectious diseases:an official publication of the Infectious Diseases Society of America 52,975-981(2011);Fowler,VG,Jr.等人,Daptomycin versus standard therapy for bacteremia and endocarditis caused by Staphylococcus aureus.The New England journal of medicine 355,653-665(2006);Yoon,YK,Kim,J.Y.,Park,D.W.,Sohn,J.W.和Kim,M.J.,Predictors of persistent methicillin-resistant Staphylococcus aureus bacteraemia in patients treated with vancomycin.The Journal of antimicrobial chemotherapy 65:1015-1018

(2010))。因此,更成功的抗葡萄球菌治疗应包括清除细胞内的细菌。

[0012] 安莎霉素是一类抗生素,包括利福霉素、利福平(rifampin)、利福平(rifampicin)、利福布汀、利福喷汀、利福拉齐、ABI-1657及其类似物,它们抑制细菌RNA聚合酶,并且具有对革兰氏阳性菌和选择性的革兰氏阴性菌的特殊效力(Rothstein,D.M.等人(2003)Expert Opin.Invest.Drugs12(2):255-271;US 7342011;US 7271165)。

[0013] 已经报道了用于预防和治疗金黄色葡萄球菌(包括MRSA)感染的免疫疗法。US2011/0262477涉及细菌粘附蛋白Eap、Emp和AdsA作为疫苗刺激针对MRSA的免疫应答的应用。WO2000071585描述了与特异性金黄色葡萄球菌菌株分离物反应的分离的单克隆抗体。US20110059085A1提出了利用对一种或多种SA荚膜抗原特异性的IgM抗体的基于抗体的策略,但没有描述实际的抗体。

[0014] 抗体-药物缀合物(ADC)也称为免疫缀合物,是靶向的化学治疗分子,其通过将有效的细胞毒性药物靶向于表达抗原的肿瘤细胞,结合了抗体和细胞毒性药物的理想特性(Teicher,B.A.(2009)Curr.Cancer Drug Targets9:982-1004),从而通过使效力最大化和使靶点外毒性最小化来增强治疗指数(Carter,P.J.和Senter P.D.(2008)The Cancer J.14(3):154-169;Chari,R.V.(2008)Acc.Chem.Res.41:98-107)。ADC包含通过接头单元与细胞毒性药物部分共价连接的靶向抗体。当未缀合的药物的全身给药可能引起对正常细胞和试图清除的肿瘤细胞的不可接受的毒性水平时,免疫缀合物允许将药物部分靶向递送至肿瘤并在其中的细胞内蓄积(Polakis P.(2005)Curr.Opin.Pharmacol.5:382-387)。

[0015] 描述了通过用于治疗败血症的抗生素结合目标细菌表面的非特异性免疫球蛋白-抗生素缀合物(US 5545721;US 6660267)。描述了抗生素缀合的抗体,其具有对细菌抗原(例如SA荚膜多糖)特异性的抗原结合部分,但缺乏与细菌Fc结合蛋白例如葡萄球菌蛋白A反应的恒定区域(US 7569677)。

[0016] 鉴于MRSA对常规抗生素的令人震惊的抗药率,以及侵袭性MRSA感染引起的死亡率和发病率,很需要治疗金黄色葡萄球菌感染的新的治疗药物。本发明通过提供克服当前治疗组合物限制的组合物和方法以及提供从下面详述中显而易见的额外优点而满足了这一需求。

[0017] 发明概述

[0018] 本发明提供了包括消除细胞内细菌的独特治疗剂。本发明证实,当常规抗生素如万古霉素无效时,这种治疗剂在体内是有效的。

[0019] 本发明提供称为"抗体-抗生素缀合物"或"AAC"的组合物,其包含通过共价连接与一种或多种利福霉素型抗生素部分缀合的抗体。

[0020] 本发明的一个方面是抗体-抗生素缀合物化合物,其包含rF1抗体,所述rF1抗体通过蛋白酶可切割的非肽类接头共价连接利福霉素型抗生素。

[0021] 本发明的示例性实施方案是具有下式的抗体-抗生素缀合物:

[0022] $Ab-(PML-abx)_p$

[0023] 其中:

[0024] Ab是rF1抗体;

[0025] PML是具有下式的蛋白酶可切割的非肽类接头:

[0026] -Str-PM-Y-

[0027] 其中Str是延伸单元;PM是拟肽单元,和Y是间隔单元;

[0028] abx是利福霉素型抗生素;并且

[0029] p是从1到8的整数。

[0030] 任何前述实施方案的抗体-抗生素缀合化合物可以包含本文所述的抗SDR抗体和特异性rF1抗体中的任何一种。这些rF1抗体结合金黄色葡萄球菌。在示例性的rF1抗体中,抗体是包含轻(L)链和重(H)链的单克隆抗体,L链包含CDR L1、CDR L2和CDR L3,H链包含CDR H1、CDR H2和CDR H3,其中CDR H1、CDR H2和CDR H3与CDR L1、CDR L2和CDR L3,分别包含每个抗体F1(SEQ ID NO.1-6)、rF1(SEQ ID NO.1-5,7)、rF1.v1(SEQ ID NO.1,18,3,4-6)的CDR的氨基酸序列,如表4A和4B所示。

[0031] 在一些实施方案中,rF1抗体包含重链可变区(VH),其中VH在选自SEQ ID NO.13的VH序列的VH区长度上,包含至少95%的序列同一性。抗体还可以包含L链可变区(VL),其中VL在选自分别为抗体rF1和rF1.v6的SEQ ID NO.14和SEQ ID NO.15的VL序列的VL区域长度上,包含至少95%的序列同一性。

[0032] 在具体实施方案中,rF1抗体包含如下的L和H链对:包含SEQ ID NO.9序列的L链与包含SEQ ID NO.10序列的H链配对;包含SEQ ID NO.11序列的L链与包含SEQ ID NO.10序列的H链配对;包含SEQ ID NO.11序列的L链与包含SEQ ID NO.12序列的H链配对。

[0033] 在任何前述实施方案中,抗体可以是缺乏Fc区的抗原结合片段。在一些实施方案中,抗体是F(ab)或$F(ab')_2$。在一些实施方案中,抗体还包含重链恒定区和/或轻链恒定区,其中重链恒定区和/或轻链恒定区包含被半胱氨酸残基取代的一个或多个氨基酸。在一些实施方案中,重链恒定区包含氨基酸取代A118C和/或S400C,和/或轻链恒定区包含氨基酸取代V205C,其中所述编号系统根据EU编号。

[0034] 在上述任何抗体的一些实施方案中,抗体不是IgM同种型。在上述任何抗体的一些实施方案中,抗体是IgG(例如,IgG1、IgG2、IgG3、IgG4)、IgE、IgD或IgA(例如,IgA1或IgA2)同种型。

[0035] 本发明的一个示例性实施方案是包含抗体-抗生素缀合化合物和药学上可接受的载体、助流剂、稀释剂或赋形剂的药物组合物。

[0036] 本发明的另一方面是治疗细菌感染的方法,其包括向感染的患者施用治疗有效量的任何前述实施方案的抗体-抗生素缀合物。本发明的另一方面是治疗患者中葡萄球菌感染的方法,包括向患者施用治疗有效量的本发明的抗体-抗生素缀合物。在一个实施方案中,患者是人。在一个实施方案中,患者感染了金黄色葡萄球菌和/或表皮葡萄球菌感染。在一些实施方案中,患者被诊断患有金黄色葡萄球菌感染。在一些实施方案中,治疗细菌感染包括减少细菌负荷或计数。

[0037] 本发明的另一方面是在感染患者中治疗葡萄球菌感染的方法,包括向患者施用治疗有效量的前述实施方案中任一项的抗体-抗生素缀合物。在一个实施方案中,患者是人。在一个实施方案中,细菌感染是金黄色葡萄球菌感染。在一些实施方案中,患者被诊断患有金黄色葡萄球菌感染。在一些实施方案中,治疗细菌感染包括减少细菌负荷或计数。

[0038] 在任何前述治疗方法的一个实施方案中,向包括金黄色葡萄球菌在内的细菌感染导致菌血症的患者施用。在具体实施方案中,该方法用于治疗葡萄球菌性心内膜炎或骨髓炎。在一个实施方案中,抗体-抗生素缀合化合物以约50mg/kg至100mg/kg的剂量施用于感

染的患者。

[0039] 还提供了在金黄色葡萄球菌感染的患者细胞中杀死细胞内金黄色葡萄球菌而不杀死宿主细胞的方法，其通过施用任何上述实施方案的rF1抗生素缀合化合物。通过使耐药株(persister)细菌与任何前述实施方案的AAC接触，提供了另一种用于在体内杀死耐药株葡萄球菌细菌细胞(例如金黄色葡萄球菌)的方法。

[0040] 在另一个实施方案中，所述治疗方法还包括施用第二治疗剂。在另一个实施方案中，第二治疗剂是抗生素，其包括普遍针对金黄色葡萄球菌或特别是MRSA的抗生素。

[0041] 在一个实施方案中，与本发明的抗体-抗生素缀合化合物组合施用的第二抗生素选自以下结构类别：(i)氨基糖苷类；(ii)β-内酰胺类；(iii)大环内酯/环肽类；(iv)四环素类；(v)氟代喹啉/氟喹诺酮类；(vi)和噁唑烷酮。

[0042] 在一个实施方案中，与本发明的抗体-抗生素缀合化合物组合施用的第二抗生素选自克林霉素、新生霉素、瑞他帕林(retapamulin)、达托霉素、GSK-2140944、CG-400549、西他沙星、替考拉宁、三氯生、萘啶酮(napthyridone)、雷得唑来(radezolid)、多柔比星、氨苄西林、万古霉素、亚胺培南、多利培南、吉西他滨、达巴万星(dalbavancin)和阿奇霉素。

[0043] 在本文的一些实施方案中，感染患者中的细菌负荷在治疗后已经降低到不可检测的水平。在一个实施方案中，与治疗前的阳性血培养物相比，患者的血培养物在治疗后是阴性的。在本文的一些实施方案中，个体中的细菌耐药性是不可检测的或低的。在本文的一些实施方案中，患者对甲氧西林或万古霉素的治疗无反应。

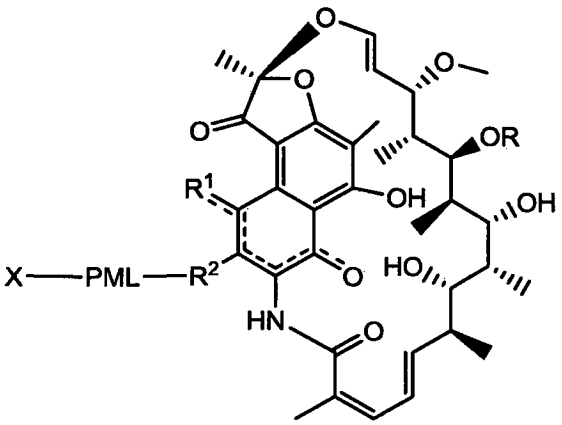
[0044] 本发明的示例性实施方案是制备抗体-抗生素缀合物的方法，包含将利福霉素型抗生素与rF1抗体缀合。

[0045] 本发明的一个示例性实施方案是用于治疗细菌感染的药盒，其包含：

[0046] a)药物组合物，包含抗体-抗生素缀合化合物和药学上可接受的载体、助流剂、稀释剂或赋形剂；和

[0047] b)使用说明。

[0048] 本发明的一个方面是具有式II的抗生素-接头中间体：

[0049] 

II

[0050] 其中：

[0051] 虚线表示任选的键；

[0052] R是H、C_1-C_{12}烷基或C(O)CH_3；

[0053] R^1是OH；

[0054] R^2是CH=N-(杂环基)，其中所述杂环基任选地被一个或多个独立地选自C(O)CH_3、C_1-C_{12}烷基、C_1-C_{12}杂芳基、C_2-C_{20}杂环基、C_6-C_{20}芳基和C_3-C_{12}碳环基的基团取代；

[0055] 或R^1和R^2形成五元或六元稠合杂芳基或杂环基，并且任选地形成螺或稠合的六元杂芳基、杂环基、芳基或碳环基环，其中所述螺或稠合的六元杂芳基、杂环基、芳基或碳环基环任选地被H、F、Cl、Br、I、C_1-C_{12}烷基或OH取代；

[0056] PML是连接到R^2或由R^1和R^2形成的稠合杂芳基或杂环基的蛋白酶可切割的非肽类接头；并具有下式：

[0057] -Str-PM-Y-

[0058] 其中Str是延伸单元；PM是拟肽单元，和Y是间隔单元；并且

[0059] X是选自马来酰亚胺、硫醇、氨基、溴化物、溴乙酰氨基、碘乙酰氨基、对甲苯磺酸酯、碘化物、羟基、羧基、吡啶基二硫化物和N-羟基琥珀酰亚胺的反应性官能团。

[0060] 应当理解，本文描述的各种实施方案的一个、一些或全部特性可以组合以形成本发明的其他实施方案。本发明的这些和其它方面对于本领域技术人员是很明显的。

附图说明

[0061] 图1A-1F：在体内和体外，MRSA的细胞内储存物被保护免于万古霉素杀死。图1A显示了产生游离细菌(浮游)与细胞内细菌的实验设计的示意图。通过静脉注射大约相同剂量的活的游离细菌或细胞内细菌感染四组小鼠，并且选择的组在感染后立即用万古霉素处理，然后每天一次(参见实施例2)。图1B和图1C分别显示感染后4天的感染小鼠的肾脏和脑部细菌负荷。虚线表示测定的检测限。图1D显示当在单层感染细胞上培养时，MRSA被保护免于万古霉素杀死。(ND=未检测到)。图1E和图1F显示当在单层感染细胞上培养时，MRSA能够在万古霉素存在下生长。将MRSA(游离细菌)接种在培养基、培养基+万古霉素、或培养基+万古霉素并接种在单层MG63成骨细胞(图1E)或人脑微血管内皮细胞(HBMEC，图1F)中。细胞外细菌(游离细菌)在仅有培养基时生长良好，但被万古霉素杀死。在含有单层哺乳动物细胞(细胞内+万古霉素)的孔中，一部分细菌在感染后的前8个小时被保护免于万古霉素杀死，并能在24小时内在细胞内的区室内扩张。误差线显示一式三份的孔的标准偏差。

[0062] 图2显示了抗体抗生素缀合物(AAC)的概念。在一个实例中，AAC由针对金黄色葡萄球菌表面上的表位的抗体组成，其通过溶酶体蛋白酶切割的接头连接到有效的利福霉素型抗生素(例如Rifalog)上。

[0063] 图3显示抗体-抗生素缀合物(AAC)的药物活化的可能机制。AAC通过抗体的抗原结合结构域(Fab)与细胞外细菌结合，并通过Fc介导的吞噬作用促进调理化细菌的摄取。接头被溶酶体蛋白酶例如组织蛋白酶B切割。在切割接头之后，接头被水解，在吞噬溶酶体内释放游离抗生素。游离的抗生素杀死了调理化细胞和被吞噬的细菌，以及任何以前存在于同一区室中的内化的细菌。

[0064] 图4A和4B显示丝氨酸-天冬氨酸(SDR)蛋白的方面。图4A显示通过来自金黄色葡萄球菌和表皮葡萄球菌的质谱法揭示的SDR蛋白的比对。SDR区域用阴影线表示。由于在金黄色葡萄球菌上有多种SDR蛋白，并且每个蛋白质有多个表位，所以rF1表位以丰度表达。图4A公开了SEQ ID NO:27的“SDSDSDSD”。图4B是显示通过SdgA和SdgB的SDR蛋白逐步糖基化的

模型。首先，SdgB将GlcNAc部分附加到SDR蛋白上的SD区域，随后通过SdgA进行额外的GlcNAc修饰。对于mAb rF1的表位包括SdgB依赖性GlcNAc部分。数据表明rF1结合GlcNac和SD骨架的一部分。图4B公开了SEQ ID NO:28的“SDSDSD”。

[0065] 图5A、5B和5C显示mAb rF1表现出对金黄色葡萄球菌的稳定结合和杀伤作用。(图A-C)用huIgG1mAbs rF1(正方形)、4675抗-ClfA(三角形)或抗疱疹病毒gD(圆圈)对细菌进行预调理。(图5A)：通过流式细胞术评估mAb与WT(USA300-Δspa)细菌的结合，并表示为平均荧光强度(MFI)。(图5B)：将CFSE标记的预调理的WT(USA300-Δspa)细菌与人PMN一起孵育。通过流式细胞术门控CD11b阳性细胞后，细菌摄取量表示为CFSE阳性PMN的百分比%。(图5C)：将预调理的WT(USA300-Δspa)细菌与PMN一起孵育以评估细菌杀伤。每ml活菌CFU的数目是至少三个实验的代表。

[0066] 图6显示了来自各种感染组织的rF1与金黄色葡萄球菌结合的流式细胞术分析。均质化的组织用mAb rF1(X轴)和抗肽聚糖mAb 702双重染色，以将细菌与组织碎片(Y轴)(左图；箭头所示的门)区分开，然后门控细菌以产生直方图图形(另见，Hazenbos等人(2013)PLOS Pathogens 9(10):1-18，图1D)。

[0067] 图7显示通过流式细胞术将rF1与各种葡萄球菌和非葡萄球菌的革兰氏阳性菌种结合(另见，Hazenbos等人(2013)PLOS Pathogens 9(10):1-18，图1E)。

[0068] 图8显示了选择强效的利福霉素型抗生素(rifalog)二甲基pipBOR，因为其杀死非复制性MRSA的能力。

[0069] 图9：生长抑制测定证明完整的TAC(一种形式的AAC)不会杀死浮游细菌，除非通过用组织蛋白酶B处理而释放抗生素。将TAC在仅有缓冲液(空心圆圈)中孵育或用组织蛋白酶B处理(实心圆圈)。完整的TAC在过夜孵育后不能防止细菌生长。用组织蛋白酶B预处理TAC释放了足够的抗生素活性，以防止细菌在6ug/mL TAC中生长，其预计含有0.006μg/mL的抗生素。

[0070] 图10显示了rF1-AAC在体外巨噬细胞测定中的功效，如实施例19所述。

[0071] 图11A和11B显示了如实施例20所述的rF1-AAC在体内的功效。与裸抗体相比，用rF1-AAC治疗金黄色葡萄球菌感染的小鼠，大大降低或消除了感染器官中的细菌计数。图11A显示用含有每个抗体2个抗生素分子的AAC(AAR2)治疗，肾脏中的细菌负荷降低约30倍，并且用含有每个抗体4个抗生素分子的AAC(AAR4)治疗，细菌负荷降低超过30,000倍。图11B显示用AAC AAR2治疗使心脏中的细菌负荷降低约70倍，其中8只小鼠中有6只在心脏中具有检测不到的细菌水平；用AAC AAR4治疗彻底根除了心脏感染，导致8只小鼠中全部8只都具有检测不到的细菌水平。

[0072] 本发明的实施方案详述

[0073] 现在将详细参考本发明的某些实施方案，其实例在所附的结构和分子式中示出。虽然将结合列举的实施方案，包括方法、材料和实例来描述本发明，但是这样的描述是非限制性的，并且本发明旨在覆盖所有替代、修改和等同物，无论它们是一般已知的，还是并入本文。如果一个或多个并入的文献、专利和类似材料与本申请不同或相矛盾，包括但不限于定义的术语、术语使用、所描述的技术等，以本申请为准。除非另有定义，本文使用的所有技术和科学术语具有与本发明所属领域的普通技术人员通常理解的相同含义。本领域技术人员将认识到可以用于本发明的实践中的与本文所述类似或等同的许多方法和材料。本发明

在任何情况下都不限于所述的方法和材料。

[0074] 本文提及的所有出版物、专利申请、专利和其它参考文献通过引用整体并入本文。

[0075] I.一般技术

[0076] 本文描述或参考的技术和方法，通常被本领域技术人员很好的理解和使用常规方法普遍采用，例如，下面描述的广泛使用的方法：Sambrook等人，分子克隆：实验室手册第三版(2001)冷泉港实验室出版社，冷泉港，纽约(Molecular Cloning:A Laboratory Manual 3d edition(2001)Cold Spring Harbor Laboratory Press,Cold Spring Harbor,N.Y.)；分子生物学现代方法(Current Protocols in Molecular Biology)(F.M.Ausubel等人编辑，(2003))；酶学系列方法(the series Methods in Enzymology)(Academic Press,Inc.)；PCR 2：实用方法(PCR 2:A Practical Approach)(M.J.MacPherson,B.D.Hames和G.R.Taylor编辑(1995))，Harlow和Lane编辑(1988)抗体、实验室手册、和动物细胞培养(Antibodies,A Laboratory Manual,and Animal Cell Culture)(R.I.Freshney编辑(1987))；寡核苷酸合成(Oligonucleotide Synthesis)(M.J.Gait编辑，1984)；分子生物学方法(Methods in Molecular Biology)，Humana Press；细胞生物学：实验室手册(Cell Biology:A Laboratory Notebook)(J.E.Cellis编辑，1998)Academic Press；动物细胞培养(Animal Cell Culture)(R.I.Freshney)编辑，1987)；细胞和组织培养介绍(Introduction to Cell and Tissue Culture)(J.P.Mather和P.E.Roberts,1998)Plenum Press；细胞和组织培养：实验室方法(Cell and Tissue Culture:Laboratory Procedures)(A.Doyle,J.B.Griffiths,和D.G.Newell编辑，1993-8)J.Wiley和Sons；实验免疫学手册(Handbook of Experimental Immunology)(D.M.Weir和C.C.Blackwell编辑)；哺乳动物细胞的基因转移载体(Gene Transfer Vectors for Mammalian Cells)(J.M.Miller和M.P.Calos编辑，1987)；PCR：聚合酶链氏反应(PCR:The Polymerase Chain Reaction)，(Mullis等人编辑，1994)；现代免疫学方法(Current Protocols in Immunology)(J.E.Coligan等人编辑，1991)；分子生物学简要方法(Short Protocols in Molecular Biology)(Wiley和Sons，1999)；免疫学(Immunobiology)(C.A.Janeway和P.Travers，1997)；抗体(Antibodies)(P.Finch，1997)；抗生素：实用方法(Antibodies:A Practical Approach)(D.Catty.编辑，IRL Press，1988-1989)；单克隆抗体：实用方法(Monoclonal Antibodies:A Practical Approach)(P.Shepherd和C.Dean编辑，牛津大学出版社(Oxford University Press)，2000)；抗体应用：实验室手册(Using Antibodies:A Laboratory Manual)(E.Harlow和D.Lane，冷泉港实验室出版社(Cold Spring Harbor Laboratory Press)，1999)；抗体(The Antibodies)(M.Zanetti和J.D.Capra编辑，Harwood Academic Publishers，1995)；和癌症：肿瘤学原理与实践(Cancer:Principles and Practice of Oncology)(V.T.DeVita等人编辑，J.B.Lippincott Company，1993)。

[0077] 本申请中使用的术语是基于IUPAC系统命名法，除非另有说明。除非另有定义，本文所用的技术和科学术语具有与本发明所属领域的普通技术人员通常理解的相同含义，并且符合：Singleton等人(1994)微生物和分子生物学词典，第二版(Microbiology and Molecular Biology Dictionary,2nd Ed.)，J.Wiley&Sons,New York,NY；和Janeway,C.,Travers,P.,Walport,M.,Shlomchik(2001)免疫学，第五版(Immunobiology,5th Ed.)，Garland Publishing,New York。

[0078] II.定义

[0079] 金黄色葡萄球菌(Staphylococcus aureus)在本文也简称为Staph A或S.aureus。同样,表皮葡萄球菌(Staphylococcus epidermidis)在本文也称为Staph E或S.epidermidis。

[0080] "抗体抗生素缀合物"或AAC是由通过接头与抗生素化学连接的抗体组成的化合物。抗体结合细菌表面上的抗原或表位,例如细菌细胞壁组分。如本发明所用,接头是蛋白酶可切割的非肽类接头,其被设计为被蛋白酶切割,包括组织蛋白酶B,其是大多数哺乳动物细胞类型中发现的溶酶体蛋白酶(Dubowchik等人(2002)Bioconj.Chem.13:855-869)。具有3个组分的AAC图描述于图2中。"THIOMAB™抗生素缀合物"或"TAC"是AAC的一种形式,其中抗体通过一个或多个半胱氨酸与接头-抗生素单元化学缀合,所述半胱氨酸通常是在抗体的特定位点被重组操作到抗体中的半胱氨酸,以不干扰抗原结合功能。

[0081] 当指示取代基的数目时,术语"一个或多个"是指从一个取代基到最高可能取代数量的范围,即,通过取代基替换一个氢直到替换所有氢。术语"取代基"表示替换母体分子上的氢原子的一个原子或一组原子。术语"取代的"表示指定的基团具有一个或多个取代基。当任何基团可以携带多个取代基且提供多种可能的取代基时,取代基独立地选择并且不必相同。术语"未取代的"是指指定的基团不含取代基。术语"任选取代的"是指指定基团是未取代的或被一个或多个取代基取代,其独立地选自可能的取代基的组。当指示取代基的数目时,术语"一个或多个"是指从一个取代基到最高可能的取代数量,即通过取代基替换一个氢直到替换所有氢。

[0082] 术语"抗生素"(abx或Abx)包括在给药浓度和给药间隔下特异性抑制微生物生长或杀死微生物例如细菌、但是对宿主是非致死性的任何分子。在具体方面,抗生素在给药浓度和给药间隔下对宿主无毒性。对细菌有效的抗生素可以广泛地分类为杀菌(即直接杀死)或抑菌(即防止分裂)。抗细菌抗生素可进一步分类为窄谱或广谱。与窄谱抗生素相反,广谱抗生素对于广泛的细菌是有效的,包括革兰氏阳性和革兰氏阴性细菌,而窄谱抗生素对于较小范围或特定的细菌家族有效。抗生素的例子包括:(i)氨基糖苷类,例如阿米卡星、庆大霉素、卡那霉素、新霉素、奈替米星、链霉素、妥布霉素、巴龙霉素,(ii)安莎霉素类,例如格尔德霉素、除莠霉素,(iii)碳头孢烯类,例如氯碳头孢,(iv)碳青霉烯类,例如厄他培南、多利培南、亚胺培南/西司他丁、美罗培南,(v)头孢菌素类(第一代),例如头孢羟氨苄、头孢唑啉、头孢噻吩、头孢氨苄,(vi)头孢菌素类(第二代),例如头孢克洛、头孢羟唑、头孢西丁、头孢丙烯、头孢呋辛,(vi)头孢菌素类(第三代),例如头孢克肟、头孢地尼、头孢托仑、头孢哌酮、头孢噻肟、头孢泊肟、头孢他啶、头孢布烯、头孢唑肟、头孢曲松,(vii)头孢菌素类(第四代),例如头孢吡肟,(viii)头孢菌素类(第五代),例如头孢吡普(ceftobiprole),(ix)糖肽类,例如替考拉宁、万古霉素,(x)大环内酯类,例如阿奇霉素、克拉霉素、地红霉素、红霉素、罗红霉素、醋竹桃霉素、泰利霉素、壮观霉素,(xi)内酰胺类,例如氨曲南,(xii)青霉素类,例如阿莫西林、氨苄西林、阿洛西林、羧苄西林、氯唑西林、双氯西林、氟氯西林、美洛西林、甲氧西林、萘夫西林、苯唑西林、青霉素、哌拉西林、替卡西林,(xiii)抗生素多肽类,例如杆菌肽、粘菌素、多粘菌素B,(xiv)喹诺酮类,例如环丙沙星、依诺沙星、加替沙星、左氧氟沙星、洛美沙星、莫西沙星、诺氟沙星、氧氟沙星、曲伐沙星,(xv)磺酰胺类,例如磺胺米隆、偶氮磺胺、磺胺乙酰胺、磺胺甲二唑、磺胺、柳氮磺吡啶、磺胺异噁唑、甲氧苄啶、甲氧苄啶-磺

胺甲噁唑(TMP-SMX)，(xvi)四环素类，例如地美环素、多西环素、米诺环素、土霉素、四环素，和(xvii)其它如肼凡纳明、氯霉素、克林霉素、林可霉素、乙胺丁醇、磷霉素、夫西地酸、呋喃唑酮、异烟肼、利奈唑胺、甲硝唑、莫匹罗星、呋喃妥因、平板霉素、吡嗪酰胺、奎奴普丁/达福普汀、利福平(rifampin)/利福平(rifampicin)或替硝唑。

[0083] 术语“耐甲氧西林金黄色葡萄球菌”(MRSA)也称为多药耐药性金黄色葡萄球菌或耐苯唑西林金黄色葡萄球菌(ORSA)，是指对β-内酰胺抗生素有抗性的金黄色葡萄球菌菌株，其中包括青霉素(如甲氧西林、双氯西林、萘夫西林、苯唑西林等)和头孢菌素类。“甲氧西林敏感型金黄色葡萄球菌”(MSSA)是指对β-内酰胺抗生素敏感的金黄色葡萄球菌的任何菌株。

[0084] 术语“最小抑菌浓度”(MIC)是指在过夜孵育后，抑制微生物的可见生长的抗微生物剂的最低浓度。测定MIC的试验是已知的。一种方法如下面的实施例部分所述。

[0085] 术语“抗金黄色葡萄球菌抗体”和“与金黄色葡萄球菌结合的抗体”是指能够以足够的亲和力结合金黄色葡萄球菌(“S.aureus”)上的抗原的抗体，使得抗体可用作靶向于金黄色葡萄球菌的诊断和/或治疗剂。在一个实施方案中，例如通过放射免疫测定(RIA)测量，抗金黄色葡萄球菌抗体与非相关的非金黄色葡萄球菌蛋白的结合程度不超过抗体与MRSA结合程度的约10％。在某些实施方案中，结合金黄色葡萄球菌的抗体具有≤1μM、≤100nM、≤10nM、≤5Nm、≤4nM、≤3nM、≤2nM、≤1nM、≤0.1nM、≤0.01nM或≤0.001nM(例如10^{-8}M或更小，例如10^{-8}M至10^{-13}M，例如10^{-9}M至10^{-13}M)的解离常数(Kd)。在某些实施方案中，抗金黄色葡萄球菌抗体结合来自不同物种的葡萄球菌中保守的金黄色葡萄球菌的表位。本文中的抗葡萄球菌抗体是指结合除金黄色葡萄球菌外至少一种葡萄球菌物种的抗体。

[0086] “SDR”是指丝氨酸-天冬氨酸重复序列;SDR存在于细胞壁蛋白家族中，其特征在于葡萄球菌中存在的粘附性A-结构域相邻的大量丝氨酸-天冬氨酸二肽重复序列(Foster TJ,Hook M(1998)Trends Microbiol6:484-488)。参与粘附的蛋白质包括凝集因子(Clf)A和ClfB。除了ClfA和ClfB之外，金黄色葡萄球菌还表达了三种SDR蛋白，SdrC、SdrD和SdrE，该家族的另外三个成员SrdF、SdrG和SdrH存在于大多数表皮葡萄球菌菌株中(McCrea KW等人(2000)丝氨酸天冬氨酸重复(Sdr)蛋白家族在表皮葡萄球菌中的表达。微生物学(The Serine-aspartate repeat(Sdr)protein family in Staphylococcus epidermidis.Microbiology)146(Pt 7):1535-1546)。在这些蛋白质的每一个中，含有25-275个SD-二肽重复序列的SDR区域(SEQ ID NO:24)位于N-末端配体结合A结构域和C-末端LPXTG-基序(SEQ ID NO:25)之间。

[0087] 称为“F1”的抗体具有如US 8,617,556的图1所示的重链和轻链可变区序列，其全部内容通过引用并入本文。具体地讲，负责F1的抗原结合性质的F1的CDR序列也在图1显示。抗体F1完全是人的，能够特异性结合葡萄球菌属物种如金黄色葡萄球菌和表皮葡萄球菌。重要的是，抗体F1能够在体内和体外结合整个细菌。此外，抗体F1能够结合已经在例如动物的感染组织中生长的细菌。重组产生的F1也称为“rF1”。rF1(和F1)抗体是抗SDR单克隆抗体。对于mAb rF1的表位包括SdgB依赖性GlcNAc部分。数据表明rF1结合GlcNac和SD骨架的一部分。本文所用的“rF1抗体”包括F1抗体、rF1抗体以及相对于rF1的含有氨基酸改变的rF1所有变体。rF1和变体抗体的氨基酸序列提供如下。

[0088] 本文中的术语“抗体”以最广泛的含义使用，并且具体涵盖单克隆抗体、多克隆抗

体、二聚体、多聚体、多特异性抗体(例如双特异性抗体)及其抗原结合的抗体片段(Miller等人(2003)J.Immunology 170:4854-4861)。抗体可以是鼠、人、人源化、嵌合或衍生自其他物种。抗体是由能够识别和结合特异性抗原的免疫系统产生的蛋白质(Janeway,C.,Travers,P.,Walport,M.,Shlomchik(2001)Immuno Biology,第五版,Garland Publishing,New York)。靶抗原通常具有多个结合位点,也称为表位,由多个抗体上的CDR识别。特异性结合不同表位的每种抗体具有不同的结构。因此,一种抗原可被多于一种相应的抗体识别和结合。抗体包括全长免疫球蛋白分子或全长免疫球蛋白分子的免疫活性部分,即包含抗原结合位点的分子,其免疫特异性地结合关注的靶点的抗原或其部分,这样的靶点包括但不限于癌细胞或产生与自身免疫疾病相关的自身免疫抗体的细胞、感染的细胞、或微生物如细菌。本文公开的免疫球蛋白(Ig)可以是除IgM以外的任何同种型(例如IgG、IgE、IgD和IgA)和亚类(例如IgG1、IgG2、IgG3、IgG4、IgA1和IgA2)。免疫球蛋白可以来自任何物种。在一方面,Ig是人、鼠或兔来源的。在一个具体实施方案中,Ig是人来源的。

[0089] 抗体的"类"指由其重链拥有的恒定结构域或恒定区的类型。存在五个主要类别的抗体:IgA、IgD、IgE、IgG和IgM,并且这些类别中的几种可以进一步划分成亚类(同种型),例如IgG1、IgG2、IgG3、IgG4、IgA1和IgA2。对应于不同类别免疫球蛋白的重链恒定结构域分别称作α、δ、ε、γ和μ。

[0090] "天然抗体"是指具有不同结构的天然存在的免疫球蛋白分子。举例来说,天然IgG抗体为约150,000道尔顿的异四聚体糖蛋白,由二硫键键合的两个相同轻链和两个相同重链构成。自N末端至C末端,各重链具有可变区(VH),还被称为可变重结构域或重链可变结构域,随后为三个恒定结构域(CH1、CH2及CH3)。类似地,自N末端至C末端,各轻链具有可变区(VL),还被称为可变轻结构域或轻链可变结构域,随后为恒定轻(CL)结构域。抗体的轻链可基于其恒定结构域的氨基酸序列而指定为称为κ(κ)和λ(λ)的两种类型之一。

[0091] 术语"全长抗体"、"完整抗体"和"全抗体"在本文中可互换地用于指具有与天然抗体结构基本相似的抗体,或具有包含本文定义的Fc区的重链的抗体。

[0092] 抗体的"抗原结合片段"是指非完整抗体的分子,其包含完整抗体的一部分,能结合与完整抗体结合的抗原。抗体片段的实例包括但不限于Fv、Fab、Fab'、Fab'-SH、F(ab')2;双体;线性抗体;单链抗体分子(例如scFv);以及由抗体片段形成的多特异性抗体。

[0093] 如本文中所用的术语"单克隆抗体"是指从基本上同质的抗体群体获得的抗体,即,组成群体的单个抗体是相同的和/或结合相同的表位,除了可能的变体抗体之外,例如,含有天然发生的突变或在产生单克隆抗体制备物期间出现(例如,糖基化的天然变异),这类变体通常以微小的量存在。IgG1抗体的一个这样的可能变体是重链恒定区的C末端赖氨酸(K)的切割。与通常包括针对不同决定簇(表位)的不同抗体的多克隆抗体制备物不同,单克隆抗体制备物的每个单克隆抗体针对抗原上的单一决定簇。因此,修饰语"单克隆的"指示该抗体的特征为从基本上同质的抗体群体获得,并且不得解释为要求通过任何特定方法产生该抗体。例如,可以通过多种技术产生根据本发明使用的单克隆抗体,所述技术包括但不限于杂交瘤方法、重组DNA方法、噬菌体展示方法和利用含有全部或部分的人免疫球蛋白基因座的转基因动物的方法,在本文中描述了用于产生单克隆抗体的这类方法和其他示例性方法。除了它们的特异性之外,单克隆抗体是有利的,因为它们可以在不被其它抗体污染的情况下合成。

[0094] 术语“嵌合抗体”是指其中一部分重链和/或轻链衍生自特定来源或物种而重链和/或轻链的其余部分衍生自不同来源或物种的抗体。

[0095] “人抗体”是这样一种抗体，其拥有对应于人或人细胞产生的或衍生自利用人抗体库或其他编码人抗体的序列的非人来源的抗体的氨基酸序列。人抗体的这种定义特别排除包含非人抗原结合残基的人源化抗体。

[0096] “人源化抗体”指包含来自非人HVR的氨基酸残基和来自人FR的氨基酸残基的嵌合抗体。在某些实施方案中，人源化抗体将包含至少1个、并且通常2个可变结构域中的基本上全部，其中HVR(例如，CDR)中的全部或基本上全部与非人抗体中的那些对应，并且FR中的全部或基本上全部与人抗体中的那些对应。人源化抗体任选地可以包含从人抗体衍生的抗体恒定区的至少一部分。抗体(例如，非人抗体)的“人源化形式”指已经进行过人源化的抗体。

[0097] 术语“可变区”或“可变域”是指抗体的重或轻链中牵涉抗体结合抗原的结构域。天然抗体的重链和轻链可变域(分别为VH和VL)一般具有类似的结构，其中每个域包含4个保守的框架区(FR)和3个高变区(HVR)。(参见，例如，Kindt等人Kuby Immunology，第6版，W.H.Freeman和Co.，第91页(2007))。单个VH或VL域可足以赋予抗原结合特异性。此外，可以分别使用来自结合抗原的抗体的VH或VL域以筛选互补VL或VH域的文库，从而分离结合特定抗原的抗体。参见，例如Portolano等人J.Immunol.150:880-887(1993)；Clarkson等人，Nature 352:624-628(1991)。

[0098] 在本文中术语“高变区”，“HVR”或“HV”是指抗体可变区的区域，其在序列上是高变的(“互补决定区”或“CDR”)和/或形成结构上定义的环和/或含有抗原接触残基(“抗原接触”)。通常，抗体包含六个HVR；三个在VH(H1、H2、H3)中，三个在VL(L1、L2、L3)中。在天然抗体中，H3和L3显示了六个HVR中的最大多样性，特别是H3被认为在赋予抗体的特异性方面发挥独特的作用。参见，例如，Xu等人，Immunity 13:37-45(2000)；Johnson和Wu，分子生物学方法(Methods in Molecular Biology)248:1-25(Lo编辑，Human Press，Totowa，NJ，2003)。实际上，仅由重链组成的天然存在的骆驼抗体在轻链不存在的情况下是功能性的和稳定的(Hamers-Casterman等人，(1993)Nature 363:446-448；Sheriff等人，(1996)Nature Struct.Biol.3:733-736)。

[0099] 许多HVR描绘正在使用中并被包括在本文中。Kabat互补决定区(CDRs)基于序列变异性并且是最常用的(Kabat等人，Sequences of Proteins of Immunological Interest，5th Ed.Public Health Service，National Institutes of Health)，Bethesda，MD.(1991))。Chothia改为指结构环的位置(Chothia和Lesk，(1987)J.Mol.Biol.196:901-917)。对于抗原接触，请参阅MacCallum等人J.Mol.Biol.262:732-745(1996)。AbM HVRs代表Kabat HVRs和Chothia结构环之间的折衷，并由Oxford Molecular的AbM抗体建模软件使用。“接触”HVR是基于对可用的复杂晶体结构的分析。这些HVR中每一个的残基如下所述。

[0100]

环	Kabat	AbM	Chothia	接触
L1	L24-L34	L24-L34	L26-L32	L30-L36
L2	L50-L56	L50-L56	L50-L52	L46-L55
L3	L89-L97	L89-L97	L91-L96	L89-L96
H1	H31-H35B	H26-H35B	H26-H32	H30-H35B (Kabat 编号)
H1	H31-H35	H26-H35	H26-H32	H30-H35 (Chothia 编号)
H2	H50-H65	H50-H58	H53-H55	H47-H58
H3	H95-H102	H95-H102	H96-H101	H93-H101

[0101] HVR可以包含以下的“延伸HVR”：在VL中的24-36或24-34(L1)、46-56或50-56(L2)和89-97或89-96(L3)，以及在VH中的26-35(H1)、50-65或49-65(H2)和93-102、94-102或95-102(H3)。除非另有说明，在本文中根据Kabat等人的以上文章来编号HVR残基、CDR残基和可变结构域中的其它残基(例如FR残基)。

[0102] 术语“依照Kabat的可变结构域残基编号”或“依照Kabat的氨基酸位置编号”及其变化形式指Kabat等人的以上文章中的用于抗体编制的重链可变结构域或轻链可变结构域编辑的编号系统。使用此编号系统，实际的线性氨基酸序列可包含较少或另外的氨基酸，对应于可变结构域FR或HVR的缩短或插入。例如，重链可变结构域可包含H2的残基52后的单一氨基酸插入(依照Kabat为残基52a)及重链FR残基82后的插入残基(例如依照Kabat为残基82a、82b和82c等)。对于给定抗体的残基Kabat编号可通过将抗体序列与“标准的”Kabat编号序列对比同源区域来确定。

[0103] “框架”或“FR”是指除高变区(HVR)残基外的可变结构域残基。一般地，可变结构域的FR由4个FR结构域组成：FR1、FR2、FR3和FR4。因此，HVR和FR序列在VH(或VL)中一般以如下顺序出现：FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4。

[0104] 为了本文的目的，“受体人框架”为包含由人免疫球蛋白框架或如下文定义的人共有框架衍生的轻链可变域(VL)框架或重链可变域(VH)框架的氨基酸序列的框架。由人免疫球蛋白框架或人共有框架“衍生”的受体人框架可以包含与其相同的氨基酸序列，或者它可以含有氨基酸序列改变。在一些实施方案中，氨基酸改变的数目是10或更少、9或更少、8或更少、7或更少、6或更少、5或更少、4或更少、3或更少、或2或更少。在一些实施方案中，VL受体人框架与VL人免疫球蛋白框架序列或人共有框架序列在序列上相同。

[0105] “人共有框架”是表示人免疫球蛋白VL或VH框架序列选集中最常存在的氨基酸残基的框架。通常，人免疫球蛋白VL或VH序列选集来自可变域序列亚组。通常，序列的亚组是如Kabat等人，Sequences of Proteins of Immunological Interest,Fifth Edition,NIH Publication 91-3242,Bethesda MD(1991)，第1-3卷中的亚组。在一个实施方案中，对于VL，亚组是如Kabat等人的上述文章中的亚组κI。在一个实施方案中，对于VH，亚组是如Kabat等人的上述文章中的亚组III。

[0106] 本文中的术语“Fc区”用于定义免疫球蛋白重链的C末端区域。该术语包括天然序

列Fc区和变体Fc区。尽管免疫球蛋白重链的Fc区的边界可能变化，但人IgG重链Fc区通常定义为从Cys226或从Pro230位置的氨基酸残基延伸到其羧基末端。Fc区的C末端赖氨酸(根据EU编号系统的残基447-也称为EU指数，如在Kabat等人，Sequences of Proteins of Immunological Interest,5th Ed.Public Health Service,National Institutes of Health,Bethesda,MD,1991中所述)可以除去，例如，在抗体的制备或纯化期间，或通过重组工程化改造编码抗体重链的核酸。因此，完整抗体的组合物可以包含除去所有K447残基的抗体群体、未除去K447残基的抗体群体以及具有和不具有K447残基的抗体混合物的抗体群体。术语"Fc受体"或"FcR"还包括新生儿受体FcRn，其负责将母体IgG转移至胎儿。Guyer等人，J.Immunol.117:587(1976)和Kim等人，J.Immunol.24:249(1994)。测量与FcRn结合的方法是已知的(参见，例如，Ghetie和Ward,Immunol.Today 18:(12):592-8(1997)；Ghetie等人，自然生物技术(Nature Biotechnology)15(7):637-40(1997)；Hinton等人，J.Biol.Chem.279(8):6213-6(2004)；WO 2004/92219Hinton等人)。可以测定FcRn在体内的结合和人FcRn高亲和力结合多肽的血清半衰期，例如，在转基因小鼠或表达人FcRn的转染的人细胞系中，或在其中施用具有变体Fc区的多肽的灵长类动物中。WO 2004/42072(Presta)描述了抗体变体，其改善或减少与FcR的结合。另见，例如Shields等人，J.Biol.Chem.9(2):6591-6604(2001)。

[0107] "亲和力成熟的"抗体是指在一个或多个高变区(HVR)中具有一个或多个改变的抗体，与不具有这种改变的亲本抗体相比，这些改变导致抗体对抗原亲和力的改善。

[0108] 术语"表位"是指抗原分子上与抗体结合的特定位点。

[0109] "结合相同表位的抗体"作为参照抗体，是指在竞争测定中阻断参照抗体与其抗原结合50％以上的抗体，相反地，在竞争测定中参照抗体阻断该抗体与其抗原结合50％以上。本文提供了示例性的竞争测定。

[0110] "裸抗体"是指未与异源部分(例如细胞毒性部分)或放射性标记物缀合的抗体。裸抗体可以存在于药物制剂中。

[0111] "效应子功能"是指归因于抗体Fc区的那些生物活性，其随抗体同种型而变化。抗体效应子功能的实例包括：C1q结合和补体依赖性细胞毒性(CDC)；Fc受体结合；抗体依赖性细胞介导的细胞毒性(ADCC)；吞噬；细胞表面受体(例如B细胞受体)的下调；和B细胞激活。

[0112] "抗体依赖性细胞介导的细胞毒性"或ADCC是指细胞毒性的形式，其中分泌的Ig结合到某些细胞毒性细胞(例如，天然杀伤(NK)细胞、嗜中性粒细胞和巨噬细胞)上存在的Fc受体(FcR)上，使得这些细胞毒性效应细胞特异性结合携带抗原的靶细胞，随后用细胞毒素杀死靶细胞。抗体"武装"细胞毒性细胞，并且是通过这种机制杀死靶细胞所必需的。用于介导ADCC、NK细胞的初级细胞仅表达Fcγ(gamma)RIII，而单核细胞表达Fcγ(gamma)RI、Fcγ(gamma)RII和Fcγ(gamma)RIII。在Ravetch和Kinet，Annu.Rev.Immunol.9:457-92(1991)的第464页表3中总结了造血细胞上的Fc表达。为了评估关注的分子的ADCC活性，可以进行体外ADCC测定，例如可以执行在US 5,500,362或US 5,821,337中描述的那些。用于这种测定的有用的效应细胞包括外周血单核细胞(PBMC)和天然杀伤(NK)细胞。或者或另外，可以在体内评价关注的分子的ADCC活性，例如在Clynes等人，PNAS USA 95:652-656(1998)中公开的动物模型中。

[0113] "吞噬作用"是指病原体被宿主细胞(例如巨噬细胞或嗜中性粒细胞)吞噬或内化

的过程。吞噬细胞通过三种途径介导吞噬作用：(i)直接细胞表面受体(例如，凝集素、整联蛋白和清道夫受体)，(ii)补体增强-使用补体受体(包括CRI、C3b、CR3和CR4的受体)结合和摄取补体调理的病原体，和(iii)抗体增强-使用Fc受体(包括Fc γ gammaRI，Fc γ gammaRIIA和Fc γ gammaRIIIA)结合抗体调理的粒子，然后其被内化并与溶酶体融合成为吞噬溶酶体。在本发明中，认为途径(iii)在将抗MRSA AAC治疗剂递送到感染的白细胞(例如嗜中性粒细胞和巨噬细胞)中起重要作用(微生物的吞噬作用：行动的复杂性(Phagocytosis of Microbes:complexity in Action)D.Underhill和A Ozinsky.(2002)Annual Review of Immunology,Vol20:825)。

[0114] “补体依赖性细胞毒性”或“CDC”是指在补体存在下裂解靶细胞。经典补体途径的激活由补体系统的第一组分(C1q)与结合于其同源抗原的抗体(适当亚类)结合引发。为了评估补体活化，可以使用CDC测定法，如Gazzano-Santoro等人，J.Immunol.Methods 202:163(1996)中所述。

[0115] 可以改变与Fc区连接的碳水化合物。由哺乳动物细胞产生的天然抗体通常包含支链的双分枝寡糖，其通常通过N-连接与Fc区的CH2结构域的Asn297连接。参见，例如Wright等人(1997)TIBTECH 15:26-32。寡糖可以包括各种碳水化合物，例如甘露糖、N-乙酰氨基葡萄糖(GlcNAc)、半乳糖和唾液酸，以及连接到双分枝寡糖结构的“茎”中的GlcNAc的岩藻糖。在一些实施方案中，可以制备在IgG中的寡糖修饰，以便产生具有某些另外改进特性的IgG。例如，提供具有碳水化合物结构的抗体修饰，其缺乏与Fc区(直接或间接)连接的岩藻糖。这样的修改可能具有改善的ADCC功能。参见，例如US 2003/0157108(Presta,L.)；US 2004/0093621(Kyowa Hakko Kogyo有限公司)。与“去岩藻糖化”或“岩藻糖缺乏”的抗体修饰相关的出版物的实例包括：US 2003/0157108；WO 2000/61739；WO 2001/29246；US 2003/0115614；US 2002/0164328；US 2004/0093621；US 2004/0132140；US 2004/0110704；US 2004/0110282；US 2004/0109865；WO 2003/085119；WO 2003/084570；WO 2005/035586；WO 2005/035778；WO2005/053742；WO2002/031140；Okazaki等人，J.Mol.Biol.336:1239-1249(2004)；Yamane-Ohnuki等人Biotech.Bioeng.87:614(2004)。能够产生去岩藻糖化抗体的细胞系的实例包括缺乏蛋白质岩藻糖基化的Lee 13 CHO细胞(Ripka等人，Arch.Biochem.Biophys.249:533-545(1986)；美国专利申请公开号2003/0157108A1，Presta,L；和WO 2004/056312A1,Adams等人，特别是实施例11)和敲除细胞系，如α-1,6-岩藻糖基转移酶基因、FUT8、敲除的CHO细胞(参见例如Yamane-Ohnuki等人，Biotech.Bioeng.87:614(2004)；Kanda,Y.等人，Biotechnol.Bioeng.,94(4):680-688(2006)；和WO2003/085107)。

[0116] “分离的抗体”是已经与其天然环境的一种成分分开的抗体。在一些实施方案中，将抗体纯化至超过95％或99％纯度，如通过例如电泳(例如，SDS-PAGE、等电聚焦(IEF)、毛细管电泳)或色谱(例如，离子交换或反相HPLC)所测定的。关于评估抗体纯度的方法的综述参见，例如Flatman等人，J.Chromatogr.B 848:79-87(2007)。

[0117] “分离的核酸”是指已经与其天然环境的一种成分分开的核酸分子。分离的核酸包括正常情况下包含该核酸分子的细胞中含有的该核酸分子，但是该核酸分子存在于染色体外或与其天然染色体位置不同的染色体位置。

[0118] “分离的编码rF1抗体的核酸”是指一种或多种编码抗体重链和轻链的核酸分子，

包括在单一载体或分开的载体中的所述核酸分子，以及存在于宿主细胞中一个或多个位置处的所述核酸分子。

[0119] 如本文所用，术语“特异性结合”或“特异性”是指可测量和可重复的相互作用，例如靶点和抗体之间的结合，其在包括生物分子的异质分子群体存在下确定靶点的存在。例如，特异性结合靶点（其可以是表位）的抗体是以更高的亲和力、亲合力、更容易和/或具有比其它靶点结合更长的持续时间来结合该靶点的抗体。在一个实施方案中，例如通过放射免疫测定法（RIA）测量，抗体与和rF1无关的靶点的结合程度低于抗体与靶点结合程度的约10％。在某些实施方案中，特异性结合rF1的抗体具有≤1μM、≤100nM、≤10nM，≤1nM或≤0.1nM的解离常数（Kd）。在某些实施方案中，抗体特异性结合来自不同物种的表位。在另一个实施方案中，特异性结合可以包括，但不是必需排他性结合。

[0120] “结合亲和力”通常是指分子（例如，抗体）的单一结合位点与其结合配偶体（例如，抗原）之间的非共价相互作用的总和强度。除非另有说明，如本文所用，“结合亲和力”是指反映结合对成员（例如，抗体和抗原）之间的1:1相互作用的固有结合亲和力。分子X对其配偶体Y的亲和力通常可以由解离常数（Kd）表示。亲和力可以通过本领域已知的常规方法测量，包括本文所述的那些。低亲和力抗体通常缓慢地结合抗原并易于解离，而高亲和力抗体通常更快地结合抗原并且易于保持结合更长时间。测量结合亲和力的各种方法是本领域已知的，其中任何一种可以用于本发明的目的。以下描述用于测量结合亲和力的具体说明性和示例性实施方案。

[0121] 在一个实施方案中，根据本发明的“Kd”或“Kd值”，通过用目标抗体的Fab型及其抗原进行的放射性标记抗原结合测定（RIA）来测量，如以下测定所述。通过在存在未标记抗原的滴定系列的情况中用最小浓度的（^{125}I）-标记抗原而平衡Fab，然后用抗Fab抗体包被的板子捕捉结合的抗原来测量Fab对抗原的溶液结合亲和力（参见例如Chen等人，J.Mol.Biol.293:865-881(1999)）。为了建立测定法的条件，微量滴定板（DYNEX Technologies,Inc.）用5μg/ml捕获性抗Fab抗体（Cappel Labs）在50mM碳酸钠（pH9.6）中包被过夜，随后在PBS中用2％（w/v）牛血清白蛋白于室温（约23℃）封闭2-5小时。在非吸附板（Nunc#269620）中，将100pM或26pM[^{125}I]-抗原与连续稀释的目标Fab混合（例如与Presta等人，Cancer Res.57:4593-4599(1997)中抗VEGF抗体Fab-12的评估一致）。然后将目标Fab孵育过夜；然而，孵育可持续更长时间（例如，约65小时）以确保达到平衡。此后，将混合物转移至捕获板，用于室温孵育（例如，1小时）。然后除去溶液，并用PBS中的0.1％吐温-20™表面活性剂洗板8次。平板干燥后，加入150μl/孔的闪烁液（MICROSCINT-20™；Packard），然后在TOPCOUNT™γ计数器（Packard）上对平板计数10分钟。选择给出小于或等于最大结合的20％的各Fab浓度，用于竞争性结合测定法。

[0122] 根据另一个实施方案，采用表面等离子共振测定法，使用**BIACORE®**-2000或**BIACORE®**-3000仪器（BIAcore,Inc.,Piscataway,NJ）于25℃用固定的抗原CM5芯片以约10个响应单位（RU）测量Kd。简言之，根据供应商的说明书，用N-乙基-N'-(3-二甲基氨基丙基)-碳二亚胺盐酸盐（EDC）和N-羟基琥珀酰亚胺（NHS）活化羧甲基化的右旋糖酐生物传感器芯片（CM5,BIAcore公司）。将抗原用10mM乙酸钠pH4.8稀释至5μg/ml（～0.2μM），之后以5μl/min的流速注射以实现大约10个响应单位（RU）的偶联蛋白质。注射抗原之后，注射1M乙

醇胺以封闭未反应基团。对于动力学测量，于25℃以大约25μl/min的流速注射在含0.05%吐温20™表面活性剂的PBS(PBST)中两倍连续稀释的Fab(0.78nM至500nM)。使用简单的一对一Langmuir结合模型(**BIAcore**®Evaluation Software version 3.2)，通过同时拟合结合和解离传感图来计算结合速率(k_{on})和解离速率(k_{off})。以k_{off}/k_{on}比率计算平衡解离常数(Kd)。参见，例如Chen等人，J.Mol.Biol.293:865-881(1999)。如果通过上述表面等离子共振测定法，结合速率超过$10^6M^{-1}s^{-1}$，那么可使用荧光淬灭技术来测定结合速率，所述技术在分光计例如带有断流装置的分光光度计(Aviv Instruments)或8000系列SLM-AMINCO™分光光度计(ThermoSpectronic)中用搅拌比色杯测量的浓度渐增的抗原存在下，测量在PBS pH7.2中20nM抗-抗原的抗体(Fab形式)于25℃的荧光发射强度(激发＝295nm；发射＝340nm，16nm带通)的升高或降低。

[0123] 根据本发明的“结合速率”或“k_{on}”还可以使用**BIACORE**®-2000或**BIACORE**®-3000系统(BIAcore，Inc.，Piscataway，NJ)按如上所述测定。

[0124] 术语“宿主细胞”、“宿主细胞系”和“宿主细胞培养物”可互换使用，并且指已经导入外源核酸的细胞，包括所述细胞的后代。宿主细胞包括“转化体”和“经转化的细胞”，其包括原代的经转化的细胞及由其衍生的后代而不考虑传代的次数。后代在核酸内容物上可以与亲本细胞不完全相同，而是可以含有突变。本文中包括具有与在初始转化细胞中筛选或选择的相同功能或生物学活性的突变体后代。

[0125] 如本文使用的术语“载体”是指能够增殖与其连接的另一种核酸的核酸分子。该术语包括作为自身复制型核酸结构的载体及整合到其已导入的宿主细胞的基因组中的载体。某些载体能够指导与其可操作连接的核酸的表达。所述载体在本文中称为“表达载体”。

[0126] 关于参照多肽序列的“氨基酸序列同一性百分比(%)”定义为候选序列中与参照多肽序列中的氨基酸残基相同的氨基酸残基的百分比，在比对序列并引入间隙之后，如果需要，可以达到最大百分比序列同一性，并且不考虑作为序列同一性的一部分的任何保守取代。用于确定氨基酸序列同一性百分比的比对可以通过本领域技术范围内的各种方式实现，例如，使用诸如BLAST、BLAST-2、ALIGN或Megalign(DNASTAR)软件的公众可用的计算机软件。本领域技术人员可以确定用于比对序列的适当参数，包括在待比较的序列的全长上实现最大比对所需的任何算法。然而，为了本文的目的，使用序列比较计算机程序ALIGN-2产生%氨基酸序列同一性值。Genentech公司编写了ALIGN-2序列比较计算机程序，并且源代码已经在华盛顿特区20559号美国版权局提交了用户文档，以美国版权注册号TXU510087注册。ALIGN-2程序可从加利福尼亚州南旧金山的Genentech公司公开获得，或者可以从源代码编译。ALIGN-2程序应编译为在UNIX操作系统上使用，包括数字UNIX V4.0D。所有序列比较参数由ALIGN-2程序设置，并且不变。

[0127] 在使用ALIGN-2用于氨基酸序列比较的情况下，给定的氨基酸序列A与或相对于给定的氨基酸序列B的氨基酸序列同一性%(其也可被称为具有或包含与或相对于给定氨基酸序列B的某一氨基酸序列同一性%的给定的氨基酸序列A)的计算如下：100乘以分数X/Y，其中X是在该程序的A和B的比对中，通过序列比对程序ALIGN-2评价为相同匹配的氨基酸残基数，并且其中Y是B中氨基酸残基的总数。应当理解，如果氨基酸序列A的长度不等于氨基酸序列B的长度，A与B的氨基酸序列同一性%将不等于B与A的氨基酸序列同一性%。除非另有特别说明，否则本文使用的所有氨基酸序列同一性%值如上所述获得。

[0128] 术语“利福霉素型抗生素”是指具有利福霉素结构或类似结构的抗生素类或组。

[0129] 术语“利福拉齐型抗生素”是指具有利福拉齐结构或类似结构的抗生素类或组。

[0130] 当指示取代基的数目时，术语“一个或多个”是指从一个取代基到最高可能取代数的范围，即，替换一个氢到通过取代基替换所有氢。术语“取代基”表示替换母体分子上的氢原子的一个原子或一组原子。术语“取代的”表示指定的基团具有一个或多个取代基。当任何基团可以携带多种取代基且提供多种可能的取代基时，独立地选择取代基并且不必相同。术语“未取代的”是指指定的基团不含取代基。术语“任选取代的”是指指定基团是未取代的或被一个或多个取代基取代，其独立地选自可能的取代基的组。当指示取代基的数目时，术语“一个或多个”是指从一个取代基到最高可能的取代数量，即通过取代基替换一个氢直到替换所有氢。

[0131] 本文所用的术语“烷基”是指具有1至12个碳原子(C_1-C_{12})的饱和直链或支链单价烃基，其中烷基可以任选地被一个或多个下述取代基独立地取代。在另一个实施方案中，烷基为1至8个碳原子(C_1-C_8)或1至6个碳原子(C_1-C_6)。烷基的实例包括但不限于甲基(Me，-CH_3)、乙基(Et，-CH_2CH_3)、1-丙基(n-Pr，正丙基，-$CH_2CH_2CH_3$)、2-丙基(i-Pr，异丙基，-$CH(CH_3)_2$)、1-丁基(n-Bu，正丁基，-$CH_2CH_2CH_2CH_3$)、2-甲基-1-丙基(i-Bu，异丁基，-$CH_2CH(CH_3)_2$)、2-丁基(s-Bu，仲丁基，-$CH(CH_3)CH_2CH_3$)、2-甲基-2-丙基(t-Bu，叔丁基，-$C(CH_3)_3$)、1-戊基(正戊基，-$CH_2CH_2CH_2CH_2CH_3$)、2-戊基(-$CH(CH_3)CH_2CH_2CH_3$)、3-戊基(-$CH(CH_2CH_3)_2$)、2-甲基-2-丁基(-$C(CH_3)_2CH_2CH_3$)、3-甲基-2-丁基(-$CH(CH_3)CH(CH_3)_2$)、3-甲基-1-丁基(-$CH_2CH_2CH(CH_3)_2$)、2-甲基-1-丁基(-$CH_2CH(CH_3)CH_2CH_3$)、1-己基(-$CH_2CH_2CH_2CH_2CH_2CH_3$)、2-己基(-$CH(CH_3)CH_2CH_2CH_2CH_3$)、3-己基(-$CH(CH_2CH_3)(CH_2CH_2CH_3)$)、2-甲基-2-戊基(-$C(CH_3)_2CH_2CH_2CH_3$)、3-甲基-2-戊基(-$CH(CH_3)CH(CH_3)CH_2CH_3$)、4-甲基-2-戊基(-$CH(CH_3)CH2CH(CH_3)_2$)、3-甲基-3-戊基(-$C(CH_3)(CH_2CH_3)_2$)、2-甲基-3-戊基(-$CH(CH_2CH_3)CH(CH_3)_2$)、2,3-二甲基-2-丁基(-$C(CH_3)_2CH(CH_3)_2$)、3,3-二甲基-2-丁基(-$CH(CH_3)C(CH_3)_3$)、1-庚基、1-辛基等。

[0132] 本文所用的术语“亚烷基”是指1至12个碳原子(C_1-C_{12})的饱和直链或支链二价烃基，其中亚烷基可以任选地被一个或多个下述取代基独立地取代。在另一个实施方案中，亚烷基是1-8个碳原子(C_1-C_8)或1-6个碳原子(C_1-C_6)。亚烷基的实例包括但不限于亚甲基(-CH_2-)、亚乙基(-CH_2CH_2-)、亚丙基(-$CH_2CH_2CH_2$-)等。

[0133] 术语“烯基”是指具有至少一个不饱和位点即碳-碳sp^2双键的2-8个碳原子(C_2-C_8)的直链或支链一价烃基，其中烯基可以任选地被本文所述的一个或多个取代基独立地取代，并且包括具有“顺式”和“反式”取向，或者“E”和“Z”取向的基团。实例包括但不限于亚乙基或乙烯基(-CH=CH_2)、烯丙基(-CH_2CH=CH_2)等。

[0134] 术语“亚烯基”是指具有至少一个不饱和位点即碳-碳sp^2双键的2-8个碳原子(C_2-C_8)的直链或支链二价烃基，其中亚烯基可以任选地被本文所述的一个或多个取代基独立地取代，并且包括具有“顺式”和“反式”取向，或者“E”和“Z”取向的基团。实例包括但不限于亚乙烯基(-CH=CH-)、烯丙基(-CH_2CH=CH-)等。

[0135] 术语“炔基”是指具有至少一个不饱和位点即碳-碳sp叁键的2-8个碳原子(C_2-C_8)的直链或支链一价烃基，其中炔基可以任选地被本文所述的一个或多个取代基独立地取代。实例包括但不限于乙炔基(-C≡CH)、丙炔基(炔丙基，-CH_2C≡CH)等。

[0136] 术语“亚炔基”是指具有至少一个不饱和位点即碳-碳sp叁键的2-8个碳原子(C_2-C_8)的直链或支链二价烃基，其中亚炔基可以任选地被本文所述的一个或多个取代基独立地取代。实例包括但不限于亚乙炔基(-C≡C-)、亚丙炔基(亚炔丙基，-CH_2C≡C-)等。

[0137] 术语“碳环”、“碳环基”和“环烷基”是指单价非芳香族饱和或部分不饱和环，具有3-12个碳原子(C_3-C_{12})的作为单环，或具有7至12个碳原子作为双环。具有7至12个原子的双环碳环可以排列成，例如双环[4,5]、[5,5]、[5;6]或[6,6]系统，并且具有9或10个环原子的双环碳环可以排列成双环[5,6]或[6,6]系统，或成桥连系统如双环[2.2.1]庚烷、双环[2.2.2]辛烷和双环[3.2.2]壬烷。螺环部分也包括在本定义的范围内。单环碳环的实例包括但不限于环丙基、环丁基、环戊基、1-环戊-1-烯基、1-环戊-2-烯基、1-环戊-3-烯基、环己基、1-环己-1-烯基、1-环己-2-烯基、1-环己-3-烯基、环己二烯基、环庚基、环辛基、环壬基、环癸基、环十一烷基、环十二烷基等。碳环基基团任选被一个或多个本文所述的取代基独立地取代。

[0138] “芳基”是指通过从母体芳族环系统的单个碳原子除去一个氢原子而衍生的6-20个碳原子(C_6-C_{20})的单价芳族烃基。一些芳基在示例性结构中表示为“Ar”。芳基包括双环基团，其包含与饱和的，部分不饱和的环或芳族碳环稠合的芳香环。典型的芳基包括但不限于衍生自苯(苯基)、取代的苯、萘、蒽、联苯、茚基、茚满基、1,2-二氢萘、1,2,3,4-四氢萘基等的基团。芳基任选地被一个或多个本文所述的取代基独立地取代。

[0139] “亚芳基”是指通过从母体芳族环系统的两个碳原子除去两个氢原子而衍生的6-20个碳原子(C_6-C_{20})的二价芳族烃基。一些亚芳基在示例性结构中表示为“Ar”。亚芳基包括双环基团，其包含与饱和的，部分不饱和的环或芳族碳环稠合的芳香环。典型的亚芳基包括但不限于衍生自苯(亚苯基)、取代苯、萘、蒽、联苯、亚茚基、亚茚满基、1,2-二氢萘、1,2,3,4-四氢萘基等的基团。亚芳基任选地被一个或多个本文所述的取代基取代。

[0140] 术语“杂环”和“杂环基”在本文中可互换使用，是指饱和或部分不饱和(即，在环内具有一个或多个双键和/或叁键)的3至约20个环原子的碳环基，其中至少一个环原子是选自氮、氧、磷和硫的杂原子，剩余的环原子是C，其中一个或多个环原子任选地被一个或多个如下所述的取代基独立地取代。杂环可以是具有3至7个环成员(2至6个碳原子和1至4个选自N,O,P和S的杂原子)的单环或具有7至10个环成员(4至9个碳原子和1至6个选自N,O,P和S的杂原子)的双环，例如：双环[4,5]、[5,5]、[5,6]或[6,6]系统。杂环描述于Paquette,Leo A.,“现代杂环化学原理(Principles of Modern Heterocyclic Chemistry)”(W.A.Benjamin,New York,1968)，特别是第1、3、4、6、7和9章；“杂环化合物化学，一系列专著(The Chemistry of Heterocyclic Compounds,A series of Monographs)”(John Wiley&Sons,New York,1950至今)，特别是第13、14、16、19和28卷；和J.Am.Chem.Soc.(1960)82:5566。“杂环基”还包括其中杂环基与饱和的，部分不饱和环或芳族碳环或杂环稠合的基团。杂环的实例包括但不限于吗啉-4-基、哌啶-1-基、哌嗪基、哌嗪-4-基-2-酮、哌嗪-4-基-3-酮、吡咯烷-1-基、硫吗啉-4-基、S-二氧代硫吗啉-4-基、氮杂环辛烷-1-基、氮杂环丁烷-1-基、八氢吡啶并[1,2-a]吡嗪-2-基、[1,4]二氮杂环庚-1-基、吡咯烷基、四氢呋喃基、二氢呋喃基、四氢噻吩基、四氢吡喃基、二氢吡喃基、四氢噻喃基、哌啶基、吗啉代、硫代吗啉代、噻咯烷基、哌嗪基、高哌嗪基、氮杂环丁烷基、氧杂环丁烷基、硫杂环丁烷基、高哌啶基、氧杂环庚烷基、硫杂环庚烷基、氧氮杂**萘**基、二氮杂**萘**基、硫杂**萘**基、2-吡咯啉基、3-吡

咯啉基、二氢吲哚基、2H-吡喃基、4H-吡喃基、二噁烷基、1,3-二氧杂环戊烷基、吡唑啉基、二噻烷基、二硫杂环戊烷基、二氢吡喃基、二氢噻吩基、二氢呋喃基、吡唑烷基、咪唑啉基、咪唑烷基、3-氮杂双环[3.1.0]己烷基、3-氮杂二环[4.1.0]庚烷基、氮杂双环[2.2.2]己烷基、3H-吲哚基、喹嗪基和N-吡啶基脲。螺环部分也包括在本定义的范围内。其中2个环原子被氧代(=O)部分取代的杂环基团的实例是嘧啶酮基和1,1-二氧代-硫吗啉基。本文的杂环基团任选地被一个或多个本文所述的取代基独立地取代。

[0141] 术语"杂芳基"是指5-、6-或7-元环的单价芳族基团,并且包括5-20个原子的稠环体系(其中至少一个是芳族的),其独立地含有一个或多个选自氮、氧和硫的杂原子。杂芳基的实例是吡啶基(包括例如,2-羟基吡啶基)、咪唑基、咪唑并吡啶基、嘧啶基(包括例如,4-羟基嘧啶基)、吡唑基、三唑基、吡嗪基、四唑基、呋喃基、噻吩基、异噁唑基、噻唑基、噁二唑基、噁唑基、异噻唑基、吡咯基、喹啉基、异喹啉基、四氢异喹啉基、吲哚基、苯并咪唑基、苯并呋喃基、噌啉基、吲唑基、吲哚基、酞嗪基、哒嗪基、三嗪基、异吲哚基、蝶啶基、嘌呤基、噁二唑基、三唑基、噻二唑基、呋咱基、苯并呋咱基、苯并噻吩基、苯并噻唑基、苯并噁唑基、喹唑啉基、喹喔啉基、萘啶基和呋喃并吡啶基。杂芳基任选被一个或多个本文所述的取代基独立地取代。

[0142] 在可能的情况下,杂环或杂芳基可以是碳(碳连接的)或氮(氮连接的)键合的。作为举例而非限制,碳键合的杂环或杂芳基键合在吡啶的第2、3、4、5或6位,哒嗪的第3、4、5或6位,嘧啶的2、4、5或6位,吡嗪的2、3、5或6位,呋喃、四氢呋喃、噻吩(thiofuran)、噻吩(thiophene)、吡咯或四氢吡咯的2、3、4或5位,噁唑、咪唑或噻唑的2、4或5位,异噁唑、吡唑或异噻唑的3、4或5位,氮丙啶的2或3位,氮杂环丁烷的2、3或4位,喹啉的2、3、4、5、6、7或8位,或异喹啉的1、3、4、5、6、7或8位。

[0143] 举例而言而非限制,氮键合的杂环或杂芳基键合于氮丙啶、氮杂环丁烷、吡咯、吡咯烷、2-吡咯啉、3-吡咯啉、咪唑、咪唑啉、2-咪唑啉、3-咪唑啉、吡唑、吡唑啉、2-吡唑啉、3-吡唑啉、哌啶、哌嗪、吲哚、二氢吲哚、1H-吲唑的1位,异吲哚或异吲哚啉的2位,吗啉的4位,以及咔唑或β-咔啉的9位。

[0144] "代谢物"是特定化合物或其盐通过体内代谢产生的产物。可以使用本领域已知的常规技术来鉴定化合物的代谢物,并且使用本文所述的测试来确定其活性。这样的产物可以例如由所施用的化合物的氧化、还原、水解、酰胺化、脱酰胺化、酯化、脱酯化、酶裂解等产生。因此,本发明包括本发明化合物的代谢物,包括通过包含使本发明的式I化合物与哺乳动物接触足以产生其代谢产物的时间的方法产生的化合物。

[0145] 术语"药物制剂"是指处于如下的形式,使得容许其中含有的活性成分的生物学活性是有效的,且不含对将接受制剂施用的个体具有不可接受的毒性的其他组分的制剂。

[0146] "无菌"制剂是无菌的或不含所有活的微生物及其孢子。

[0147] "稳定"制剂是其中蛋白质在储存时基本上保持其物理和化学稳定性和完整性的制剂。用于测量蛋白质稳定性的各种分析技术在本领域是可获得的,并且在肽和蛋白质药物递送(Peptide and Protein Drug Delivery),247-301,Vincent Lee Ed.,Marcel Dekker,Inc.,New York,New York,Pubs.(1991)和Jones,A.Adv.Drug Delivery Rev.10:29-90(1993)中进行了综述。稳定性可以在所选择的温度下测量所选择的时间段。为了快速筛选,制剂可以在40℃保持2周至1个月,此时测定稳定性。当制剂在2-8℃下储存时,通常制

剂应在30℃或40℃下稳定至少1个月，和/或在2-8℃下稳定至少2年。当制剂在30℃下储存时，通常制剂应在30℃下稳定至少2年，和/或在40℃稳定至少6个月。例如，储存期间的聚集程度可以用作蛋白质稳定性的指标。因此，“稳定”制剂可以是其中小于约10％和优选小于约5％的蛋白质在制剂中以聚集体存在。在其它实施方案中，可以确定在制剂储存过程中聚集体形成的任何增加。

[0148] “等渗”制剂是具有与人血液基本相同的渗透压的制剂。等渗制剂通常具有约250至350mOsm的渗透压。术语“低渗”描述渗透压低于人血液的制剂。相应地，术语“高渗”用于描述渗透压高于人血液的制剂。例如，可以使用蒸气压或冰冻型渗透压计测量等渗性。作为加入盐和/或缓冲液的结果，本发明的制剂是高渗的。

[0149] 本文所用的“载体”包括药学上可接受的载体，赋形剂或稳定剂，其所使用的剂量和浓度对暴露于其中的细胞或哺乳动物是无毒的。生理上可接受的载体通常是含水pH缓冲溶液。生理上可接受的载体的实例包括缓冲液如磷酸盐、柠檬酸盐和其它有机酸；抗氧化剂，包括抗坏血酸；低分子量(少于约10个残基)多肽；蛋白质，如血清白蛋白、明胶或免疫球蛋白；亲水性聚合物如聚乙烯吡咯烷酮；氨基酸如甘氨酸、谷氨酰胺、天冬酰胺、精氨酸或赖氨酸；单糖、二糖和其他碳水化合物，包括葡萄糖、甘露糖或糊精；螯合剂如EDTA；糖醇如甘露醇或山梨糖醇；盐形成抗衡离子如钠；和/或非离子表面活性剂如**吐温®**，聚乙二醇(PEG)和PLURONICS™。

[0150] “药学上可接受的载体”是指药物制剂中活性成分以外的对个体无毒的成分。药学上可接受的载体包括但不限于缓冲剂、赋形剂、稳定剂或防腐剂。“药学上可接受的酸”包括在配制浓度和方式下无毒的无机酸和有机酸。例如，合适的无机酸包括盐酸、高氯酸、氢溴酸、氢碘酸、硝酸、硫酸、磺酸、亚磺酸、对氨基苯磺酸、磷酸、碳酸等。合适的有机酸包括直链和支链烷基、芳族、环状、脂环族、芳基脂族、杂环、饱和、不饱和、单、二和三羧酸，包括例如甲酸、乙酸、2-羟基乙酸、三氟乙酸、苯乙酸、三甲基乙酸、叔丁基乙酸、邻氨基苯甲酸、丙酸、2-羟基丙酸、2-氧代丙酸、丙二酸、环戊烷丙酸、3-苯基丙酸、丁酸、丁二酸、苯甲酸、3-(4-羟基苯甲酰基)苯甲酸、2-乙酰氧基-苯甲酸、抗坏血酸、肉桂酸、月桂基硫酸、硬脂酸、粘康酸、扁桃酸、琥珀酸、双羟萘酸、富马酸、苹果酸、马来酸、羟基马来酸、丙二酸、乳酸、柠檬酸、酒石酸、乙醇酸、葡萄糖酸、葡糖酸、丙酮酸、乙醛酸、草酸、甲磺酸、琥珀酸、水杨酸、邻苯二甲酸、扑酸、棕榈酸(palmeic acid)、硫氰酸、甲磺酸、乙磺酸、1,2-乙烷二磺酸、2-羟基乙磺酸、苯磺酸、4-氯苯磺酸、萘-2-磺酸、对甲苯磺酸、樟脑磺酸、4-甲基双环[2.2.2]辛-2-烯-1-甲酸、葡庚糖、4,4'-亚甲基双-3-(羟基-2-烯-1-甲酸)、羟基萘甲酸。

[0151] “药学上可接受的碱”包括以配制的浓度和方式无毒的无机和有机碱。例如，合适的碱包括由无机碱形成金属如锂、钠、钾、镁、钙、铵、铁、锌、铜、锰、铝、N-甲基葡糖胺、吗啉、哌啶形成的碱和有机无毒碱包括伯胺、仲胺和叔胺、取代的胺、环胺和碱性离子交换树脂，[例如，$N(R')_4^+$(其中R'是独立地H或C_{1-4}烷基，例如铵、Tris)]，例如异丙胺、三甲胺、二乙胺、三乙胺、三丙胺、乙醇胺、2-二乙氨基乙醇、三甲胺、二环己胺、赖氨酸、精氨酸、组氨酸、咖啡因、普鲁卡因、海巴明、胆碱、甜菜碱、乙二胺、葡糖胺、甲基葡糖胺、可可碱、嘌呤、哌嗪、哌啶、N-乙基哌啶、聚胺树脂等。特别优选的有机无毒碱是异丙胺、二乙胺、乙醇胺、三甲基胺、二环己胺、胆碱和咖啡因。

[0152] 可用于本发明的其它药学上可接受的酸和碱包括衍生自氨基酸的那些，例如组氨

酸，甘氨酸，苯丙氨酸，天冬氨酸，谷氨酸，赖氨酸和天冬酰胺。

[0153] “药学上可接受的”缓冲剂和盐包括衍生自上述酸和碱的酸和碱加成盐的那些。特定的缓冲液和/或盐包括组氨酸，琥珀酸盐和乙酸盐。

[0154] “药学上可接受的糖”是当与关注的蛋白质结合时，显著地防止或减少储存时蛋白质的化学和/或物理不稳定性的分子。当制剂被冻干，然后重构时，“药学上可接受的糖”也可以称为“冻干保护剂”。示例性的糖及其相应的糖醇包括：氨基酸，如谷氨酸钠或组氨酸；甲胺如甜菜碱；溶致盐如硫酸镁；多元醇，例如三元或更高分子量的糖醇，例如甘油、葡聚糖、赤藓糖醇、甘油、阿糖醇、木糖醇、山梨醇和甘露醇；丙二醇；聚乙二醇；**PLURONICS®**；及其组合。另外的示例性的冻干保护剂包括甘油和明胶，以及麦芽糖、松三糖、棉子糖、甘露三糖和水苏糖。还原糖的实例包括葡萄糖、麦芽糖、乳糖、麦芽酮糖、异麦芽酮糖和乳果糖。非还原性糖的实例包括选自糖醇和其它直链多元醇的多羟基化合物的非还原性糖苷。优选的糖醇是单糖苷，特别是通过还原二糖，例如乳糖、麦芽糖、乳果糖和麦芽酮糖，获得的那些化合物。糖苷侧基可以是糖苷或半乳糖苷。糖醇的另外的实例是葡萄糖醇、麦芽糖醇、乳糖醇和异麦芽酮糖。优选的药学上可接受的糖是非还原糖海藻糖或蔗糖。将药学上可接受的糖以“保护量”(例如预冻干)加入到制剂中，这意味着蛋白质在储存期间(例如，在重构和储存之后)基本上保持其物理和化学稳定性和完整性。

[0155] 本文所关注的“稀释剂”是药学上可接受的(施用于人类时安全无毒)，并且可用于制备液体制剂，例如冻干后重新配制的制剂。示例性的稀释剂包括无菌水、注射用抑菌水(BWFI)、pH缓冲溶液(例如磷酸盐缓冲盐水)、无菌盐水溶液、林格溶液或葡萄糖溶液。在替代实施方案中，稀释剂可以包括盐和/或缓冲液的水溶液。

[0156] “防腐剂”是可以加入到本文制剂中以减少细菌活性的化合物。例如，添加防腐剂可以促进生产多用途(多剂量)制剂。潜在防腐剂的实例包括十八烷基二甲基苄基氯化铵、氯化六烃季铵、苯扎氯铵(烷基苄基二甲基氯化铵的混合物，其中烷基是长链化合物)和苄索氯铵。其他类型的防腐剂包括芳族醇如苯酚、丁基和苄醇，对羟基苯甲酸烷基酯如对羟基苯甲酸甲酯或对羟基苯甲酸丙酯、邻苯二酚、间苯二酚、环己醇、3-戊醇和间甲酚。本文最优选的防腐剂是苄醇。

[0157] “个体”或“受试者”或“患者”是哺乳动物。哺乳动物包括但不限于驯养的动物(例如，牛、羊、猫、狗和马)，灵长类动物(例如，人类和非人类灵长类动物如猴子)，兔子和啮齿动物(例如，小鼠和大鼠)。在某些实施方案中，个体或受试者是人。

[0158] 如本文所用，“治疗”(及其语法变化例如“治疗”)是指临床干预，旨在临床病理过程中改变待治疗的个体，组织或细胞的自然过程。治疗的期望效果包括但不限于降低疾病进展的速度，改善或缓解疾病状态，以及缓解或改善的预后，所有这些都可由本领域技术人员如医师检测。在一个实施方案中，治疗可以意味着减轻症状，减轻疾病的任何直接或间接的病理后果，降低感染性疾病进展的速度，疾病状态的改善或缓解，以及缓解或改善的预后。在一些实施方案中，本发明的AAC和TAC用于延缓疾病的发展或减缓感染性疾病的进展或减少血流和/或感染的组织和器官中的细菌负荷。

[0159] 如本文所用，“联合”是指除了另一种治疗方式之外施用的一种治疗方式。因此，“联合”是指在向个人施用其他治疗方式之前，期间或之后施用的一种治疗方式。

[0160] 术语“菌血症”是指血液中存在细菌，其是通过血液培养最常检测到的。在手术期

间(特别是当涉及粘膜如胃肠道时),或由于导管和其他异物进入动脉或静脉时,细菌可以进入血液作为感染(如肺炎或脑膜炎)的严重并发症。菌血症有几个后果。对细菌的免疫应答可导致败血症和败血性休克,死亡率相对较高。细菌也可以通过血液传播到身体的其他部位,导致感染远离原始感染部位。实例包括心内膜炎或骨髓炎。

[0161] "治疗有效量"是实现特定病症的可测量改善所需的最小浓度。本文的治疗有效量可以根据诸如患者的疾病状态、年龄、性别和体重等因素,以及抗体在个体中引起所需反应的能力而变化。治疗有效量也是抗体的治疗有益效果超过任何毒性或有害作用的量。在一个实施方案中,治疗有效量是有效降低体内感染中的菌血症的量。在一方面,"治疗有效量"是至少有效地减少从患者样品(例如血液)分离出的相对于给药前至少一个对数的细菌负荷或集落形成单位(CFU)的量。在更具体的方面,减少至少为2个对数。在另一方面,减少量至少为3、4、5个对数。然而在另一方面,使用本领域已知的测定法,包括本文所例示的测定,降低到可检测水平以下。在另一个实施方案中,与感染患者治疗前或治疗开始时的阳性血培养物相比,治疗有效量是在治疗期间给予的一个或多个剂量中的AAC的量,其实现阴性的血培养物(即,不产生作为AAC靶点的细菌)。

[0162] "预防有效量"是指为达到期望的预防结果在所需的剂量和时间段内的有效量。通常但不一定是由于在疾病的早期阶段,甚至在暴露于感染风险升高的条件之前,在个体中使用预防剂量时,预防有效量可以小于治疗有效量。在一个实施方案中,预防有效量是至少有效减少、预防感染从一个细胞到另一个细胞的发生或扩散的量。

[0163] "慢性"给药是指以连续而不是急性模式施用药物,以便在较长时间内维持初始治疗效果(活性)。"间歇性"给药是并非不间断地连续进行的治疗,而是本质上是周期性的。

[0164] 术语"包装插入物"用于指通常包括在治疗产品的商业包装中的说明书,其包含关于使用这种治疗产品的适应症、用途、剂量、给药、组合疗法、禁忌症和/或警告的信息。

[0165] 术语"手性"是指具有镜像配偶体不重叠性质的分子,而术语"非手性"是指在其镜像配偶体上重叠的分子。

[0166] 术语"立体异构体"是指具有相同化学构成但空间中原子或基团的排列不同的化合物。

[0167] "非对映体"是指具有两个或更多个手性中心的立体异构体,其分子不是彼此的镜像。非对映体具有不同的物理性质,例如熔点,沸点,光谱性质和反应性。非对映异构体的混合物可以在高分辨率分析方法如电泳和色谱中分离。

[0168] "对映异构体"是指化合物的两种立体异构体,它们是彼此不重叠的镜像。

[0169] 本文使用的立体化学定义和惯例通常遵循S.P.Parker编辑,McGraw-Hill化学词典(McGraw-Hill Dictionary of Chemical Terms)(1984)McGraw-Hill Book Company,New York;和Eliel,E.和Wilen,S.,有机化合物的立体化学(Stereochemistry of Organic Compounds)(1994)John Wiley&Sons,Inc.,New York。许多有机化合物以光学活性形式存在,即它们具有旋转偏振光平面的能力。在描述光学活性化合物时,前缀D和L,或R和S用于表示分子关于其手性中心的绝对构型。使用前缀d和l或(+)和(-)来表示化合物对偏振光平面的旋转符号,其中(-)或l表示化合物是左旋的。以(+)或d为前缀的化合物是右旋的。对于给定的化学结构,这些立体异构体是相同的,除了它们是彼此的镜像。具体的立体异构体也可以称为对映异构体,这些异构体的混合物通常称为对映体混合物。将对映异构体的50:50

混合物称为外消旋混合物或外消旋物，其可能发生在化学反应或过程中不存在立体选择性或立体定向性的情况。术语“外消旋混合物”和“外消旋物”是指没有光学活性的两种对映体物质的等摩尔混合物。

[0170] 术语“保护基团”是指这样的取代基，其通常用于阻断或保护特定官能团，而其它官能团在化合物上起反应。例如，“氨基保护基”是连接到氨基上的取代基，该氨基阻断或保护化合物中的氨基官能团。合适的氨基保护基包括但不限于乙酰基、三氟乙酰基、叔丁氧羰基(BOC)、苄氧羰基(CBZ)和9-芴基亚甲氧基羰基(Fmoc)。关于保护基及其用途的一般描述，参见T.W.Greene，有机合成中的保护基(Protective Groups in Organic Synthesis)，John Wiley&Sons,New York,1991，或更新版本。

[0171] 本文所用的术语“约”是指本技术领域中的技术人员熟知的相应值的通常误差范围。在此引用“约”值或参数包括(特别描述)针对该值或参数本身的实施方案。

[0172] 如本文和所附权利要求中所使用，单数形式“一种”、“一个”和“该”包括复数参考，除非上下文另有明确指出。例如，提到“抗体”时是指从一个到多个抗体，例如以摩尔量，并且包括本领域技术人员已知的其等价物等。

[0173] III.组合物和方法

[0174] 抗生素-抗体缀合物(AAC)

[0175] 本文的实验结果强有力地表明，旨在消除细胞内细菌的疗法将提高临床成功率。为了实现这一目的，本发明提供了一种独特的治疗剂，其选择性地杀死已经侵入宿主细胞的细胞内区室的金黄色葡萄球菌。本发明证明这样的治疗剂在常规抗生素如万古霉素失败的体内模型中是有效的。

[0176] 本发明提供了一种抗菌治疗，其旨在通过靶向于逃避常规抗生素治疗的细菌群而防止抗生素逃避。该新型抗菌治疗是通过抗体抗生素缀合物(AAC)实现的，其中对在金黄色葡萄球菌(包括MRSA)上发现的细胞壁组分特异型的rF1抗体与有效的利福霉素型抗生素(利福霉素衍生物)化学连接。利福霉素型抗生素通过设计为可被蛋白酶切割的蛋白酶可切割的非肽类接头连接到抗体上，所述蛋白酶包括组织蛋白酶B，其是大多数哺乳动物细胞类型中发现的溶酶体蛋白酶(Dubowchik等人(2002)Bioconj.Chem.13:855-869)。图2中显示了具有3个组分的AAC的图。不受任何一种理论的限制，AAC的一种作用机制如图3所示。AAC作为前药，其中利福霉素型抗生素是无活性的(由于抗体的大尺寸)，直到接头被切割为止。由于在天然感染中发现的显著比例的金黄色葡萄球菌在宿主感染过程中的某一时刻被宿主细胞吸收，主要是嗜中性粒细胞和巨噬细胞，在宿主细胞内的时间为细菌提供了逃避抗生素活性的重要机会。本发明的AAC被设计为结合葡萄球菌细菌，并且在细菌被宿主细胞摄取后在吞噬体内部释放抗生素。通过这种机制，AAC能够将活性抗生素专门集中在通过常规抗生素治疗金黄色葡萄球菌不佳的地方。虽然本发明不受特定作用机制的限制或限定，但AAC通过三种潜在的机制改善抗生素活性：(1)AAC在摄取细菌的哺乳动物细胞内递送抗生素，从而增加难以扩散进入细菌隐藏的吞噬溶酶体中的抗生素的效力。(2)AAC调理细菌，从而增加吞噬细胞对游离细菌的吸收，并在局部释放抗生素以便当细菌在吞噬溶酶体中隐藏时杀死细菌。由于成千上万的AAC可以结合单个细菌，因此该平台在这些细胞内的小环境中释放出足够的抗生素，以维持最大的抗菌杀伤力。此外，随着越来越多的细菌从预先存在的细胞内储库中释放出来，这种基于抗体的治疗方法的快速结合率确保这些细菌立即被“标

记”，然后才能逃逸到相邻或远端的细胞，从而减轻感染的进一步扩散。(3)与从血清中快速清除的抗生素相比，AAC通过将抗生素与抗体连接来改善体内抗生素的半衰期(改善的药代动力学)。AAC的改善的药代动力学能够在金黄色葡萄球菌富集的区域中递送足够的抗生素，同时限制需要全身施用的抗生素的总体剂量。该属性将允许用AAC长期治疗以靶向于持续感染，且抗生素副作用最小。

[0177] 本发明的抗体-抗生素缀合化合物包含抗SDR抗体，其由重组引入的半胱氨酸通过蛋白酶可切割的非肽类接头共价连接到利福霉素型抗生素。

[0178] 在一个示例性实施方案中，抗SDR抗体(例如rF1抗体)是包含重组引入的半胱氨酸氨基酸的半胱氨酸改造的抗体。

[0179] 在一个示例性实施方案中，将蛋白酶可切割的非肽类接头通过所述rF1(抗SDR抗体)上重组引入的半胱氨酸共价连接到利福霉素型抗生素。

[0180] 示例性实施方案是具有下式的抗体-抗生素缀合物：

[0181] Ab-(PML-abx)$_p$

[0182] 其中：

[0183] Ab是rF1抗体；

[0184] PML是具有下式的蛋白酶可切割的非肽类接头：

[0185] -Str-PM-Y-

[0186] 其中Str是延伸单元；PM是拟肽单元，和Y是间隔单元；

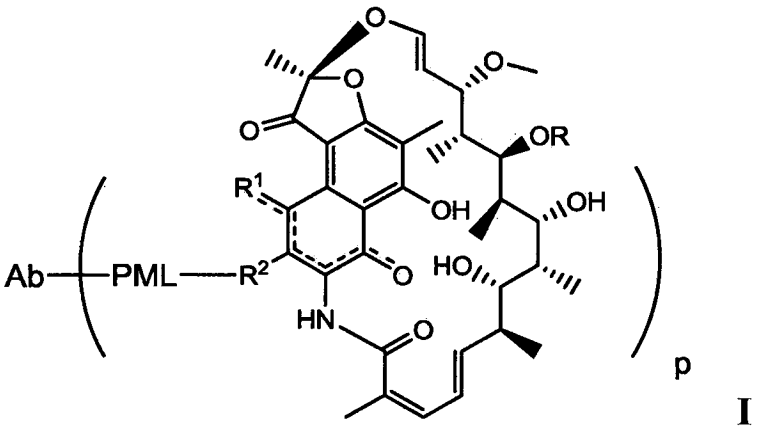
[0187] abx是利福霉素型抗生素；并且

[0188] p是从1到8的整数。

[0189] 利福霉素型抗生素可以是利福拉齐型抗生素。

[0190] 利福霉素型抗生素可以包含与蛋白酶可切割的非肽类接头连接的季胺。

[0191] 抗体-抗生素缀合物的示例性实施方案具有式I：

[0192] 

I

[0193] 其中：

[0194] 虚线表示任选的键；

[0195] R是H、C_1-C_{12}烷基或C(O)CH_3；

[0196] R^1是OH；

[0197] R^2是CH=N-(杂环基)，其中所述杂环基任选地被一个或多个独立地选自C(O)CH_3、C_1-C_{12}烷基、C_1-C_{12}杂芳基、C_2-C_{20}杂环基、C_6-C_{20}芳基和C_3-C_{12}碳环基的基团取代；

[0198] 或R^1和R^2形成五元或六元稠合杂芳基或杂环基，并且任选地形成螺或稠合的六元

杂芳基、杂环基、芳基或碳环基环，其中所述螺或稠合的六元杂芳基、杂环基、芳基或碳环基环任选地被H、F、Cl、Br、I、C_1-C_{12}烷基或OH取代；

[0199] PML是连接到R^2或由R^1和R^2形成的稠合杂芳基或杂环基的蛋白酶可切割的非肽类接头；并且

[0200] Ab是rF1抗体。

[0201] 抗生素部分可以通过反应性接头部分与抗体分子缀合，其中抗生素部分的数目可以通过本文所述的方法引入的游离半胱氨酸残基的数量来限制。示例性AAC包含具有1、2、3或4个工程化改造的半胱氨酸氨基酸的抗体(Lyon,R.等人(2012)Methods in Enzym.502:123-138)。

[0202] 为了有效瞄准MRSA，表位优选是高度丰富的、在感染期间稳定表达的、并且在所有临床MRSA菌株中高度保守的。rF1抗体可满足这些要求，另外也与表皮葡萄球菌结合。

[0203] 抗SDR和rF1抗体

[0204] 如下所述产生抗SDR抗体以生成F1抗体。本文提供了抗SDR抗体的几个实例，包括rF1、SD2、SD3和SD4。

[0205] 这里将详细描述rF1抗体。

[0206] rF1抗体是一种完全人类的，能够特异性结合葡萄球菌物种，如金黄色葡萄球菌和表皮葡萄球菌。重要的是，rF1能够在体内和体外结合整个细菌。此外，抗体rF1能够结合已经在例如动物的感染组织中生长的细菌。本文提供的rF1抗体或其功能等同物能够结合金黄色葡萄球菌表面蛋白ClfA、ClfB、SdrC、SdrD和SdrE。

[0207] 表4A和表4B显示亲本抗体F1、rF1抗体及其变体的H链和L链CDR序列的比对。F1和rF1在FW1和LC CDR3中的顺序不同(QHYXRFPYT，其中X可以是I或M(SEQ ID NO:26))；F1是I(SEQ ID NO:6)和rF1是M(SEQ ID NO:7))。

[0208] 表4A：重链CDR序列

[0209]

抗体	HC CDR1	HC CDR2	HC CDR3
F1	RFAMS (SEQ ID NO:1)	SINNGNNPYYARSVQY (SEQ ID NO: 2)	DHPSSGWPTFDS (SEQ ID NO: 3)
rF1	RFAMS (SEQ ID NO:1)	SINNGNNPYYARSVQY (SEQ ID NO: 2)	DHPSSGWPTFDS (SEQ ID NO: 3)
rF1.v1	RFAMS (SEQ ID NO:1)	SINSGNNPYYARSVQY (SEQ ID NO: 8)	DHPSSGWPTFDS (SEQ ID NO: 3)

[0210] 表4B:轻链CDR序列

[0211]

抗体	LC CDR1	LC CDR2	LC CDR3
F1	RASENVGDWLA (SEQ ID NO: 4)	KTSILES (SEQ ID NO:5)	QHYIRFPYT (SEQ ID NO:6)
rF1	RASENVGDWLA (SEQ ID NO: 4)	KTSILES (SEQ ID NO:5)	QHYMRFPYT (SEQ ID NO:7)
rF1.v6	RASENVGDWLA (SEQ ID NO: 4)	KTSILES (SEQ ID NO:5)	QHYIRFPYT (SEQ ID NO:6)

[0212] 在一个实施方案中，H和L链框架(FR)序列如下：

[0213] HC FW1 EVQLVESGGGLVQPGGSLRLSCAASGFTLS(SEQ ID NO.16)

[0214] HC FW2 WVRQAPGRGLEWVA(SEQ ID NO.17)

[0215] HC FW3 RFTVSRDVSQNTVSLQMNNLRAEDSATYFCAK(SEQ ID NO.18)

[0216] HC FW4 WGPGTLVTVSS(SEQ ID NO.19)

[0217] LC FW1 DIQLTQSPSALPASVGDRVSITC(SEQ ID NO.20)

[0218] LC FW2 WYRQKPGKAPNLLIY(SEQ ID NO.21)

[0219] LC FW3 GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC(SEQ ID NO.22)

[0220] LC FW4 FGQGTKVEIKRTV(SEQ ID NO.23)

[0221] 对rF1进行各种氨基酸修饰以改善稳定性和功能。在HC CDR2中，通过将第4残基N变为S来消除NG去酰胺化位点，从而提高抗体的稳定性。对LC骨架进行TV修复，以消除rF1中存在的严重抗体聚集。

[0222] 为了缀合形成本发明的治疗性AAC，可以进行H和L链的以下配对以形成全长四聚体抗体。框起来的是CDR1、CDR2、CDR3序列。引入的半胱氨酸(C)是以下划线标记的。粗体残基是相对于母体F1的氨基酸变化。在L链中，粗体的“RTV”之后的A是恒定区的第一个残基。H链中Kabat位置114处的带下划线的C开始恒定区。

[0223] 在1A和2A中，SEQ ID NO.9的全长(FL)L链具有在C kappa结尾附近aa 205处工程化改造的Cys，与SEQ ID NO.10(无Cys)的FL IgG1 H链配对。该抗体将具有2个Cys位点，每个L链上有一个位点，用于与接头-抗生素单元缀合以形成AAC。

[0224] 1A.rF1-V205C FL轻链

[0225]

DIQLTQSPSALPASVGDRVSITCRASENVGDWLAWYRQKPGKAPNLLIYKTSILESGVPSRFSG
SGSGTEFTLTISSLQPDDFATYYCQHYMRFPYTFGQGTK**V**EIK**RTV**AAPSVFIFPPSDEQLKSG
TASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVY
ACEVTHQGLSSPCTKSFNRGEC (SEQ ID NO.9)

[0226] 2A.rF1.v1 FL重链(无Cys),配对带有Cys205的rF1-V205C轻链

[0227]

EVQL**V**ESGGGLVQPGGSLRLSCAASGFTLSRFAMSWVRQAPGRGLEWVASIN**S**GNNPYYARSVQ
YRFTVSRDVSQNTVSLQMNNLRAEDSATYFCAKDHPSSGWPTFDSWGPGTLVTVSSASTKGPSV
FPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMI
SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
G (SEQ ID NO: 10)

[0228] 在1B与2A中,具有工程改造的Cys 205的SEQ ID NO.11的rF1.v6L链与SEQ ID NO.10(无Cys)的FL IgG1H链配对。该抗体将具有2个Cys位点,每个L链上有一个位点,用于与接头-抗生素单元的缀合。

[0229] 1B.rF1.v6-V205C轻链

[0230]

DIQLTQSPSALPASVGDRVSITCRASENVGDWLAWYRQKPGKAPNLLIYKTSILESGVPSRFSG
SGSGTEFTLTISSLQPDDFATYYCQHYIRFPYTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSG
TASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVY
ACEVTHQGLSSPCTKSFNRGEC (FL SEQ ID NO. 11)

[0231] 在1B与2B中,L和H链各自具有工程化改造的Cys,因此该四聚体抗体可以具有高达4的AAR(抗生素:抗体比例)。

[0232] 2B.具有Cys114(114 Kabat编号或118-Eu编号)的rF1.v1 FL重链

[0233]

EVQLVESGGGLVQPGGSLRLSCAASGFTLSRFAMSWVRQAPGRGLEWVASINSGNNPYYARSVQYRFTVSRDVSQNT
VSLQMNNLRAEDSATYFCAKDHPSSGWPTFDSWGPGTLVTVSSCSTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFP
EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPP
CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV
LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE
SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG(SEQ ID
NO.12)

[0234] rF1.v1 H链可变区

[0235]

EVQL**V**ESGGGLVQPGGSLRLSCAASGFTLSRFAMSWVRQAPGRGLEWVASIN**S**GNNPYY ARSVQYRFTVSRDVSQNTVSLQMNNLRAEDSATYFCAKDHPSSGWPTFDSWGPGTLVTVSS (SEQ ID NO. 13)

[0236] rF1 L链可变区

[0237]

DIQLTQSPSALPASVGDRVSITCRASENVGDWLAWYRQKPGKAPNLLIYKTSILESGVP SRFSGSGSGTEFTLTISSLQPDDFATYYCQHYMRFPYTFGQGTKVEIKRTV (SEQ ID NO. 14)

[0238] rF1.v6 L链可变区

[0239]

DIQLTQSPSALPASVGDRVSITCRASENVGDWLAWYRQKPGKAPNLLIYKTSILESGVP SRFSGSGSGTEFTLTISSLQPDDFATYYCQHYIRFPYTFGQGTKVEIKRTV (SEQ ID NO. 15)

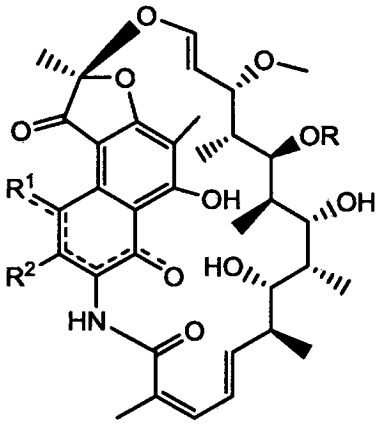
[0240] 包括rF1的抗SDR抗体可以包含非半胱氨酸的至少一个氨基酸被半胱氨酸取代。在一些实施方案中，非半胱氨酸的至少一个氨基酸在轻链位置205处为缬氨酸，和/或在轻链位置110处为缬氨酸，和/或在重链位置114处为丙氨酸，其中氨基酸编号是根据Kabat (1991)，其与根据Eu编号公约的位置118相同。

[0241] 利福霉素型抗生素

[0242] 本发明的抗体-抗生素缀合物(AAC)的抗生素部分(abx)是具有细胞毒性或细胞抑制作用的利福霉素型抗生素或基团。利福霉素是由细菌地中海诺卡氏菌(Nocardia mediterranei)、地中海拟无枝酸菌(Amycolatopsis mediterranei)或人工获得的一组抗生素。它们是抑制细菌RNA聚合酶的较大的安莎霉素家族的亚类(Fujii等人(1995) Antimicrob.Agents Chemother.39:1489-1492;Feklistov等人(2008)Proc Natl Acad Sci USA,105(39):14820-5)，并且对革兰氏阳性菌和选择性革兰氏阴性菌具有效力。利福霉素对分枝杆菌特别有效，且因此用于治疗结核病、麻风病和鸟分枝杆菌复合体(MAC)感染。利福霉素型群组包括“经典的”利福霉素药物以及利福霉素衍生物利福平(rifampicin)(利福平(rifampin)，CA登记号13292-46-1)、利福布汀(CA登记号72559-06-9;US 2011/0178001)，利福喷汀和利福拉齐(CA登记号129791-92-0，Rothstein等人(2003)Expert Opin.Investig.Drugs 12(2):255-271;Fujii等人(1994)Antimicrob.Agents Chemother 38:1118-1122)。许多利福霉素型抗生素都具有抗发育的有害性质(Wichelhaus等人(2001) J.Antimicrob.Chemother.47:153-156)。在1957年首先从地中海链霉菌(Streptomyces mediterranei)的发酵培养物中分离出利福霉素。发现约七种利福霉素，称为利福霉素A、B、C、D、E、S和SV(US 3150046)。利福霉素B是商业上首次引入的，且在二十世纪六十年代可用于治疗耐药性结核病。利福霉素已被用于治疗许多疾病，最重要的是HIV相关性结核病。由于大量可用的类似物和衍生物，利福霉素已广泛用于消除已经变得对常用抗生素具有抗性的致病菌。例如，利福平以其有效作用和预防耐药性的能力而闻名。它迅速杀死快速分裂的杆菌菌株以及“耐药株”细胞，所述细胞长时间保持生物无活性来使其逃避抗生素活性。此

外，利福布汀和利福喷汀均已被用于HIV阳性患者的获得性结核病。

[0243] 式I的抗体-抗生素缀合物的抗生素部分(abx)是具有以下结构的利福霉素型部分：

[0244] 

[0245] 其中：

[0246] 虚线表示任选的键；

[0247] R是H、C_1-C_{12}烷基或C(O)CH_3；

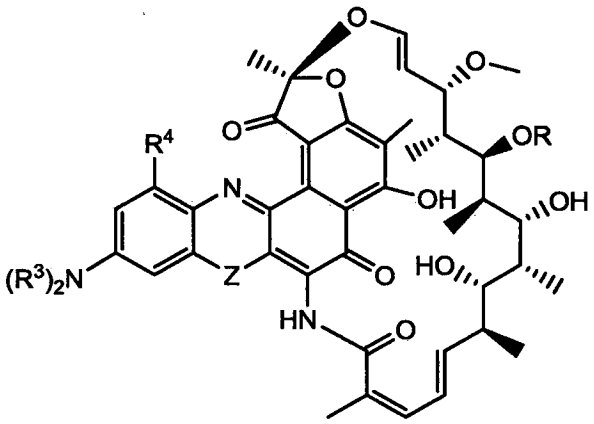
[0248] R^1是OH；

[0249] R^2是CH=N-(杂环基)，其中所述杂环基任选地被一个或多个独立地选自C(O)CH_3、C_1-C_{12}烷基、C_1-C_{12}杂芳基、C_2-C_{20}杂环基、C_6-C_{20}芳基和C_3-C_{12}碳环基的基团取代；

[0250] 或R^1和R^2形成五元或六元稠合杂芳基或杂环基，并且任选地形成螺或稠合的六元杂芳基、杂环基、芳基或碳环基环，其中所述螺或稠合的六元杂芳基、杂环基、芳基或碳环基环任选地被H、F、Cl、Br、I、C_1-C_{12}烷基或OH取代；并且

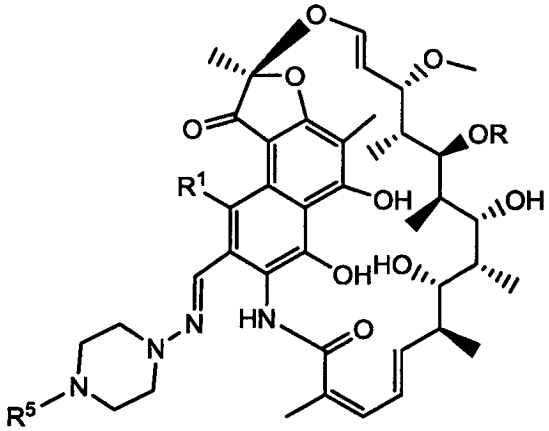
[0251] 其中非肽类接头PML共价连接到R^2上。

[0252] 利福霉素型部分的实施方案是：

[0253] 

[0254] 其中R^3独立地选自H和C_1-C_{12}烷基；R^4选自H、F、Cl、Br、I、C_1-C_{12}烷基和OH；且Z选自NH、N(C_1-C_{12}烷基)、O和S；并且其中非肽类接头PML共价连接到N(R^3)$_2$的氮原子上。

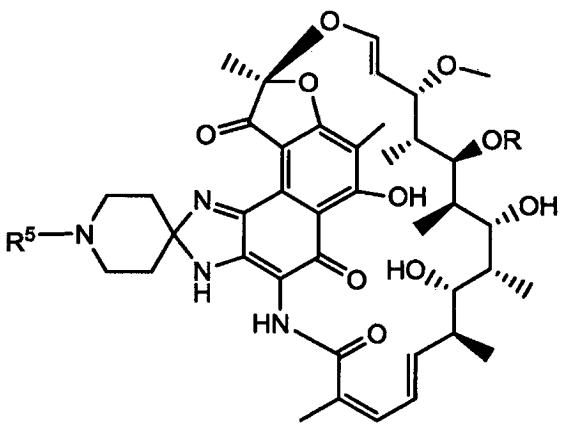
[0255] 利福平型部分的实施方案是：

[0256] 

[0257] 其中，

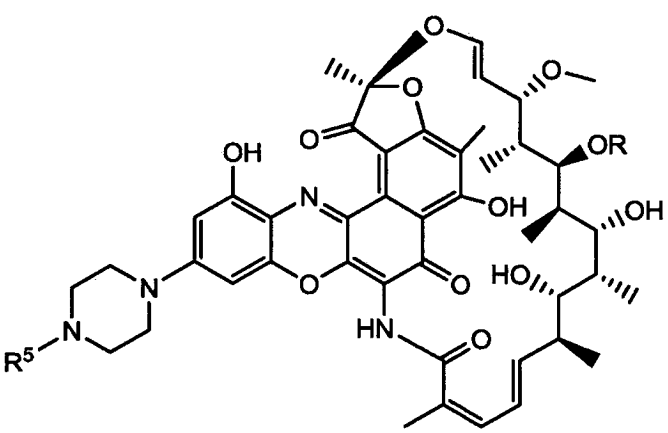
[0258] R^5选自H和C_1-C_{12}烷基；并且其中非肽类接头PML共价连接到NR^5的氮原子上。

[0259] 利福布汀型部分的实施方案是：

[0260] 

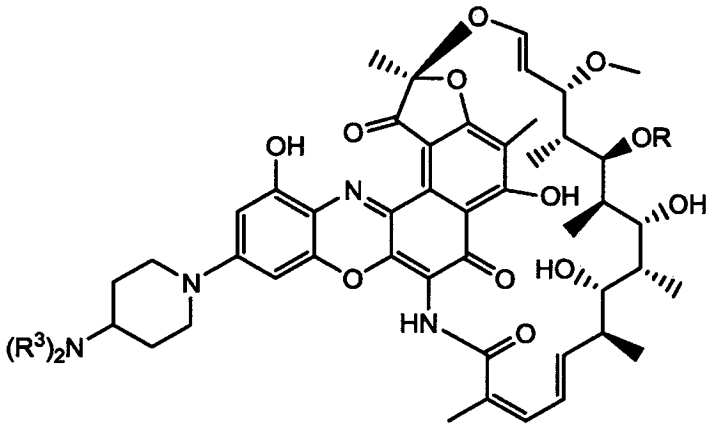
[0261] 其中R^5选自H和C_1-C_{12}烷基；并且其中非肽类接头PML共价连接到NR^5的氮原子上。

[0262] 苯并噁嗪并利福霉素型部分的实施方案是：

[0263] 

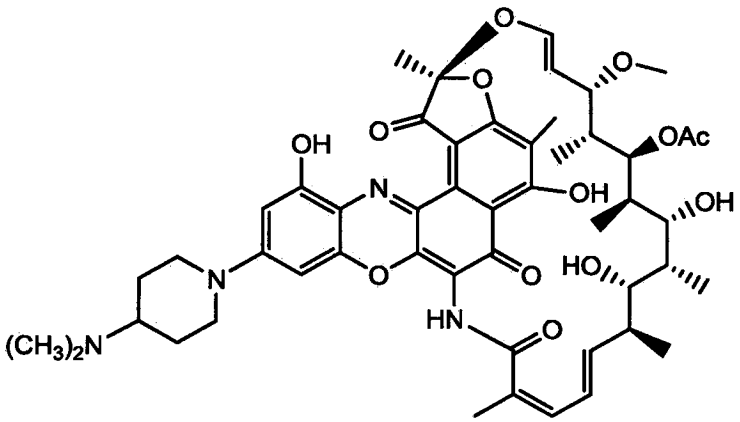
[0264] 其中R^5选自H和C_1-C_{12}烷基；并且其中非肽类接头PML共价连接到NR^5的氮原子上。

[0265] 本文称为pipBOR的苯并噁嗪并利福霉素型部分的实施方案是：

[0266] 

[0267] 其中R^3独立地选自H和C_1-C_{12}烷基；并且其中非肽类接头PML共价连接到$N(R^3)_2$的氮原子上。

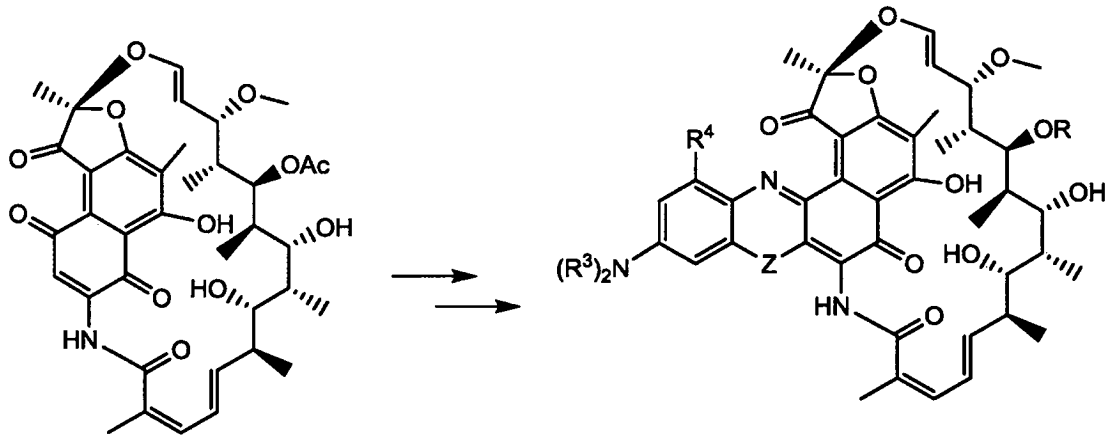
[0268] 本文称为二甲基pipBOR的苯并噁嗪并利福霉素型部分的实施方案是：

[0269] 

[0270] 其中非肽类接头PML共价连接到$N(CH_3)_2$的氮原子上。

[0271] 半合成衍生物利福霉素S，或还原的钠盐型利福霉素SV可以以几个步骤转化为利福拉齐型抗生素，其中R为H或Ac，R^3独立地选自H和C_1-C_{12}烷基；R^4选自H、F、Cl、Br、I、C_1-C_{12}烷基和OH；并且Z选自NH、N(C_1-C_{12}烷基)、O和S(参见，例如WO 2014/194247中的图23A和B，以及图25A和B)。可制备苯并噁嗪并(Z=O)、苯并噻嗪并(Z=S)、苯并二嗪并(Z=NH、N(C_1-C_{12})烷基)利福霉素(US 7271165)。含有取代基的苯并噁嗪并利福霉素(BOR)、苯并噻嗪并利福霉素(BTR)和苯并二嗪并利福霉素(BDR)类似物根据US 7271165第28栏式A中提供的编号方案编号，其通过引用并入本文。“25-O-脱乙酰基”利福霉素是指利福霉素类似物，其中25位的乙酰基已被除去。在该位置进一步衍生化的类似物称为“25-O-脱乙酰基-25-(取代基)利福霉素”，其中在完整化合物名称中用衍生基团的命名替换“取代基”。

[0272] 利福霉素型抗生素部分可以通过在US 4610919；US 4983602；US 5786349；US5981522；US 4859661；US 7271165；US 2011/0178001；Seligson等人，(2001)抗癌药物(Anti-Cancer Drugs)12:305-13；Chem.Pharm.Bull.，(1993)41:148中和在WO 2014/194247中公开的类似方法合成，它们各自通过引用并入本文。通过使用标准的MIC体外测定，通过测量其最小抑菌浓度(MIC)筛选利福霉素型抗生素部分的抗微生物活性(Tomioka等人，(1993)Antimicrob.Agents Chemother.37:67)。

[0273] 

利福霉素-S 苯并噁嗪并利福霉素

[0274] 蛋白酶可切割的非肽类接头

[0275] “蛋白酶可切割的非肽类接头”(PML)是双官能或多官能部分，其共价连接到一个或多个抗生素部分(abx)和抗体单元(Ab)以形成式I的抗体-抗生素缀合物(AAC)。AAC中的蛋白酶可切割的非肽类接头是用于细胞内蛋白酶切割的底物，包括在溶酶体条件下。蛋白酶包括各种组织蛋白酶和半胱天冬酶。细胞内AAC的非肽类接头的切割可以释放具有抗菌作用的利福霉素型抗生素。

[0276] 可以使用具有与抗生素(abx)和抗体(Ab)结合的反应官能性的接头试剂或接头-抗生素中间体，方便地制备抗体-抗生素缀合物(AAC)。在一个示例性实施方案中，半胱氨酸工程化改造的抗体(Ab)的半胱氨酸硫醇可以与接头试剂，抗生素部分或抗生素-接头中间体的官能团形成键。

[0277] AAC的PML部分可以包含一个氨基酸残基。

[0278] AAC的PML部分包含拟肽单元。

[0279] 一方面，接头试剂或接头-抗生素中间体具有反应性位点，其具有对存在于抗体上的亲核半胱氨酸具有反应性的亲电子基团。抗体的半胱氨酸硫醇与接头试剂或接头抗生素上的亲电子基团反应，形成共价键。有用的亲电子基团包括但不限于马来酰亚胺和卤代乙酰胺基团。

[0280] 根据Klussman等人(2004)，Bioconjugate Chemistry 15(4):765-773第766页的缀合方法，且根据实施例18的方法，半胱氨酸改造的抗体与接头试剂或接头-抗生素中间体，与亲电子官能团如马来酰亚胺或α-卤代羰基反应。

[0281] 在另一个实施方案中，接头试剂或接头-抗生素中间体的反应性基团含有可与抗体的游离半胱氨酸硫醇形成键的硫醇反应性官能团。硫醇反应官能团的实例包括但不限于马来酰亚胺、α-卤代乙酰基，活化酯如琥珀酰亚胺酯、4-硝基苯基酯、五氟苯基酯、四氟苯基酯、酸酐、酰氯、磺酰氯、异氰酸酯和异硫氰酸酯。

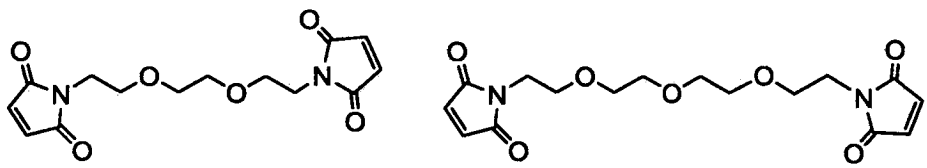
[0282] 在另一个实施方案中，接头试剂或抗生素-接头中间体具有反应性官能团，其具有对存在于抗体上的亲电子基团具有反应性的亲核基团。抗体上有用的亲电子基团包括但不限于吡啶基二硫化物、醛和酮羰基。接头试剂或抗生素-接头中间体的亲核基团的杂原子可以与抗体上的亲电子基团反应，并与抗体单元形成共价键。接头试剂或抗生素-接头中间体上有用的亲核基团包括但不限于酰肼、肟、氨基、硫醇、肼、缩氨基硫脲、肼羧酸酯和芳基酰

肼。抗体上的亲电子基团提供了用于连接到接头试剂或抗生素-接头中间体的方便位点。

[0283] PML部分可以包含一个或多个接头组分。示例性的接头组分包括单个氨基酸，例如瓜氨酸("cit")，6-马来酰亚胺基己酰基("MC")，马来酰亚胺基丙酰基("MP")和对氨基苄氧基羰基("PAB")，N-琥珀酰亚胺基4-(2-吡啶基硫基)戊酸酯("SPP")和4-(N-马来酰亚胺基甲基)环己烷-1-羧酸酯("MCC")。各种接头组分是本领域已知的，其中一些在下面描述。

[0284] 在另一个实施方案中，接头可以被调节溶解度或反应性的基团取代。例如，带电荷的取代基如磺酸根(-SO_3-)或铵可增加试剂的水溶性，且促进接头试剂与抗体或抗生素部分的偶联反应，或促进Ab-L(抗体-接头中间体)与abx或abx-L(抗生素-接头中间体)与Ab的偶联反应，这取决于用于制备AAC的合成途径。

[0285] 本发明的AAC明确地考虑但不限于用以下接头试剂制备的那些：BMPEO、BMPS、EMCS、GMBS、HBVS、LC-SMCC、MBS、MPBH、SBAP、SIA、SIAB、SMCC、SMPB、SMPH、磺基-EMPS、磺基-GMBS、磺基-KMOS、磺基-MBS、磺基-SIAB、磺基-SMCC、磺基-SMPB、SVSB(琥珀酰亚胺基-(4-乙烯基砜)苯甲酸酯)和双马来酰亚胺试剂如DTME、BMB、BMDB、BMH、BMOE、BM(PEG)$_2$和BM(PEG)$_3$。双马来酰亚胺试剂允许半胱氨酸工程化改造的抗体的硫醇基团以顺序或汇合的方式连接到含硫醇的抗生素部分、标记物或接头中间体。除马来酰亚胺之外，与半胱氨酸工程化改造的抗体、抗生素部分或接头-抗生素中间体的巯基反应的其它官能团包括碘乙酰胺、溴乙酰胺、乙烯基吡啶、二硫化物、吡啶基二硫化物、异氰酸酯和异硫氰酸酯。

[0286] 

BM(PEG)$_2$ BM(PEG)$_3$

[0287] 还可以通过其他商业来源，例如Molecular Biosciences Inc.(Boulder,CO)获得有用的接头试剂，或根据Toki等人(2002)J.Org.Chem.67:1866-1872;Dubowchik等人(1997)Tetrahedron Letters,38:5257-60;Walker,M.A.(1995)J.Org.Chem.60:5352-5355;Frisch等人(1996)Bioconjugate Chem.7:180-186;US 6214345;WO 02/088172;US 2003130189;US2003096743;WO 03/026577;WO 03/043583;和WO 04/032828中描述的方法合成。

[0288] 在另一个实施方案中，AAC的PML部分包含树突状接头，用于将一个以上的抗生素部分通过支链多官能接头部分共价连接至抗体上(Sun等人(2002)生物有机&药物化学通讯(Bioorganic&Medicinal Chemistry Letters)12:2215;Sun等人(2003)生物有机&药物化学(Bioorganic&Medicinal Chemistry)11:1761-1768)。树突状接头可以增加抗生素与抗体的摩尔比，即负载，其与AAC的效力有关。因此，当半胱氨酸改造的抗体仅具有一个反应性半胱氨酸硫醇基团时，多个抗生素部分可以通过树枝状接头连接。

[0289] 在式I AAC的某些实施方案中，蛋白酶可切割的非肽类接头PML具有下式：

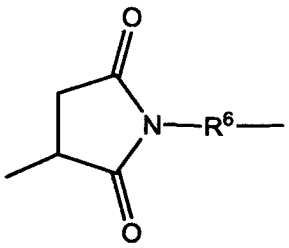
[0290] -Str-PM-Y-

[0291] 其中Str是延伸单元；PM是拟肽单元，和Y是间隔单元；

[0292] abx是利福霉素型抗生素；并且

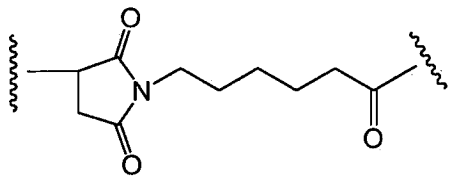
[0293] p是从1到8的整数。

[0294] 在一个实施方案中，延伸单元“Str”具有下式：

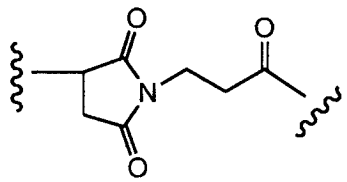
[0295] 

[0296] 其中R^6选自C_1-C_{12}亚烷基、C_1-C_{12}亚烷基-C(=O)、C_1-C_{12}亚烷基-NH、$(CH_2CH_2O)_r$、$(CH_2CH_2O)_r$-C(=O)、$(CH_2CH_2O)_r$-CH_2、和C_1-C_{12}亚烷基-NHC(=O)CH_2CH(噻吩-3-基)，其中r是1至10的整数。

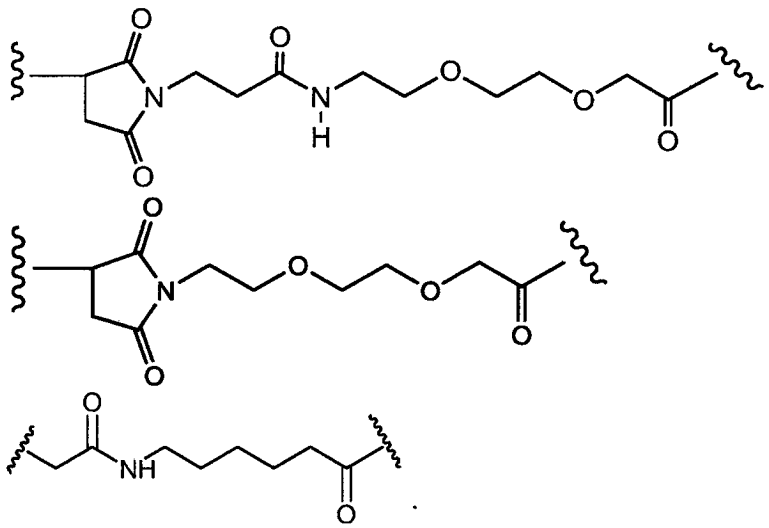
[0297] 示例性的延伸单元如下所示(其中波浪线表示与抗体共价连接的位点)：



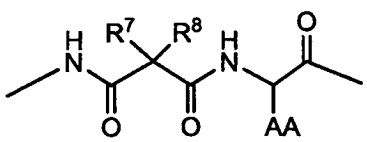
MC



MP

[0298] 

[0299] 在一个实施方案中，PM具有下式：

[0300] 

[0301] 其中R^7和R^8一起形成C_3-C_7环烷基环，和

[0302] AA是选自H、-CH_3、-CH_2(C_6H_5)、-$CH_2CH_2CH_2CH_2NH_2$、-$CH_2CH_2CH_2NHC(NH)NH_2$、-CHCH(CH_3)CH_3、和-$CH_2CH_2CH_2NHC(O)NH_2$的氨基酸侧链。

[0303] 在一个实施方案中，间隔单元Y包含对氨基苄基(PAB)或对氨基苄氧基羰基(PABC)。

[0304] 间隔单元允许释放抗生素部分而没有单独的水解步骤。间隔单元可以是“自发性的(self-immolative)”或“非自发性的”。在某些实施方案中，接头的间隔单元包含对氨基苄基单元(PAB)。在一个这样的实施方案中，对氨基苄醇通过对氨基苄基基团和抗生素部分之间的酰胺键，氨基甲酸酯，甲基氨基甲酸酯或碳酸酯连接到氨基酸单元上(Hamann等人(2005)Expert Opin.Ther.Patents(2005)15:1087-1103)。在一个实施方案中，间隔单元是对氨基苄氧基羰基(PAB)。

[0305] 在一个实施方案中，当与非肽类接头的PAB间隔单元连接时，抗生素包含季胺，例如二甲基氨基哌啶基基团。这种季胺接头-抗生素中间体(PLA)的实例是来自表2的PLA-1至4。季胺基团可调节抗生素部分的切割以优化AAC的抗菌作用。在另一个实施方案中，抗生素与非肽类接头的PABC间隔单元连接，在AAC中形成氨基甲酸酯官能团。这样的氨基甲酸酯官能团也可以优化AAC的抗菌作用。PABC氨基甲酸酯接头-抗生素中间体(PLA)的实例是来自表2的PLA-5和PLA-6。

[0306] 自发性间隔物的其它实例包括但不限于与PAB基团电子性相似的芳族化合物，例如2-氨基咪唑-5-甲醇衍生物(US7375078;Hay等人(1999)Bioorg.Med.Chem.Lett.9:2237)和邻-或对-氨基苄基缩醛。可以使用在酰胺键水解后进行环化的间隔物，例如取代和未取代的4-氨基丁酸酰胺(Rodrigues等人(1995)Chemistry Biology 2:223)，适当取代的双环[2.2.1]和双环[2.2.2]环系统(Storm等人(1972)J.Amer.Chem.Soc.94:5815)和2-氨基苯基丙酸酰胺(Amsberry等人(1990)J.Org.Chem.55:5867)。在甘氨酸处被取代的含胺药物的消除(Kingsbury等人(1984)J.Med.Chem.27:1447)也是在AAC中有用的自发性间隔物的示例。

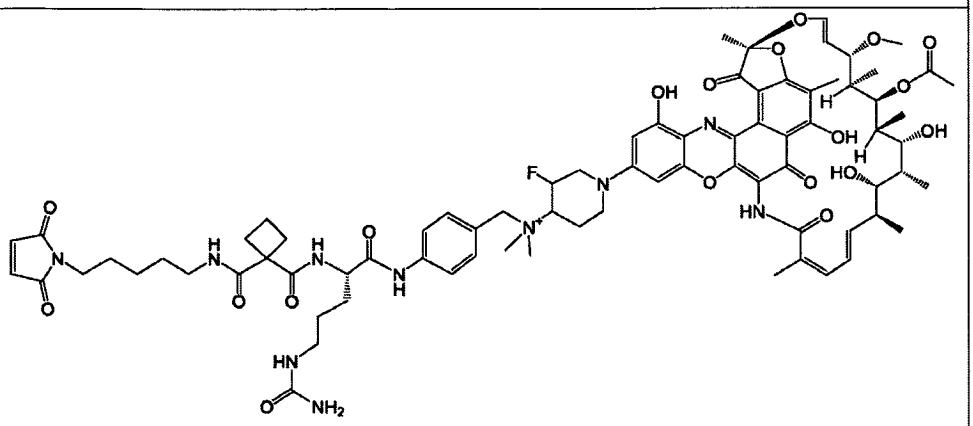
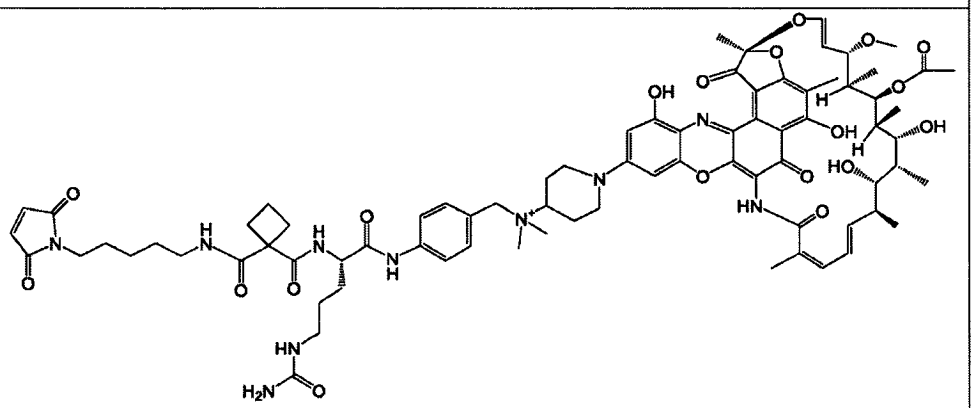
[0307] 从AAC切割释放的活性抗生素的量可以通过半胱天冬酶释放试验来测量。

[0308] 对AAC有用的接头-抗生素中间体

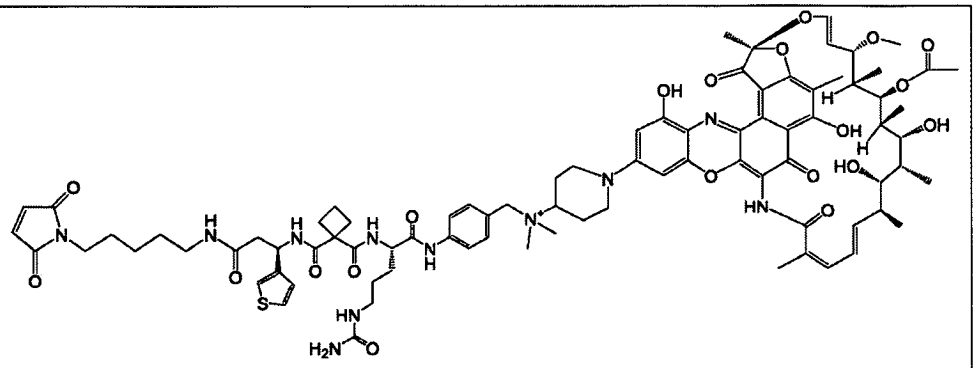
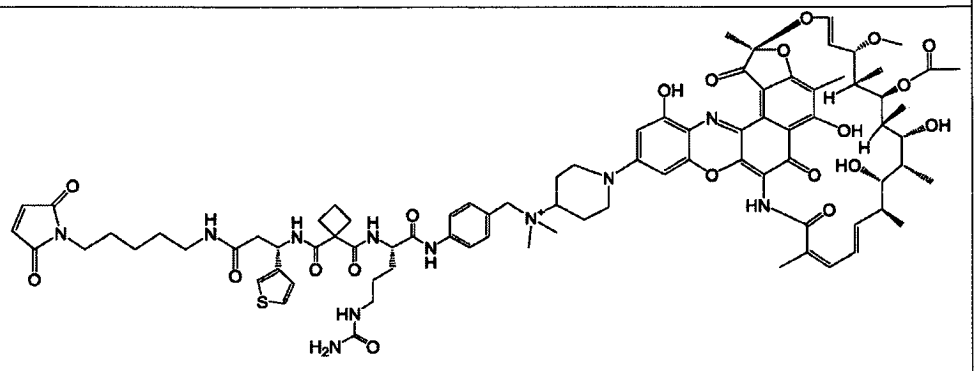
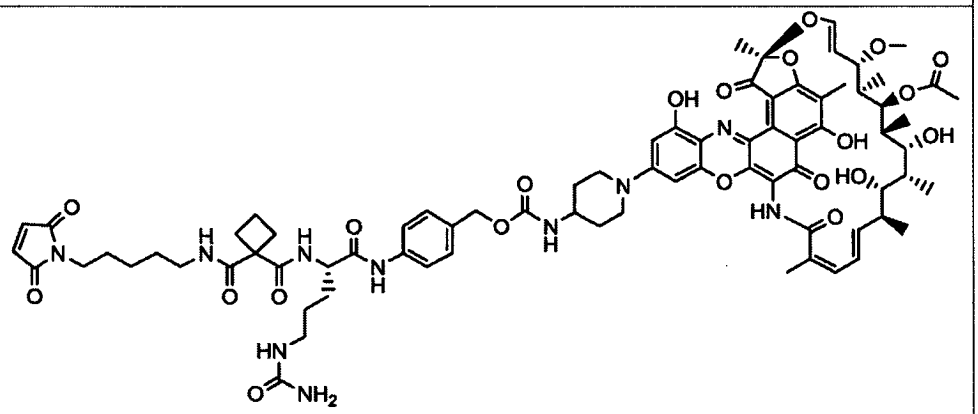
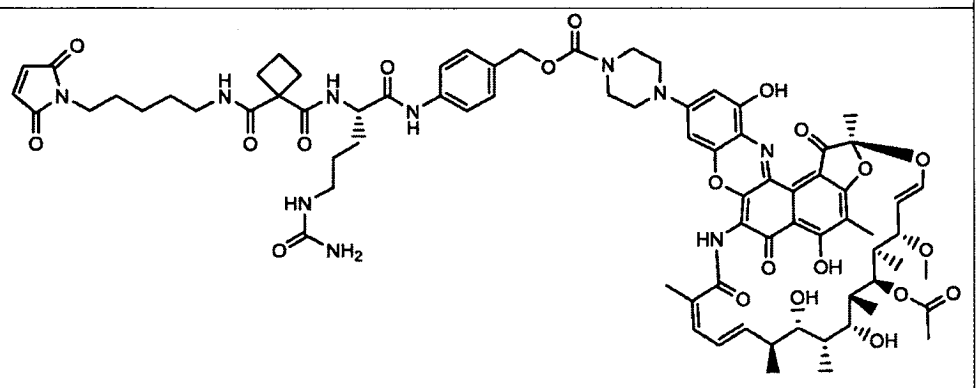
[0309] 实施例7-17通过将利福霉素型抗生素部分与接头试剂偶联制备式II和表2的PML接头-抗生素中间体(PLA)。接头试剂通过WO 2012/113847;US 7659241;US 7498298;US 20090111756;US 2009/0018086;US 6214345;Dubowchik等人(2002)Bioconjugate Chem.13(4):855-869中描述的方法制备。

[0310] 表2 PML接头-抗生素中间体

[0311]

LA 编号	结构
PLA-1	
PLA-2	

[0312]

PLA-3	
PLA-4	
PLA-5	
PLA-6	

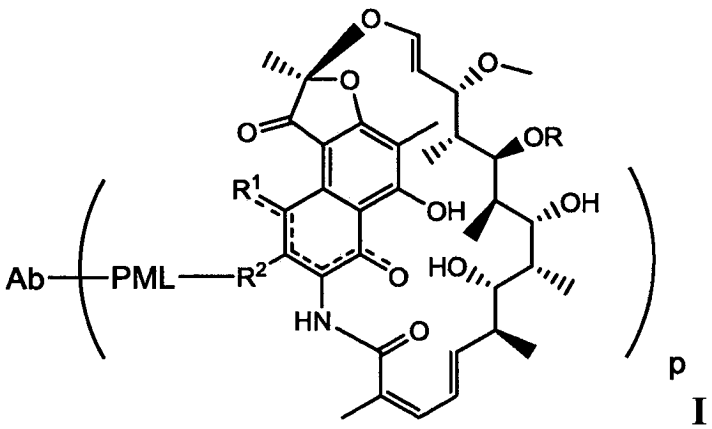
[0313] 抗体-抗生素缀合物的实施方案

[0314] 将半胱氨酸改造的rF1抗体通过游离半胱氨酸硫醇基团与利福霉素的衍生物(称为pipBOR等)经蛋白酶可切割的非肽类接头连接,形成表3中的抗体-抗生素缀合物化合物(AAC)。接头设计为被包括组织蛋白酶B、D等的溶酶体蛋白酶切割。实施例7-17详细描述了由抗生素和PML接头等组成的接头-抗生素中间体的产生。设计接头,使得在PAB部分处的酰胺键的切割将抗体与活性状态的抗生素分离。

[0315] 命名为"二甲基pipBOR"的AAC与"pipBOR"AAC相同,除了抗生素上的二甲基化的氨基和接头上的氧羰基。

[0316] 图3显示抗体-抗生素缀合物(AAC)的药物活化的可能机制。活性抗生素(Ab)仅在AAC在哺乳动物细胞内内化后释放。AAC中抗体的Fab部分结合金黄色葡萄球菌,而AAC的Fc部分通过Fc受体介导的结合吞噬细胞(包括嗜中性粒细胞和巨噬细胞)增强细菌摄取。在内化到吞噬溶酶体中后,接头可以通过溶解体蛋白酶裂解,释放吞噬溶酶体内部的活性抗生素。

[0317] 本发明的抗体-抗生素缀合物(AAC)化合物的一个实施方案包括式I:

[0318] 

I

[0319] 其中:

[0320] 虚线表示任选的键;

[0321] R是H、C_1-C_{12}烷基或C(O)CH_3;

[0322] R^1是OH;

[0323] R^2是CH=N-(杂环基),其中所述杂环基任选地被一个或多个独立地选自C(O)CH_3、C_1-C_{12}烷基、C_1-C_{12}杂芳基、C_2-C_{20}杂环基、C_6-C_{20}芳基和C_3-C_{12}碳环基的基团取代;

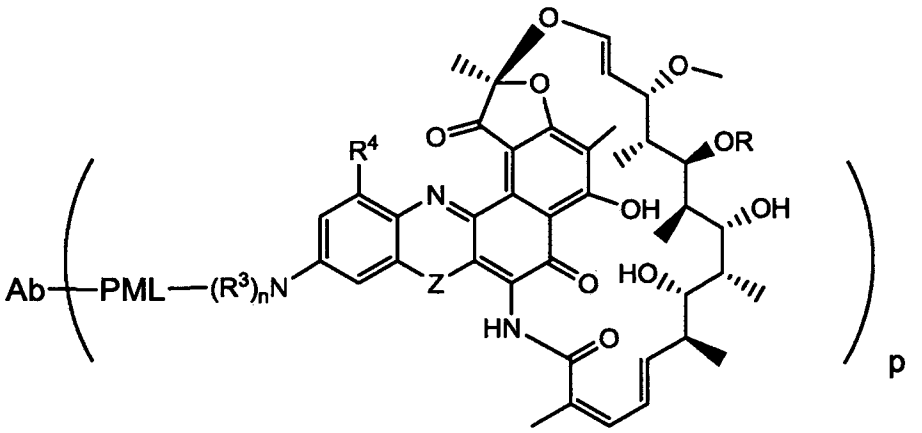
[0324] 或R^1和R^2形成五元或六元稠合杂芳基或杂环基,并且任选地形成螺或稠合的六元杂芳基、杂环基、芳基或碳环基环,其中所述螺或稠合的六元杂芳基、杂环基、芳基或碳环基环任选地被H、F、Cl、Br、I、C_1-C_{12}烷基或OH取代;

[0325] PML是连接到R^2或由R^1和R^2形成的稠合杂芳基或杂环基的蛋白酶可切割的非肽类接头;

[0326] Ab是rF1抗体;并且

[0327] p是从1到8的整数。

[0328] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式:

[0329] 

[0330] 其中，

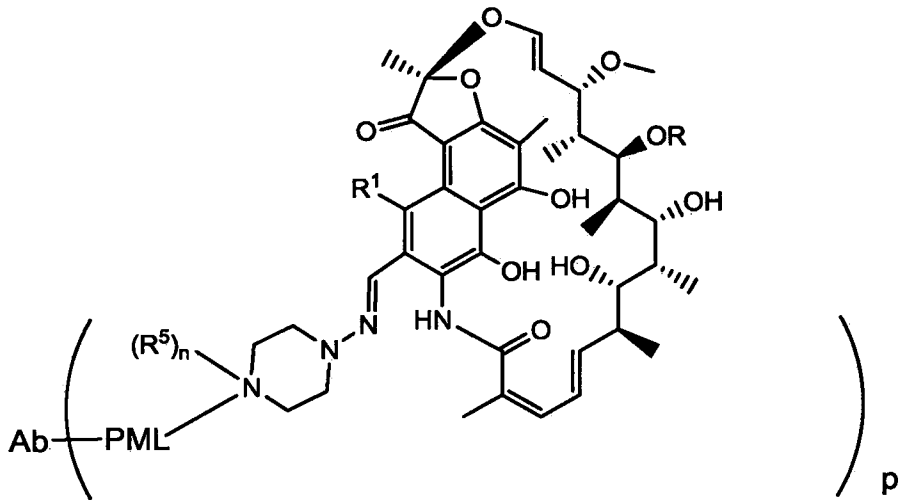
[0331] R^3独立地选自H和C_1-C_{12}烷基；

[0332] n为1或2；

[0333] R^4选自H、F、Cl、Br、I、C_1-C_{12}烷基和OH；和

[0334] Z选自NH、N(C_1-C_{12}烷基)、O和S。

[0335] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

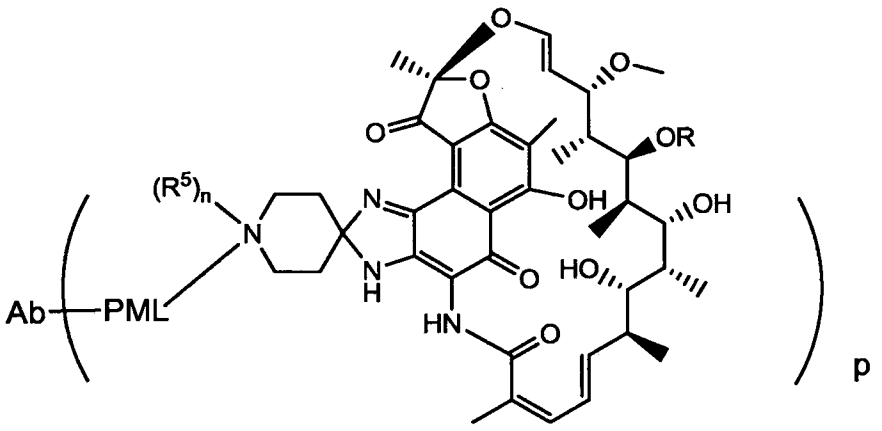
[0336] 

[0337] 其中，

[0338] R^5选自H和C_1-C_{12}烷基；和

[0339] n为0或1。

[0340] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

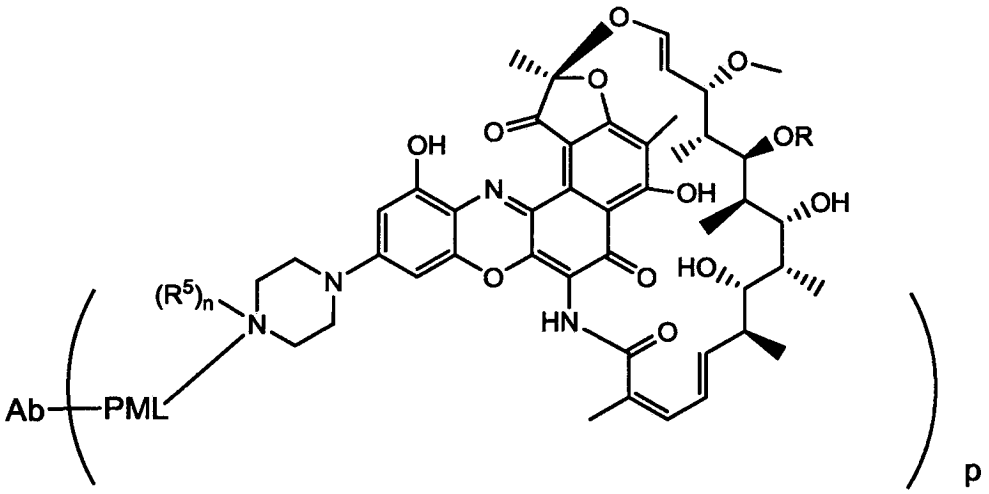
[0341] 

[0342] 其中，

[0343] R^5选自H和C_1-C_{12}烷基；和

[0344] n为0或1。

[0345] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

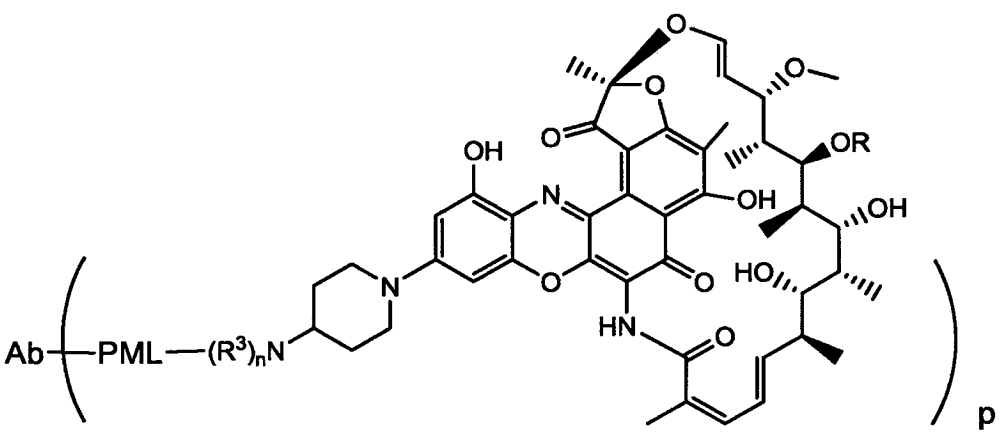
[0346] 

[0347] 其中，

[0348] R^5选自H和C_1-C_{12}烷基；和

[0349] n为0或1。

[0350] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

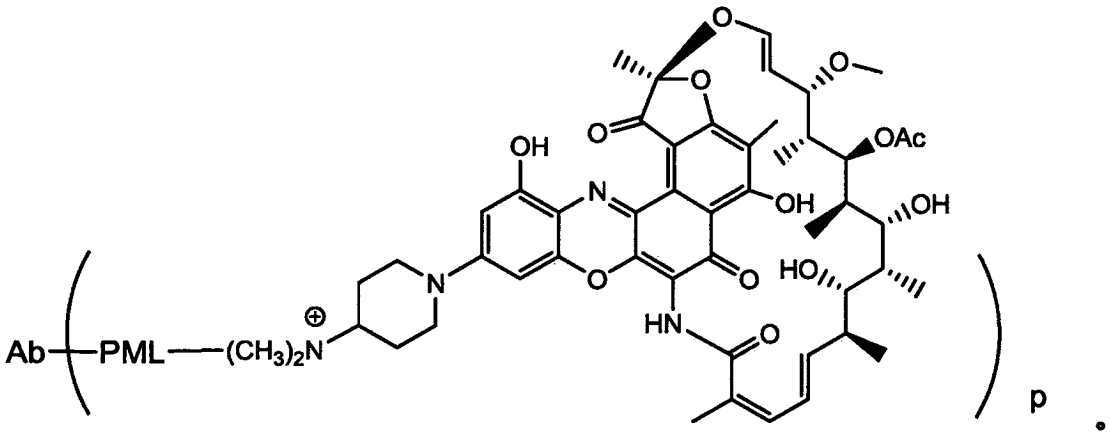
[0351] 

[0352] 其中，

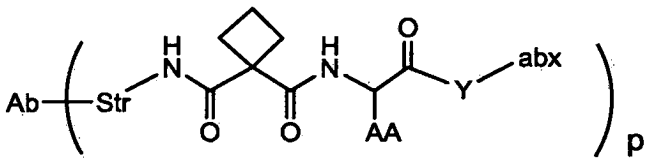
[0353] R^3独立地选自H和C_1-C_{12}烷基；和

[0354] n为1或2。

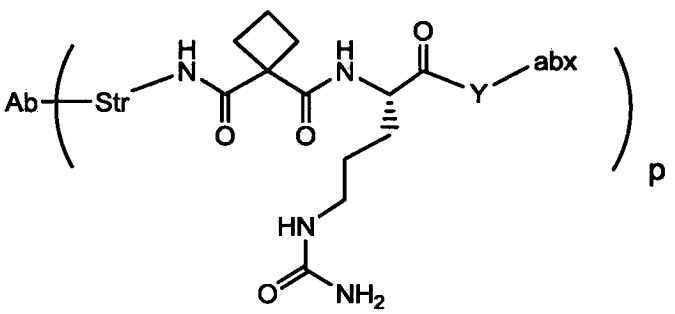
[0355] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

[0356] 

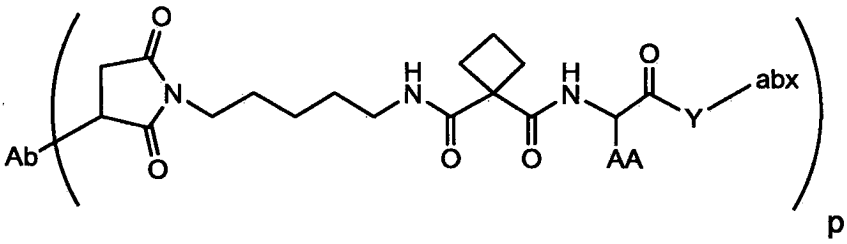
[0357] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

[0358] 

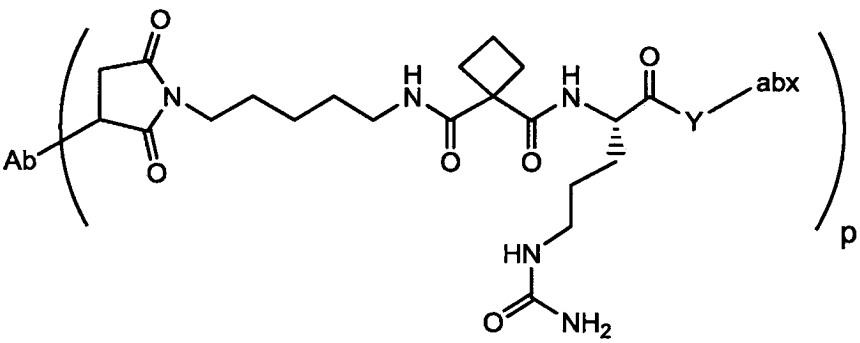
[0359] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

[0360] 

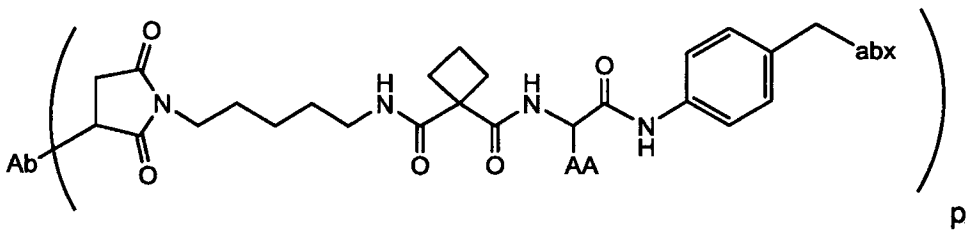
[0361] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

[0362] 

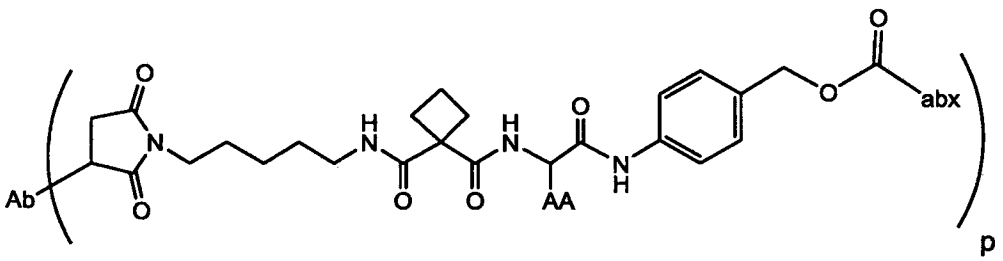
[0363] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

[0364] 

[0365] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

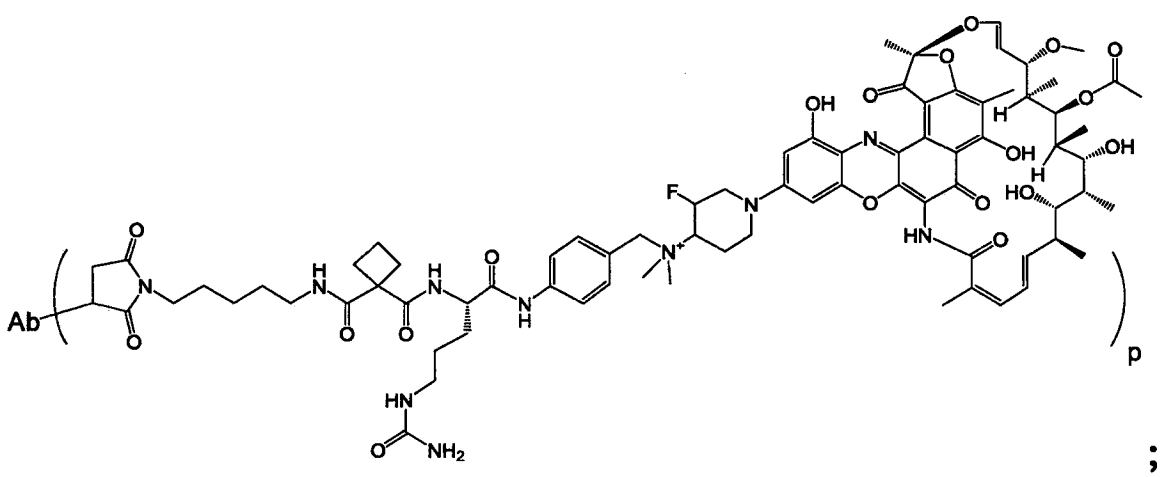


[0366] 和

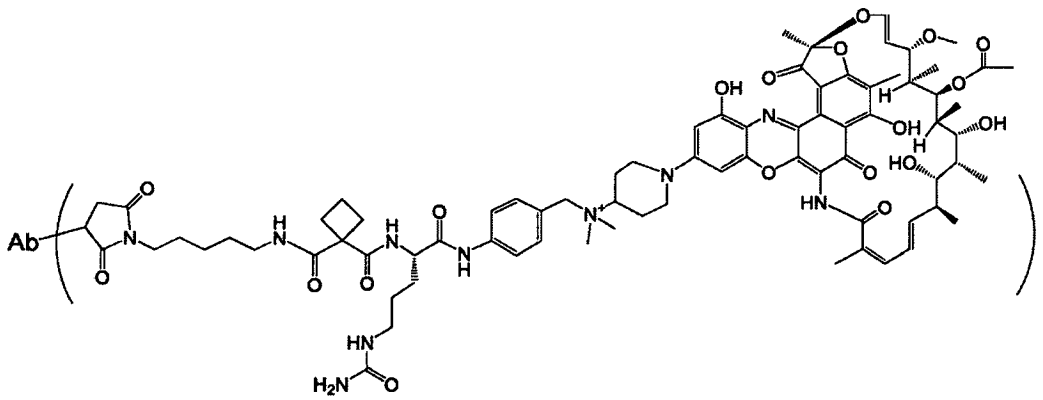


[0367] 本发明的抗体-抗生素缀合物(AAC)化合物的另一个实施方案包括下式：

[0368]



[0369]



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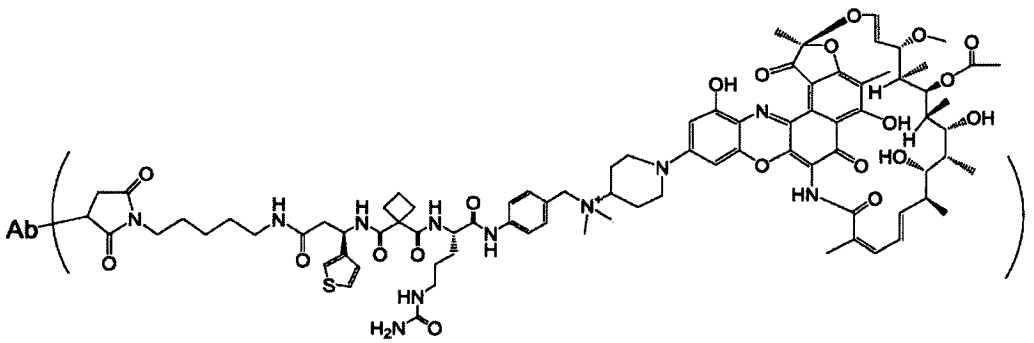

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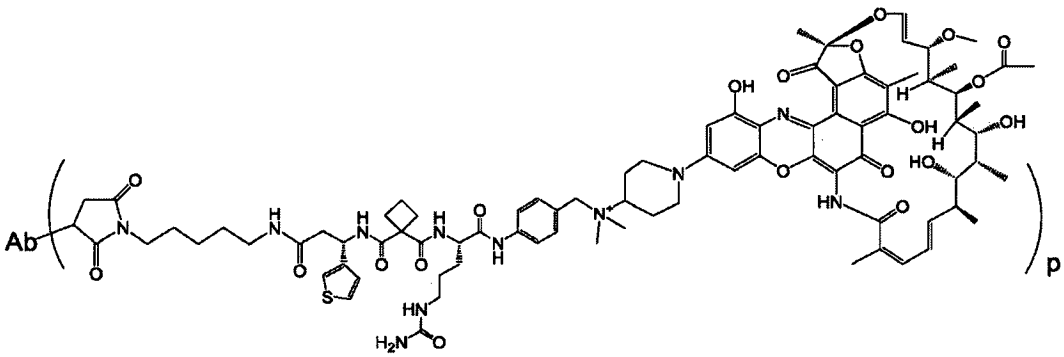
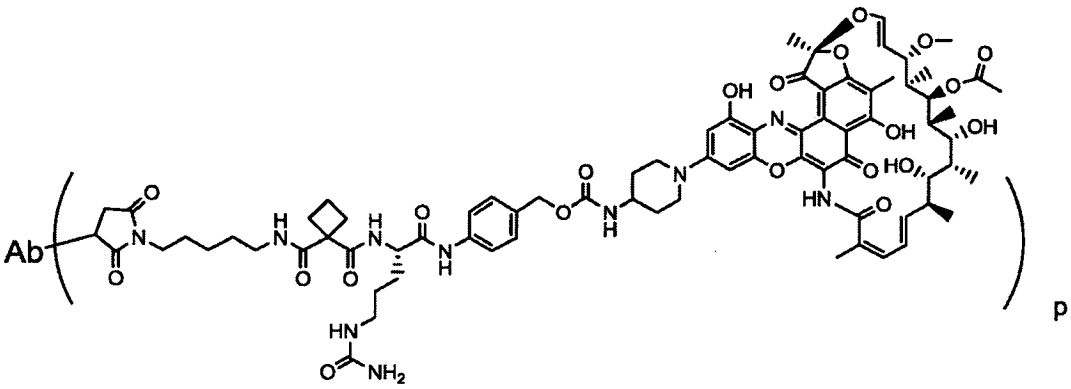


;



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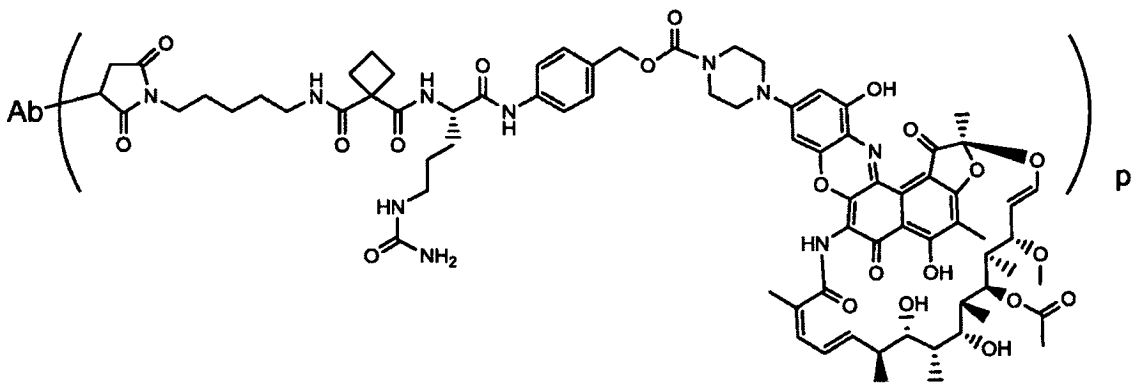
和

[0370]



[0371] AAC的抗生素负载

[0372] 抗生素负载量由p表示，即，在式I分子中每个抗体的抗生素(abx)部分的平均数。抗生素负载量可以为每个抗体1至20个抗生素部分(D)。式I的AAC包括与1至20个抗生素部分缀合的抗体集合或一组抗体。来自缀合反应的AAC制剂中每个抗体的抗生素部分的平均数可以通过常规方法表征，例如质谱、ELISA测定和HPLC。也可以确定AAC在p方面的定量分布。在一些情况下，分离、纯化和表征均质的AAC(其中p是来自具有其它抗生素负荷的AAC的某一值)可以通过诸如反相HPLC或电泳的方法来实现。

[0373] 对于一些抗体-抗生素缀合物，p可能受抗体上附着位点数目的限制。例如，如上述示例性实施方案中，当附着点是半胱氨酸硫醇，抗体可以仅具有一个或几个半胱氨酸硫醇基团，或者可以仅具有一个或几个足够反应性的硫醇基团，通过其可连接接头。在某些实施方案中，较高的抗生素负载，例如p>5，可能导致某些抗体-抗生素缀合物的聚集、不溶性、毒性或细胞通透性的丧失。在某些实施方案中，本发明的AAC的抗生素负载量为1至约8；约2至约6；约2至约4；或约3至约5；约4；或约2。

[0374] 在某些实施方案中，在缀合反应期间少于抗生素部分的理论最大值与抗体缀合。抗体可以含有例如不与抗生素-接头中间体或接头试剂反应的赖氨酸残基，如下所述。通常，抗体不含许多可与抗生素部分连接的游离和反应性半胱氨酸硫醇；实际上，抗体中大多数半胱氨酸硫醇残基作为二硫键存在。在某些实施方案中，可以在部分或全部还原条件下，用还原剂例如二硫苏糖醇(DTT)或三羰基乙基膦(TCEP)还原抗体，以产生反应性半胱氨酸硫醇基团。在某些实施方案中，将抗体进行变性条件以显示反应性亲核基团，例如赖氨酸或半胱氨酸。

[0375] 可以以不同的方式控制AAC的负载(抗生素/抗体比例，“AAR”)，例如：(i)限制抗生素-接头中间体或接头试剂相对于抗体的摩尔过量，(ii)限制缀合反应时间或温度，和(iii)部分或限制半胱氨酸硫醇修饰的还原条件。如果本文或附图中提及“DAR”，其含义与“AAR”相同。

[0376] 应当理解，当多于一个亲核基团与抗生素-接头中间体反应或与接头试剂、随后是抗生素部分试剂反应时，那么所得产物是具有连接到抗体上的一个或多个抗生素部分分布的AAC化合物的混合物。每种抗体的平均抗生素数可以通过双重ELISA抗体测定从混合物中计算出来，该测定对于抗体是特异性的并且对抗生素是特异性的。可以通过质谱在混合物中鉴定单个AAC分子，并通过HPLC分离，例如疏水相互作用层析(参见，例如McDonagh等人(2006)Prot.Engr.Pesign&Selection 19(7):299-307；Hamblett等人(2004)Clin.Cancer

Res.10:7063-7070;Hamblett,K.J.等人,"药物负荷对抗CD30抗体-药物缀合物的药理学、药代动力学和毒性的影响(Effect of drug loading on the pharmacology, pharmacokinetics,and toxicity of a anti-CD30antibody-drug conjugate)",Abstract No.624,美国癌症研究协会,2004年度会议(American Association for Cancer Research,2004Annual Meeting),March 27-31,2004,AACR学报,第45卷,2004年3月(Proceedings of the AACR,Volume 45,March 2004);Alley,S.C.等人,"控制药物连接在抗体-药物缀合物中的位置(Controlling the location of drug attachment in antibody-drug conjugates)",Abstract No.627,美国癌症研究协会,2004年度会议(American Association for Cancer Research,2004Annual Meeting),March 27-31,2004,AACR学报,第45卷,2004年3月(Proceedings of the AACR,Volume 45,March 2004))。在某些实施方案中,可以通过电泳或色谱从缀合混合物中分离具有单一负载值的均相AAC。本发明的半胱氨酸改造的抗体能够实现更均质的制备,因为抗体上的反应位点主要限于改造的半胱氨酸硫醇。在一个实施方案中,每个抗体的抗生素部分的平均数目在约1至约20的范围内。在一些实施方案中,选择和控制该范围为约1至4。

[0377] 制备抗体-抗生素缀合物的方法

[0378] 式I的AAC可以通过使用本领域技术人员已知的有机化学反应、条件和试剂的几种途径制备,包括:(1)抗体的亲核基团与二价接头试剂的反应,通过共价键形成Ab-L,随后与抗生素部分(abx)反应;和(2)抗生素部分的亲核基团与二价接头试剂反应,通过共价键形成L-abx,随后与抗体的亲核基团反应。US 7498298描述了通过后一种途径制备式I的AAC的示例性方法,其通过引用明确地并入本文。

[0379] 抗体上的亲核基团包括但不限于:(i)N-末端胺基,(ii)侧链胺基,例如赖氨酸,(iii)侧链硫醇基,例如半胱氨酸,和(iv)抗体被糖基化的糖羟基或氨基。胺、硫醇和羟基是亲核基团,并且能够与接头部分和接头试剂上的亲电子基团反应形成共价键,亲电子基团包括:(i)活性酯如NHS酯、HOBt酯、卤代甲酸酯和酰基卤;(ii)烷基和苄基卤,如卤代乙酰胺;(iii)醛、酮、羧基和马来酰亚胺基团。某些抗体具有可还原的链间二硫键,即半胱氨酸桥。通过用还原剂如DTT(二硫苏糖醇)或三羰基乙基膦(TCEP)进行处理,使得抗体完全或部分还原,抗体可以与接头试剂缀合反应。因此,每个半胱氨酸桥在理论上将形成两个反应性硫醇亲核试剂。另外的亲核基团可以通过赖氨酸残基的修饰引入抗体,例如通过使赖氨酸残基与2-亚氨基噻吩(Traut's试剂)反应,导致胺转化为硫醇。可以通过引入一个、两个、三个、四个或更多个半胱氨酸残基(例如通过制备包含一个或多个非天然半胱氨酸氨基酸残基的变体抗体),将反应性硫醇基团引入抗体。

[0380] 本发明的抗体-抗生素缀合物也可以通过抗体上的亲电子基团,例如醛或酮羰基,与接头试剂或抗生素上的亲核基团之间的反应产生。接头试剂上有用的亲核基团包括但不限于酰肼、肟、氨基、肼、缩氨基硫脲、肼羧酸酯和芳基酰肼。在一个实施方案中,修饰抗体以引入能够与接头试剂或抗生素上的亲核取代基反应的亲电子部分。在另一个实施方案中,糖基化抗体的糖可被氧化,例如,与高碘酸氧化试剂形成可与连接试剂或抗生素部分的胺基反应的醛或酮基。所得到的亚胺希夫碱基可以形成稳定的键,或可以被还原。例如由硼氢化物试剂形成稳定的胺键。在一个实施方案中,糖基化抗体的碳水化合物部分与半乳糖氧化酶或偏高碘酸钠反应,可以在抗体中产生可与抗生素上适当基团反应的羰基(醛和酮)基

团(Hermanson,Bioconjugate Techniques)。在另一个实施方案中,含有N-末端丝氨酸或苏氨酸残基的抗体可与偏高碘酸钠反应,导致产生醛而代替第一个氨基酸(Geoghegan& Stroh,(1992)Bioconjugate Chem.3:138-146;US 5362852)。这样的醛可以与抗生素部分或接头亲核试剂反应。

[0381] 抗生素部分上的亲核基团包括但不限于:胺、硫醇、羟基、酰肼、肟、肼、缩氨基硫脲、肼羧酸酯和芳基酰肼基团,其能够与接头部分和接头试剂上的亲电子基团形成共价键,包括:(i)活性酯如NHS酯、HOBt酯、卤代甲酸酯和酰基卤;(ii)烷基和苄基卤,如卤代乙酰胺;(iii)醛、酮、羧基和马来酰亚胺基团。

[0382] 根据实施例18中所述的方法,表3中的抗体-抗生素缀合物(AAC)通过所述rF1抗体与表2的接头-抗生素中间体缀合制备。通过体外巨噬细胞测定(实施例19)和体内小鼠肾模型(实施例20)测试AAC的功效。

[0383] 表3 rF1抗体-PML-抗生素缀合物(AAC)

[0384]

AAC 编号	AAC 化学式	接头-abx PLA 编号	AAR *
101	硫代-rF1-LC-V205C-MC-(CBDK-cit)-PAB-(二甲基, 氟代 pipBOR)	PLA-1	2.0
102	硫代-rF1-HC-121C, LC-V205C-MC-(CBDK-cit)-PAB-(二甲基 pipBOR)	PLA-2	3.9
103	硫代-rF1-LC-V205C-MC-(CBDK-cit)-PAB-(二甲基 pipBOR)	PLA-2	1.9
104	硫代-rF1-HC-A121C,	PLA-2	3.7

[0385]

	LC-V205C-MC-(CBDK-cit)-PAB-(二甲基 pipBOR)		

[0386] *AAR=抗生素/抗体比例平均值

[0387] 野生型("WT")、半胱氨酸改造的突变抗体("硫代")、轻链("LC")、重链("HC")、6-马来酰亚胺基己酰基("MC")、马来酰亚胺基丙酰基("MP")、环丁基二酮基("CBDK")、瓜氨酸("cit")、半胱氨酸("cys")、对氨基苄基("PAB")和对氨基苄氧羰基("PABC")

[0388] 用抗体-抗生素缀合物治疗和预防感染的方法

[0389] 本发明的rF1-AAC可用作对人和兽医的葡萄球菌,例如金黄色葡萄球菌、腐生性葡萄球菌(S.saprophyticus)和溶血性葡萄球菌(S.simulans)的有效的抗微生物剂。在具体方面,本发明的AAC可用于治疗金黄色葡萄球菌感染。

[0390] 进入血液后,金黄色葡萄球菌可以在几乎任何器官中引起转移性感染。继发感染发生在治疗开始前约三分之一的病例中(Fowler等人,(2003)Arch.Intern.Med.163:2066-2072),甚至在治疗开始后的10%的患者中(Khatib等人(2006)Scand.J.Infect.Dis.,38:

7-14)。感染的标志是大量的脓液储库、组织破坏和脓疮的形成(所有这些都含有大量的嗜中性粒细胞)。如果菌血症持续超过三天,约40%的患者会发生并发症。

[0391] 上文(在副标题抗体-抗生素缀合物下)已经描述了提出的AAC的作用机制。本发明的rF1抗体-抗生素缀合物(AAC)对于治疗细胞内病原体具有显著的治疗优势。AAC接头通过暴露于吞噬溶酶体酶而切割,释放活性抗生素。由于局限的空间和相对较高的局部抗生素浓度(每个细菌约为10^4个),结果是吞噬溶酶体不再支持细胞内病原体的存活。因为AAC基本上是无活性的前药,抗生素的治疗指数可以相对于游离(非缀合)形式扩展。抗体提供病原体特异性靶向,而可切割接头在病原体的细胞内位置特定的条件下切割。这种作用可以直接关系到调理的病原体以及共定位在吞噬溶酶体中的其他病原体。抗生素耐受性是导致疾病的病原体抵抗抗生素和其他抗菌药物杀伤的能力,并且在机制上与多药耐药性不同(Lewis K(2007)."耐药株细胞、休眠和感染性疾病"。自然微生物学综述("Persister cells,dormancy and infectious disease".Nature Reviews Microbiology)5(1):48-56.doi:10.1038/nrmicro1557)。相反,这种耐受的形式是由称为耐药株的一小部分微生物细胞引起的(Bigger JW(1944年10月14日))"通过间歇性灭菌用青霉素治疗葡萄球菌感染",柳叶刀("Treatment of staphylococcal infections with penicillin by intermittent sterilization".Lancet)244(6320):497-500)。这些细胞在经典意义上不是多药耐药性的,而是耐受抗生素治疗的休眠细胞,抗生素可以杀死其遗传上相同的兄弟姐妹。这种抗生素耐受性是由非分裂或非常缓慢分裂的生理状态引起的。当抗菌治疗无法消除这些耐药株细胞时,它们成为复发性慢性感染的储库。本发明的抗体-抗生素缀合物具有杀死这些耐药株细胞的独特性质并且抑制多药耐药细菌群体的出现。

[0392] 在另一个实施方案中,本发明的rF1-AAC可用于治疗感染,不管病原体是否存活于细胞内区室中。

[0393] 在另一个实施方案中,本发明的rF1-AAC也可用于靶向于以浮游生物或生物膜形式的葡萄球菌。用本发明的抗体-抗生素缀合物(AAC)治疗的细菌感染包括治疗细菌性肺部感染,例如金黄色葡萄球菌肺炎、骨髓炎、复发性鼻窦炎、细菌性心内膜炎、细菌性眼部感染如沙眼和结膜炎、心脏、脑或皮肤感染,胃肠道感染,一般例如旅行者腹泻、溃疡性结肠炎、肠易激综合征(IBS)、克罗恩病和IBD(炎性肠病),细菌性脑膜炎、以及任何器官如肌肉、肝、脑膜或肺中的脓肿。细菌感染可以在身体其他部位,如尿路、血流、伤口或导管插入部位。本发明的AAC可用于涉及生物膜、植入物或避孕区(例如骨髓炎和假体关节感染),以及高死亡率感染例如医院获得性肺炎和菌血症的难治疗的感染。可以治疗以预防金黄色葡萄球菌感染的易感患者组包括血液透析患者、免疫受损患者、重症监护病房的患者和某些手术患者。在另一方面,本发明提供了杀死、治疗或预防在动物,优选哺乳动物,最优选人类中的微生物感染的方法,其包括向所述动物施用rF1AAC或本发明的AAC药物制剂。本发明进一步特征在于治疗或预防与这种微生物感染相关或由其机会性地引起的疾病。这些治疗或预防的方法可包括本发明组合物的口服、局部、静脉内、肌肉内或皮下给药。例如,在手术或插入IV导管之前、在ICU护理、移植医学、癌症化疗或癌症化疗后,或其它具有高感染风险的活动中,可以施用本发明的AAC以预防感染发作或传播。

[0394] 细菌感染可以由具有活性和非活性形式的细菌引起,并且AAC以治疗细菌感染的活性和非活性潜在形式的量和足够的持续时间施用,后者持续时间长于需要治疗细菌感染

的活性形式。

[0395] 本发明的一个方面是通过施用治疗有效量的本发明的rF1-AAC来治疗感染金黄色葡萄球菌和/或单核细胞增生利斯特菌的患者的方法。本发明还考虑了一种通过在医院环境例如外科手术、烧伤患者和器官移植中施用治疗有效量的本发明的rF1-AAC，来预防金黄色葡萄球菌、或表皮葡萄球菌、或腐生性葡萄球菌、或溶血性葡萄球菌感染的方法。

[0396] 由本领域有经验的医师确定的需要治疗细菌感染的患者可能已经被感染、但不需要用他/她感染的细菌类型做诊断。由于患有细菌感染的患者可以在非常快的时间内轮流转，所以在几个小时内，进入医院的患者可以给予本发明的rF1-AAC以及一种或多种护理标准抗生素，如万古霉素或环丙沙星。当诊断结果变得可用并且指示在感染中存在例如金黄色葡萄球菌时，患者可以继续用rF1AAC进行治疗。因此，在治疗细菌感染或特别是金黄色葡萄球菌感染的方法的一个实施方案中，给予患者治疗有效量的rF1AAC。在本发明的治疗或预防方法中，本发明的AAC可以作为唯一的治疗剂或与其它药剂如下述那些联合给药。在临床前模型中，本发明的AAC显示优于万古霉素对MRSA的治疗。AAC与SOC的比较可以例如通过降低死亡率来测量。被评估的患者将通过各种可测量因素评估对AAC治疗的反应。临床医生可能用来评估患者改善的体征和症状的例子包括：诊断升高时白细胞计数的正常化、诊断升高(发热)时体温的正常化、血液培养物的清除、在伤口中可见的改善包括红斑减少和脓液引流、减少呼吸机要求、如通气的患者需要较少氧气或降低通气率，如果患者在诊断时通气，则完全从呼吸机中脱气，如果在诊断时需要这些药物，则使用较少的药物来支持稳定的血压，如果在诊断时异常，那么提示终末器官功能衰竭例如升高的肌酐或肝功能测试的实验室异常的正常化，和放射学成像(例如以前建议肺炎显示分辨率的胸部X片)的改善。在ICU的患者中，这些因素可能至少每天测量一次。密切监测发烧，包括绝对嗜中性粒细胞计数的白细胞计数以及“左移”(指示响应于主动感染的增加的嗜中性粒细胞产生爆发的出现)的证据已经解决。

[0397] 在本发明的治疗方法的上下文中，如果本领域技术人员的医师评估在至少两个或更多个以前的因素中与治疗前或治疗开始时或诊断时的值、体征或症状相比存在显著的可测量的改善，则认为具有细菌感染的患者被治疗。在一些实施方案中，在3、4、5、6或更多个上述因素中存在可测量的改善。在一些实施方案中，与治疗前的值相比，测量因素的改善为至少50％、60％、70％、80％、90％、95％或100％。通常，如果患者的可测量的改善包括以下内容，则可以将患者视为细菌感染(例如金黄色葡萄球菌感染)被完全治疗：i)重复了不会生长出最初识别的细菌的血液或组织培养物(通常为几种)；ii)发烧正常化；iii)WBC正常化；和iv)对于患者先前存在的并发症，表明终末器官衰竭(心脏、肺、肝、肾、血管塌陷)的证据已经完全或部分地解决。

[0398] 给药。在任何上述方面中，在治疗感染的患者中，AAC的剂量通常为约0.001至1000mg/kg/天。在一个实施方案中，细菌感染的患者以约1mg/kg至约150mg/kg范围内的AAC剂量治疗，通常为约5mg/kg至约150mg/kg，更具体地为25mg/kg至125mg/kg，50mg/kg至125mg/kg，甚至更具体地为约50mg/kg至100mg/kg。AAC可以每天给予(例如，单剂量为5至50mg/kg/天)或较不频繁(例如，单剂量为5、10、25或50mg/kg/周)。一个剂量可以分开2天，例如一天25mg/kg，第二天25mg/kg。患者可以每3天服用一次剂量(q3D)，每周一次至每隔一周一次(qOW)，持续1-8周。在一个实施方案中，患者通过具有护理标准(SOC)的IV每周一次、

持续2-6周给予本发明的AAC，以治疗细菌感染如金黄色葡萄球菌感染。治疗长度将由患者的状况或感染的程度决定，例如，不复杂的菌血症的治疗持续时间为2周，或菌血症伴心内膜炎为6周。

[0399] 在一个实施方案中，AAC以2.5至100mg/kg的初始剂量给药连续1-7天，然后每1-7天一次0.005至10mg/kg的维持剂量，持续一个月。

[0400] 给药途径。为了治疗细菌感染，本发明的AAC可以静脉内(i.v.)或皮下的任何前述剂量给药。在一个实施方案中，静脉内给药rF1-AAC。在一个具体实施方案中，通过静脉内给药rF1-AAC，其中rF1抗体是选自SDR和rF1抗体和表4A和4B中公开的具有氨基酸序列的抗体组。

[0401] 联合疗法。AAC可以酌情与一种或多种另外的、例如第二治疗或预防剂一起施用，其由治疗患者的医师确定。

[0402] 在一个实施方案中，与本发明的抗体-抗生素缀合化合物组合施用的第二抗生素选自以下结构类别：(i)氨基糖苷类；(ii)β-内酰胺类；(iii)大环内酯/环肽；(iv)四环素；(v)氟喹啉/氟喹诺酮类；(vi)和噁唑烷酮类。参见：Shaw,K.和Barbachyn,M.(2011)Ann.N.Y.Acad.Sci.1241:48-70;Sutcliffe,J.(2011)Ann.N.Y.Acad.Sci.1241:122-152。

[0403] 在一个实施方案中，与本发明的抗体-抗生素缀合化合物组合施用的第二抗生素选自克林霉素、新生霉素、瑞他帕林(retapamulin)、达托霉素、GSK-2140944、CG-400549、西他沙星、替考拉宁、三氯生、萘啶酮(napthyridone)、雷得唑来、多柔比星、氨苄西林、万古霉素、亚胺培南、多利培南、吉西他滨、达巴万星和阿奇霉素。

[0404] 这些另外的治疗或预防剂的其它实例是抗炎剂(例如非甾体抗炎药(NSAID，例如detoprofen、双氯芬酸、二氟尼柳、依托度酸、非诺洛芬、氟比洛芬、布洛芬、吲哚美辛、酮洛芬、甲氯灭酸盐、甲芬那酸、美洛昔康、nabumeone、萘普生钠、奥沙普秦、吡罗昔康、舒林酸、托美汀、塞来昔布、罗非昔布、阿司匹林、水杨酸胆碱、salsalte和水杨酸钠和水杨酸镁)和类固醇(例如，可的松、地塞米松、氢化可的松、甲基强的松龙、泼尼松龙、强的松、去炎松))、抗菌药(例如阿奇霉素、克拉霉素、红霉素、加替沙星、左氧氟沙星、阿莫西林、甲硝唑、青霉素G、青霉素V、甲氧西林、苯唑西林、氯唑西林、双氯西林、萘夫西林、氨苄西林、羧苄西林、替卡西林、美洛西林、哌拉西林、阿洛西林、替莫西林、头孢噻吩、头孢匹林、头孢拉定、头孢噻啶、头孢唑啉、头孢羟唑、头孢呋辛、头孢氨苄、头孢丙烯、头孢克洛、氯碳头孢、头孢西丁、头孢奈唑、头孢噻肟、头孢唑肟、头孢曲松、头孢哌酮、头孢他啶、头孢克肟、头孢泊肟、头孢布烯、头孢地尼、头孢匹罗、头孢吡肟、BAL5788、BAL9141、亚胺培南、厄他培南、美罗培南、氨曲南、克拉维酸、舒巴坦、他唑巴坦、链霉素、新霉素、卡那霉素、巴龙霉素、庆大霉素、妥布霉素、阿米卡星、奈替米星、壮观霉素、西索米星、dibekalin、异帕米星、四环素、金霉素、地美环素、米诺环素、土霉素、甲烯土霉素、多西环素、泰利霉素、ABT-773、林可霉素、克林霉素、万古霉素、奥利万星、达巴万星、替考拉宁、奎奴普丁和达福普汀、磺胺、对氨基苯甲酸、磺胺嘧啶、磺胺异噁唑、磺胺甲噁唑、酞磺胺噻唑、利奈唑胺、萘啶酸、噁喹酸、诺氟沙星、培氟沙星、依诺沙星、氧氟沙星、环丙沙星、替马沙星、洛美沙星、氟罗沙星、格帕沙星、司帕沙星、曲伐沙星、克林沙星、莫西沙星、吉米沙星、西他沙星、达托霉素、加雷沙星、雷莫拉宁、法罗培南、多粘菌素、替加环素、AZD2563、甲氧苄氨嘧啶)、抗细菌抗体，包括来自AAC靶向Ag的相同或不同抗原的抗体、血小板聚集抑制剂(例如阿昔单抗、阿司匹林、西洛他唑、氯吡格雷、双

嘧达莫、依替巴肽、噻氯匹定或替罗非班)、抗凝血剂(例如,达肝素、达那肝素、依诺肝素、肝素、亭扎肝素或华法林)、退烧药(例如,对乙酰氨基酚)或降脂药(例如,考来烯胺、考来替泊、烟酸、吉非贝齐、普罗布考、依泽替米贝或他汀类药物如阿托伐他汀、瑞舒伐他汀、洛伐他汀、辛伐他汀、普伐他汀、西立伐他汀和氟伐他汀)。在一个实施方案中,本发明的AAC与金黄色葡萄球菌(包括耐甲氧西林和甲氧西林敏感菌株)的护理标准(SOC)一起施用。MSSA通常通常用萘夫西林或苯唑西林治疗,MRSA通常用万古霉素或头孢唑啉治疗。

[0405] 这些另外的试剂可以在给予AAC的14天、7天、1天、12小时或1小时内或与其同时施用。另外的治疗剂可以与AAC相同或不同的药物组合物存在。当存在于不同的药物组合物中时,可以使用不同的给药途径。例如,AAC可以静脉内或皮下给药,而第二药剂可以口服给药。

[0406] 药物制剂

[0407] 本发明还提供含有rF1-AAC的药物组合物,以及使用含有AAC的药物组合物治疗细菌感染的方法。这样的组合物可以进一步包含合适的赋形剂,例如药学上可接受的赋形剂(载体),包括缓冲剂、酸、碱、糖、稀释剂、助流剂、防腐剂等,这在本领域是公知的并且在本文中描述。本发明的方法和组合物可以单独使用或与其它常规方法和/或治疗感染性疾病的试剂组合使用。在一些实施方案中,药物制剂包含:1)本发明的rF1-AAC,和2)药学上可接受的载体。在一些实施方案中,药物制剂包含:1)本发明的AAC,和任选的2)至少一种另外的治疗剂。

[0408] 通过将具有所需纯度的AAC与任选的生理上可接受的载体、赋形剂或稳定剂(Remington's Pharmaceutical Sciences,16th edition,Osol,A.Ed.(1980))以水溶液或冻干的或其他干燥的制剂形式混合,制备包含本发明的AAC的药物制剂用于储存。可接受的载体、赋形剂或稳定剂在所使用的剂量和浓度上对受者无毒,并且包括缓冲液如磷酸盐、柠檬酸盐、组氨酸和其它有机酸;抗氧化剂,包括抗坏血酸和甲硫氨酸;防腐剂(如十八烷基二甲基苄基氯化铵、氯化六甲双铵、苯扎氯铵、苄索氯铵);苯酚、丁基或苄基醇;对羟基苯甲酸烷基酯如对羟基苯甲酸甲酯或丙酯;邻苯二酚;间苯二酚;环己醇;3-戊醇;和间甲酚);低分子量(小于约10个残基)多肽;蛋白质,如血清白蛋白、明胶或免疫球蛋白;亲水性聚合物如聚乙烯吡咯烷酮;氨基酸如甘氨酸、谷氨酰胺、天冬酰胺、组氨酸、精氨酸或赖氨酸;单糖、二糖和其他碳水化合物,包括葡萄糖、甘露糖或糊精;螯合剂如EDTA;糖类如蔗糖、甘露醇、海藻糖或山梨醇;形成盐的反荷离子如钠;金属络合物(例如Zn-蛋白复合物);和/或非离子表面活性剂如吐温™、PLURONICS™或聚乙二醇(PEG)。用于体内施用的药物制剂通常是无菌的,通过无菌过滤膜过滤容易地完成。

[0409] 活性成分也可以被包埋在例如通过凝聚技术或通过界面聚合制备的微胶囊中,例如分别在胶体药物递送系统(例如,脂质体、白蛋白微球、微乳液、纳米颗粒和纳米胶囊)或在大乳剂中的羟甲基纤维素或明胶微胶囊和聚(甲基丙烯酸甲酯)微胶囊。这种技术在Remington's Pharmaceutical Sciences第16版,Osol,A.Ed.(1980)中公开。

[0410] 可以制备缓释制剂。缓释制剂的合适实例包括含有本发明的抗体或AAC的固体疏水性聚合物的半透性基质,该基质是成形制品例如薄膜或微胶囊形式。缓释基质的实例包括聚酯、水凝胶(例如,聚(2-羟乙基-甲基丙烯酸酯)或聚(乙烯醇))、聚交酯(U.S.专利号3,773,919)、L-谷氨酸和γ乙基-L-谷氨酸盐的共聚物、不可降解的乙烯-乙酸乙烯酯、可降解

乳酸-乙醇酸共聚物如LUPRON DEPOT™(由乳酸-乙醇酸共聚物和醋酸亮丙瑞林组成的可注射微球)和聚-D-(-)-3-羟基丁酸。虽然聚合物如乙烯-乙酸乙烯酯和乳酸-乙醇酸能够释放分子超过100天,但某些水凝胶在较短的时间段释放蛋白质。当封装的抗体或AAC长时间保留在体内时,由于在37℃暴露于水分中,它们可能会变性或聚集,导致生物活性的丧失和免疫原性的可能变化。根据所涉及的机制,可以设计出稳定的合理策略。例如,如果发现聚集机制是通过硫醇-二硫化物交换形成分子间S-S键,则可以通过修饰巯基残基、用酸性溶液冻干、控制含水量、使用合适的添加剂和开发特定的聚合物基质组合物来实现稳定化。

[0411] AAC可以以任何合适的形式配制以递送至靶细胞/组织。例如,AAC可以配制成脂质体,由各种类型的脂质、磷脂和/或表面活性剂组成的小囊泡,其可用于向哺乳动物递送药物。脂质体的组分通常排列成双层形成,类似于生物膜的脂质排列。含有抗体的脂质体通过本领域已知的方法制备,例如在Epstein等人(1985)Proc.Natl.Acad.Sci.USA 82:3688;Hwang等人,(1980)Proc.Natl Acad.Sci.USA 77:4030;US 4485045;US 4544545;WO 97/38731;US 5013556中描述的。

[0412] 特别有用的脂质体可以通过含有磷脂酰胆碱、胆固醇和PEG衍生的磷脂酰乙醇胺(PEG-PE)的脂质组合物的反相蒸发法产生。脂质体通过限定孔径的过滤器挤出以产生具有所需直径的脂质体。

[0413] 材料和方法

[0414] 细菌菌株和培养:

[0415] 所有实验都是使用从NARSA(http://www.narsa.net/control/member/repositories)获得的MRSA-USA300NRS384进行的,除非另有说明。

[0416] 细菌在补充有5%绵羊血的胰酶解大豆琼脂平板(TSA板)上在37℃下生长18小时。对于液体培养物,将来自TSA板的单个菌落接种到胰酶解大豆肉汤培养基(TSB)中,并在37℃下孵育,同时以200rpm摇动18小时;将这些培养物在新鲜TSB中的100倍稀释液进一步传代培养多次。

[0417] 细胞外细菌的MIC测定

[0418] 通过在胰酶解大豆肉汤培养基中连续2倍稀释制备的抗生素来测定细胞外细菌的MIC。抗生素的稀释液在96孔培养皿中一式四份制成。从指数生长培养物取MRSA(USA300的NRS384菌株)并稀释至1×10^4CFU/mL。细菌在抗生素存在下培养18-24小时并在37℃下振荡,通过读取630nM的光密度(OD)测定细菌生长。MIC被确定为将细菌生长抑制>90%的抗生素剂量。

[0419] 细胞内细菌的MIC测定

[0420] 对鼠腹膜巨噬细胞内隐藏的细菌测定细胞内MIC(见下文,用于产生鼠腹膜巨噬细胞)。将巨噬细胞以4×10^5个细胞/mL的密度接种在24孔培养皿中,并以每个巨噬细胞10-20个细菌的比例感染MRSA。将巨噬细胞培养物保持在补充有50μg/mL庆大霉素(仅对细胞外细菌有活性的抗生素)的生长培养基中,以抑制细胞外细菌的生长,并在感染后1天将测试的抗生素加入到生长培养基中。在加入抗生素后24小时评估细胞内细菌的存活。用补充有0.1%牛血清白蛋白和0.1%Triton-X的Hanks缓冲盐水溶液裂解巨噬细胞,并在含有0.05%吐温-20的磷酸缓冲盐水溶液中进行裂解物的连续稀释。通过在含有5%去纤维蛋白的羊血的胰酶解大豆琼脂平板上接种来确定存活的细胞内细菌数。

[0421] 细菌细胞壁制剂(CWP)、免疫印迹和ELISA

[0422] 通过将每毫升补充有30%棉子糖、100μg/ml溶葡萄球菌素(Cell Sciences,Canton,MA)的10mM Tris-HCl(pH 7.4)中40mg片状金黄色葡萄球菌或表皮葡萄球菌与无EDTA的蛋白酶抑制剂混合物(Roche,Pleasanton,CA)在37℃下孵育30分钟来产生CWP。将裂解物以11,600×g离心5分钟,收集含有细胞壁成分的上清液。对于免疫沉淀,将CWP在含有1μg/mL指示一抗的NP-40缓冲液(120mM NaCl、50mM Tris-HCl pH8.0、1%NP-40、完全蛋白酶抑制剂混合物(Roche)和2mM二硫苏糖醇)中稀释4倍,并在4℃孵育2小时,随后与蛋白A/G琼脂糖(Thermo,Waltham,MA)孵育1小时。通过在37℃下在20mM Tris-HCl(pH 7.4)、150mM NaCl、100μg/ml溶葡萄球菌素、1%Triton-X100(Thermo)和无EDTA的蛋白酶抑制剂混合物中孵育30分钟产生全细胞裂解物(WCL)。对于免疫印迹分析,在4-12%Tris-甘氨酸凝胶上分离蛋白质,并转移到硝酸纤维素膜(Invitrogen,Carlsbad,CA)上,然后用指示的一抗(1μg/mL)印迹。使用的抗体列于表1中。通过用补充有0.1mM $CaCl_2$和0.01mM $MnCl_2$的伴刀豆球蛋白A(ConA)或sWGA-琼脂糖珠(Vector Labs,Burlingame,CA)的培养基上清液免疫沉淀过滤(0.2微米)过夜来进行凝集素研究。

[0423] 使用标准方案进行ELISA实验。简言之,将预涂有CWP的板与人IgG制备物,即,纯化的人IgG(Sigma)、静脉内免疫球蛋白制剂(Gammagard Liquid)(Baxter,Westlake Village,CA))、来自健康供体或MRSA患者(均由实验室产生)的库存血清反应。通过使用已知浓度的抗-肽聚糖的mAb28.9.9产生的校准曲线,计算血清或纯化IgG中存在的抗葡萄球菌IgG的浓度。

[0424] 用来自人嗜中性粒细胞和培养细胞的人嗜中性粒细胞蛋白酶或溶酶体提取物处理细菌

[0425] 使用溶酶体富集试剂盒(Thermo)从人嗜中性粒细胞、THP-1细胞和RAW细胞中分离出溶酶体提取物。总共5×10^7个细胞用于在溶酶体中获得300至500微克的总蛋白。在所有步骤中省略了蛋白酶抑制剂以维持溶酶体中的蛋白酶活性。使用杜恩斯匀浆器(Wheaton,Millville,NJ)将细胞的质膜破碎30次。将匀浆物以500×g离心5分钟以获得核后上清液,将其加载到碘克沙醇的8%、20%、23%、27%和30%(从上至下)的梯度的顶部。在4℃下以145,000×g超速离心2小时后,我们获得了在8%和20%的碘克沙醇之间分层的溶酶体。将该溶酶体组分稀释到PBS中,并在4℃以18,000×g离心30分钟使其沉淀。用PBS洗涤溶酶体沉淀,并在含Tris缓冲盐水的2%CHAPS中裂解,得到溶酶体提取物。

[0426] 为了分析宿主蛋白酶对SDR蛋白的切割,将金黄色葡萄球菌用50mM的纯化人嗜中性粒细胞丝氨酸蛋白酶或0.1mg/ml嗜中性粒细胞溶酶体提取物,在含150mM NaCl和2mM $CaCl_2$的50mM Tris(pH 8.0)中处理;或用0.1mg/ml的RAW或THP-1溶酶体提取物,在含100mM NaCl和2mM DTT(pH 5.5)的50mM柠檬酸钠中处理。以100μg/ml加入组织蛋白酶G抑制剂(Calbiochem,Billerica,MA)。当使用纯化的蛋白酶时,将这些混合物在37℃下孵育30分钟,当使用溶酶体裂解物时孵育1小时,并离心以沉淀细菌。通过免疫印迹分析上清液以检测裂解产物。在一些实验中,从剩余的细菌沉淀中获得细胞壁制备物,并通过免疫印迹进行分析。

实施例

[0427] 实施例1细胞内MRSA受到对抗常规抗生素的保护

[0428] 为了证实哺乳动物细胞在抗生素治疗存在下为金黄色葡萄球菌提供保护性小环境的假说，将目前用作侵入性MRSA感染的护理标准(SOC)的三种主要抗生素(万古霉素、达托霉素和利奈唑胺)对细胞外浮游细菌与小鼠巨噬细胞内隔离细菌的效力进行比较(表1)。

[0429] 对于细胞外细菌，将MRSA在胰酶解大豆肉汤培养基中培养过夜，并确定MIC是防止生长的最小抗生素剂量。对于细胞内细菌，小鼠腹膜巨噬细胞用MRSA感染并在庆大霉素存在下培养，以杀死细胞外细菌。感染后一天将测试抗生素加入到培养基中，24小时后测定存活的细胞内细菌总数。临床相关抗生素的预期血清浓度在抗菌药物(Antimicrobial Agents)，Andre Bryskier.ASM Press，Washington DC(2005)中被报道。

[0430] 表1：几种抗生素对在液体培养物中生长的细胞外细菌与小鼠巨噬细胞内隔离的细胞内细菌的最小抑菌浓度(MIC)。

[0431]

抗生素(Abx)	细胞外 MRSA MIC (μg/mL)	细胞内 MRSA MIC (μg/mL)	血清 Cmax (μg/mL)
万古霉素	1	>100	50
达托霉素	4	>100	60
利奈唑胺	0.3	>20	20
利福平	0.004	50	20

[0432] 具有高毒力的社区获得性MRSA菌株USA300的分析显示，虽然细胞外MRSA对液体培养中低浓度的万古霉素、达托霉素和利奈唑胺的生长抑制高度敏感，但所有三种抗生素都不能杀死暴露于临床可达到的抗生素浓度的在巨噬细胞内部隔离的MRSA相同菌株。即使是利福平，被认为相对有效地消除细胞内病原体(Vandenbroek，P.V.(1989)抗菌药物、微生物、吞噬细胞。传染病综述(Antimicrobial Drugs，Microorganisms，Phagocytes.Reviews of Infectious Diseases)11，213-245)，与抑制浮游细菌生长(MIC)所需的剂量比较，仍需要6，000倍更高的剂量来消除细胞内MRSA(表1)，这与其他研究一致，表明大多数现有抗生素在体外和体内杀死细胞内金黄色葡萄球菌都是无效的(Sandberg，A.，Hessler，J.H.，Skov，R.L.，Blom，J.&Frimodt-Moller，N.(2009)“鼠腹膜炎模型中抗生素对金黄色葡萄球菌的细胞内活性(Intracellular activity of antibiotics against Staphylococcus aureus in a mouse peritonitis model)”Antimicrob Agents Chemother53，1874-1883)。

[0433] 实施例2细胞内MRSA感染的传播

[0434] 这些实验比较了细胞内细菌与等量的游离浮游细菌的毒性，并确定细胞内细菌是否能够在体内在万古霉素存在下建立感染。通过静脉内注射大致相当剂量的金黄色葡萄球

菌游离细菌(2.9×10^6)感染四组小鼠，其细菌直接从肉汤培养物，或在由供体小鼠腹膜感染产生的宿主巨噬细胞和嗜中性粒细胞内隔离的细胞内细菌(1.8×10^6)中取出(图1A)，并且选择的组在感染后立即用万古霉素处理，然后每天一次。在感染后4天检查小鼠，细菌定殖在肾脏中，其是在小鼠中一致地被金黄色葡萄球菌定殖的器官[23]。在三次独立实验中，观察到感染细胞内细菌的小鼠肾脏中与那些感染了相同量的浮游细菌相比的相同或更高的细菌负荷(图1B)。令人惊讶的是，发现细胞内细菌的感染导致脑的更一致的定殖，这是在该模型中感染浮游细菌后没有有效定殖的器官(图1C)。此外，在该模型中，细胞内细菌而不是浮游细菌能够在万古霉素治疗的情况下建立感染(图1B、图1C)

[0435] 体外的进一步分析更定量地解决了细胞内存活促进抗生素逃避的程度。为此，在万古霉素存在下，MG63成骨细胞被浮游MRSA或细胞内MRSA感染。

[0436] 成骨细胞或HBMEC感染。从ATCC获得MG63细胞系(CRL-1427)，并保持在补充有10mM Hepes和10％胎牛血清的RPMI 1640组织培养基(RPMI-10)中。从Scienc Cell Research Labs(Carlsbad,CA)获得HBMEC细胞(目录号#1000)和ECM培养基(目录号#1001)。将细胞接种在24孔组织培养板中并培养以获得汇合层。在实验当天，细胞在RPMI(无补充物)中洗涤一次。将MRSA或感染的腹膜细胞在完全RPMI-10中稀释，并在感染前立即加入5ug/mL万古霉素。将腹膜细胞以1×10^6个腹膜细胞/mL加入到成骨细胞中。用0.1％triton-x裂解细胞样品以确定感染时活的细胞内细菌的实际浓度。所有感染的实际滴度通过在含5％去纤维蛋白的羊血的胰酶解大豆琼脂上连续稀释的细菌接种来测定。

[0437] 将MRSA(游离细菌)接种在培养基、培养基+万古霉素或培养基+万古霉素并接种在单层MG63成骨细胞(图1E)或人脑微血管内皮细胞(HBMEC，图1F)上。将板离心以促进细菌与单层接触。在每个时间点，收集培养物上清液以回收细胞外细菌或裂解粘附细胞以释放细胞内细菌。

[0438] 暴露于仅有的万古霉素的浮游细菌被有效地杀死。在培养一天后，存活细菌未恢复(图1D)。当将类似数量的浮游细菌接种在MG63成骨细胞上时，回收了通过入侵成骨细胞而抵抗万古霉素的感染后一天与MG63细胞相关的少量存活细菌(约0.06％的输入)。

[0439] 隔离在腹膜细胞内的MRSA在万古霉素存在下显示出存活率和感染效率的显著增加。在万古霉素杀死浮游细菌培养物的相同条件下，白细胞中约15％的细胞内MRSA存活。在万古霉素存在下，细胞内的细菌也能更好地感染MG63成骨细胞的单层，导致在暴露于万古霉素后一天回收的细菌增加一倍(图1D)。此外，在MG63细胞(图1E)、原代人脑内皮细胞(图1F)和A549支气管上皮细胞(未显示)中，在持续暴露于杀死游离活细菌的万古霉素浓度下，细胞内金黄色葡萄球菌在24小时内能够增加近10倍。虽然保护免受抗生素杀伤，但在感染的腹膜巨噬细胞和嗜中性粒细胞的培养物中未发生细菌生长(未显示)。这些数据一起支持髓系细胞中MRSA的细胞内储库可以促进感染传播到新的位点，甚至在活性抗生素治疗存在的情况下，并且甚至在持续的抗生素治疗的条件下，细胞内生长可以在内皮和上皮细胞中发生。

[0440] 实施例3抗SDR和其他抗体的产生

[0441] 为了产生mAb rF1，使用FACSAria细胞分选仪(BD,San Jose,CA)从MRSA感染的供体外周血中分离$CD19^+CD3^-CD27^+IgD^-IgA^-$记忆B细胞。在用B细胞淋巴瘤(Bcl)-xL和Bcl-6基因病毒转导之前，如以前在Kwakkenbos MJ,等人(2010)Nat Med 16:123-128中所述，在白

细胞介素-21存在的情况下，记忆细胞在表达CD40L的小鼠L成纤维细胞上被激活。转导的B细胞保持在相同的培养系统中。供体血液的使用由机构委员会批准。通过ELISA测定与MSSA菌株Newman的裂解物的反应性，从培养物上清液中选择单克隆抗体(mAb)rF1；阳性孔是亚克隆并通过ELISA重新测试两次。通过使用pcDNA3.1(Invitrogen)克隆具有人IgG1κ恒定区的重链和轻链可变区，并转染到293T细胞(ATCC)中来产生重组rF1。使用蛋白A偶联**SEPHAROSE®**(Invitrogen)从培养物上清液获得纯化的IgG。在US 8,617,556(Beaumont等人)和Hazenbos等人(2103)PLOS Pathogens9(10):1-18中描述了mAb rF1及其变体的产生。其全部内容通过引用并入本文。

[0442] 使用Symplex™技术从金黄色葡萄球菌感染患者的外周B细胞中克隆人IgG1mAbs SD2、SD3和SD4(全部针对糖基化SDR蛋白)和4675(人IgG1抗ClfA)，该技术保护抗体重链和轻链的同源配对[34]。血浆和记忆B细胞都用作重组全长IgG库的基因来源(手稿准备中)。通过转染哺乳动物细胞表达单个抗体克隆[35]。7天后收获含有全长IgG1抗体的上清液，用于筛选通过ELISA结合的抗原。抗体4675、SD2、SD3和SD4对结合来自USA300或Newman金黄色葡萄球菌菌株的细胞壁制剂是阳性的。随后在200ml瞬时转染中产生抗体，并用蛋白A色谱(MabSelect SuRe,GE Life Sciences,Piscataway,NJ)纯化，用于进一步测试。这些抗体的分离和使用经区域伦理审查委员会批准。生成rF1变体。

[0443] 通过用各自的重组蛋白免疫小鼠产生针对ClfA(9E10)、ClfB、(10D2)、SdrD(17H4)、IsdA(2D3)和未修饰的SDR蛋白(9G4)的小鼠mAb，所述重组蛋白使用标准方案在大肠杆菌中表达后纯化；通过蛋白A亲和层析纯化杂交瘤上清液。通过用肽聚糖(PGN)衍生的肽CKKGGG-(L-Ala)-(D-γ-Glu)-(L-Lys)-(D-Ala)-D-Ala)免疫兔、然后克隆IgG来产生兔单克隆抗体28.9.9。

[0444] 实施例4从MRSA感染的供体分离的高调理单克隆抗体(rF1)的表征

[0445] 如上所述，分离来自MRSA感染供体的外周血记忆B细胞的几种金黄色葡萄球菌反应性单克隆抗体(mAb)。当表征这些抗体时，鉴定出一种IgG1 mAb(以下称为rF1)，其对一组金黄色葡萄球菌菌株具有广泛的反应性，其通过人多形核白细胞(PMN)诱导强烈的调理性吞噬细胞杀伤(OPK)。

[0446] 来自临床MRSA菌株USA300的mAb rF1与细菌的最大结合比同种型匹配的抗ClfA mAb高约10倍(图5A)。与增加的结合一致，rF1的调理作用导致通过PMN的USA300的增加的摄取(图5B)和杀伤(图5C)。相比之下，人类抗ClfA的预调理对细菌活力没有影响(图5C)。在没有PMN的情况下，rF1抗体不影响USA300的生存能力。因此，rF1是具有结合MRSA的能力的mAb并且通过PMN诱导MRSA的有效杀伤。

[0447] 实施例5 rF1与葡萄球菌菌株的结合

[0448] 与来自培养物或感染组织的完整细菌结合的rF1的FACS分析

[0449] 从TSA平板或TSB培养物中收获完整细菌，并用不含酚红的补充有0.1％IgG游离BSA(Sigma)和10mM Hepes、pH 7.4(HB缓冲液)的HBSS进行洗涤。细菌(20×10^8CFU/mL)与300μg/mL兔IgG(Sigma)在HB缓冲液中在室温(RT)下孵育1小时以阻断非特异性IgG结合。细菌用2μg/mL的一抗进行染色，包括rF1或同种型对照IgG1mAb gD:5237(Nakamura GR等人(1993)J Virol 67:6179-6191)，接下来用荧光的抗人IgG二抗(Jackson Immunoresearch,West Grove,PA)染色。洗涤细菌并通过**FACSCalibur®**(BD)分析。

[0450] 对于来自感染的小鼠组织的细菌的抗体染色，将6-8周龄的雌性C57Bl/6小鼠(Charles River,Wilmington,MA)静脉内注射在PBS中的10^8CFU的对数期生长的USA300。感染后两天收获小鼠器官。如Tattevin P等人(2010)抗菌药物和化学治疗(Antimicrobial Agents and chemotherapy)54:610-613所述，建立了兔感染的心内膜炎(IE)。兔被静脉注射$5x10^7$CFU的稳定期生长的MRSA菌株COL，并在18小时后收获心脏赘生物。在用7×10^7CFU的稳定期COL感染后18小时，静脉内给予30mg/kg万古霉素治疗，每日两次。

[0451] 为了裂解小鼠或兔细胞，使用**gentleMACS®**细胞分离器(Miltenyi)，在M管(Miltenyi,Auburn,CA)中将组织匀浆，随后在含有0.1%Triton-X100(Thermo)、10μg/mL DNAseI(Roche)和Complete Mini蛋白酶抑制剂混合物(Roche)的PBS中在室温下温育10分钟。将悬浮液通过40微米过滤器(BD)，细菌用如上所述的mAb染色。通过用20μg/mL鼠mAb 702抗金黄色葡萄球菌肽聚糖(abcam,Cambridge,MA)和荧光染料标记的抗鼠IgG二抗(Jackson Immunoresearch)进行双重染色，将细菌与小鼠器官碎片区分。在流式细胞术分析过程中，从双荧光图中选择用mAb 702染色阳性的细菌。所有动物实验均由Genentech机构审查委员会和加州大学旧金山分校批准。

[0452] 流式细胞术(FCM)分析显示了rF1与测试的所有15种金黄色葡萄球菌菌株的有效结合活性(图7)。这些菌株广泛分布在金黄色葡萄球菌种系发生中[8]。由于细菌细胞表面抗原的表达水平在体外和体内生长之间可能不同，我们还测试了rF1在全身感染后识别从各种小鼠组织中分离出来的USA300的能力。rF1mAb与感染的小鼠肾脏、肝脏和肺部的USA300强烈结合(图6)。来自小鼠肾脏的USA300结合rF1持续至感染后至少8天(未显示)，表明感染期间rF1表位的强烈长期表达。另外，在感染性心内膜炎兔模型中，rF1与来自心脏赘生物的MRSA COL细菌强烈结合。用万古霉素治疗不影响rF1与MRSA的反应性(图6)。因此，rF1识别的抗原在各种菌株中是保守的，并且在各种生长和感染条件下稳定表达。

[0453] 鉴于rF1对所有金黄色葡萄球菌菌株的反应性的普遍性质，进行实验以观察这种反应性是否延伸至其他革兰氏阳性细菌。值得注意的是，只有凝固酶阴性的人类病原体表皮葡萄球菌才能检测到rF1结合(图7)。rF1mAb不与任何其他所测试的葡萄球菌物种结合，包括腐生性葡萄球菌、路邓葡萄球菌(S.lugdunensis)、溶血性葡萄球菌和肉葡萄球菌(S.carnosus)，或其他革兰氏阳性物种如酿脓链球菌(Streptococcus pyogenes)、枯草芽孢杆菌(Bacillus subtilis)、粪肠球菌(Enterococcus faecalis)、和单核细胞增生利斯特菌(Listeria monocytogenes)(图7)。因此，rF1是人抗体，其结合人适应的葡萄球菌病原体上稳定表达的表面抗原，并促进通过人PMN的细菌杀伤。

[0454] 实施例6 rF1抗体的氨基酸修饰

[0455] 总之，将每个rF1Ab的VH区域克隆出来并连接到人H链γ1恒定区，VL连接到κ恒定区，以表达作为IgG1的Ab。野生型序列在某些位置被改变以提高抗体的稳定性，同时保持如下所述的抗原结合。然后生成半胱氨酸改造的抗体(ThioMabs，也称为THIOMAB™)。

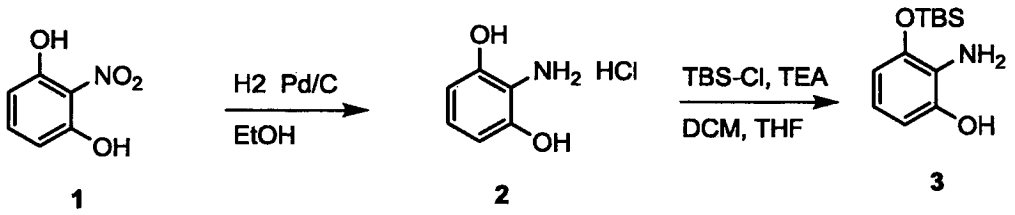
[0456] i.产生稳定性变体

[0457] 工程化改造rF1抗体以改善某些性质(避免脱酰胺、天冬氨酸异构化、氧化或N-连接糖基化)，并测试氨基酸置换后抗原结合的保留和化学稳定性。氨基酸改变如US8,617,556中所述。

[0458] iii.生成Cys工程改造的突变体(ThioMabs)

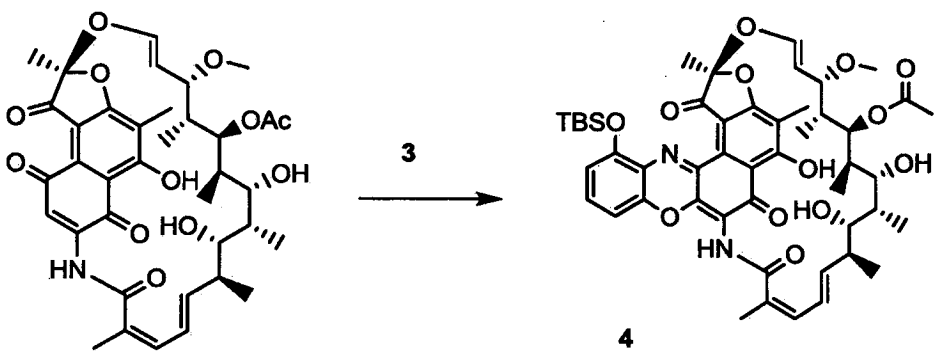
[0459] 如前所述在预定位置将半胱氨酸引入H链(CH1)或L链(Cκ)中,产生全长ThioMabs,例如在L链的κ恒定区中的V205和人γ1H链的位置A118(根据Eu协议的氨基酸位置编号),以允许抗体与接头-抗生素中间体缀合。然后将H和L链克隆到不同的质粒中,并将编码H和L的质粒共转染到293细胞中,其中它们被表达并组装成完整的抗体。H和L链也可以克隆到相同的表达质粒中。IgG1具有2个工程化改造的Cys,每个H链中有一个;或2个工程化改造的Cys,每个L链中有一个;或在每个H和L链中的工程化改造的Cys的组合,导致每个抗体四聚体有4个工程化改造的Cys,这些都是通过表达cys突变体链和野生型链的所需组合而产生。

[0460] 实施例7哌啶基苯并噁嗪并利福霉素(pipBOR)5

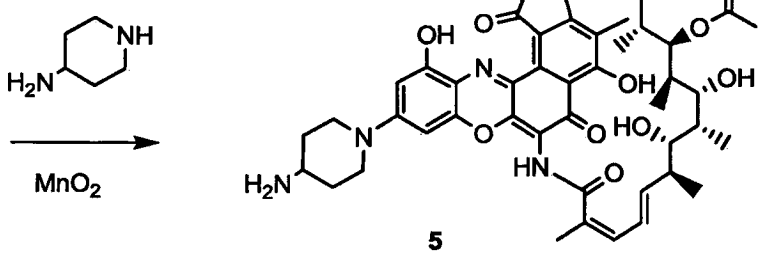


1 2 3

[0461]



利福霉素 S 4

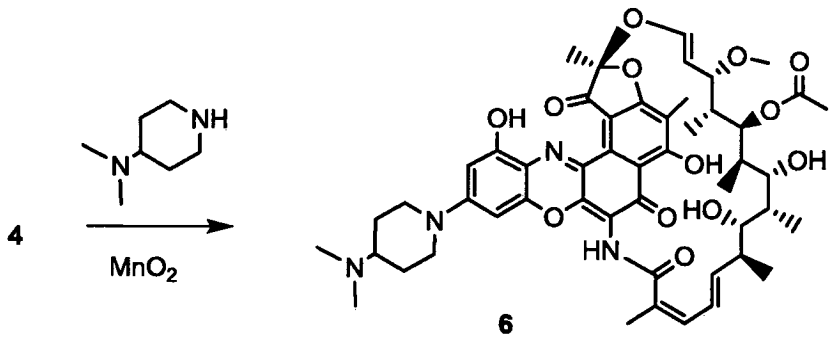


5

[0462] 将2-硝基苯-1,3-二醇1在氢气下用钯/碳催化剂在乙醇溶剂中氢化,得到2-氨基苯-1,3-二醇2,分离为盐酸盐。用叔丁基二甲基硅烷基氯和三乙胺在二氯甲烷/四氢呋喃中单保护2,得到2-氨基-3-(叔丁基二甲基硅烷氧基)苯酚3。利福霉素S(ChemShuttle Inc.,Fremont,CA,US 7342011;US 7271165;US 7547692)与3通过用氧化锰或氧气在甲苯中氧化缩合,在室温下反应得到TBS保护的苯并噁嗪并利福霉素4。LCMS(ESI):$M+H^+$=915.41。4与哌啶-4-胺和氧化锰反应得到哌啶基苯并噁嗪并利福霉素(pipBOR)5。LCMS(ESI):$M+H^+$=899.40

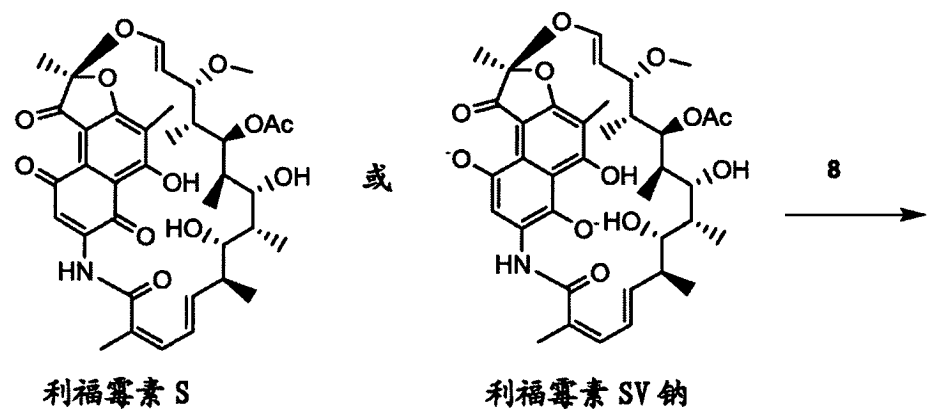
[0463] 实施例8二甲基pipBOR 6

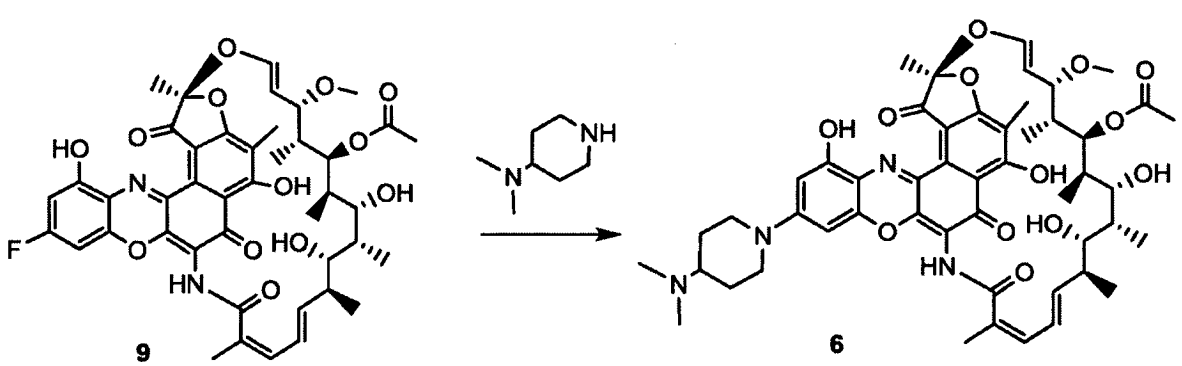
[0464]



[0465] N,N-二甲基哌啶-4-胺与TBS-保护的苯并噁嗪并利福霉素4反应得到二甲基哌啶基苯并噁嗪并利福霉素(二甲基pipBOR)6

[0466]

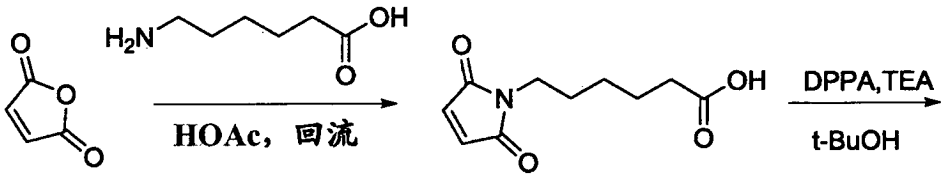




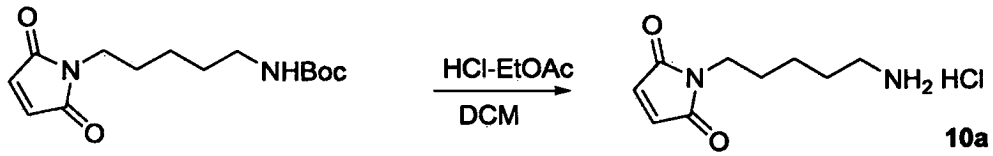
[0467] 或者,(5-氟-2-硝基-1,3-亚苯基)双(氧基)双(亚甲基)二苯7在氢气下用钯/碳催化剂在四氢呋喃/甲醇溶剂中氢化以除去苄基,得到2-氨基-5-氟苯-1,3-二醇8。LCMS(ESI):$M+H^+$=144.04。将市售的利福霉素S或利福霉素SV钠盐(ChemShuttle Inc.,Fremont,CA)与2-氨基-5-氟苯-1,3-二醇8通过在乙酸乙酯中在空气或铁氰化钾中氧化缩合而在60℃下反应,得到氟苯并噁嗪并利福霉素9。用N,N-二甲基哌啶-4-胺置换氟化物得到二甲基pipBOR 6。LCMS(ESI):$M+H^+$=927.43

[0468] 实施例9(S)-N-(5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基)-N-(1-(4-(羟甲基)苯基氨基)-1-氧代-5-脲基戊烷-2-基)环丁烷-1,1-二甲酰胺10

[0469] 步骤1:1-(5-氨基戊基)-1H-吡咯-2,5-二酮盐酸盐10a的制备



[0470]

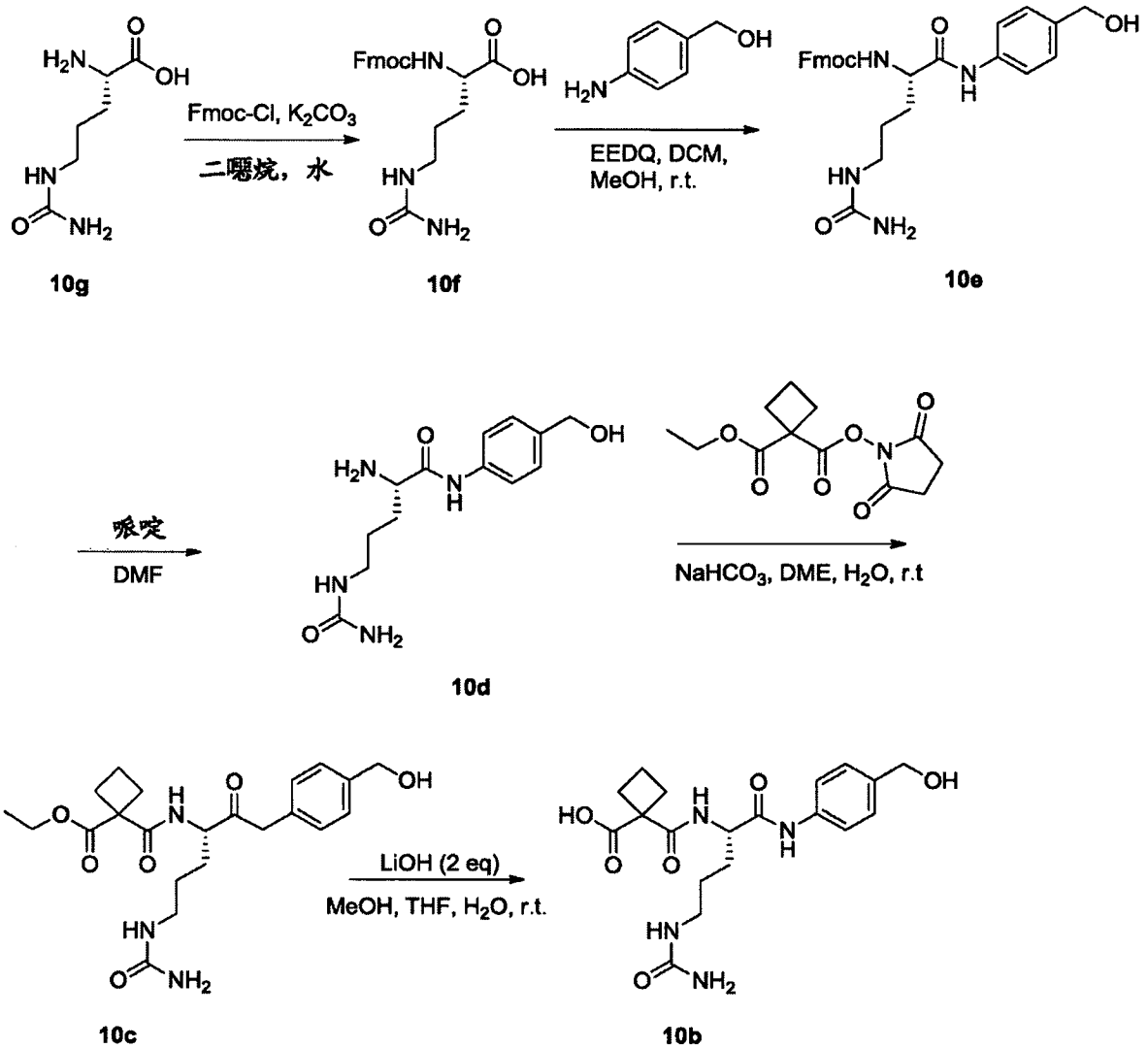


10a

[0471] 将马来酸酐、呋喃-2,5-二酮(150g,1.53mol)加入到在HOAc(1000mL)中搅拌的6-氨基己酸(201g,1.53mol)溶液中。混合物在室温下搅拌2小时后,回流加热8小时。在减压下除去有机溶剂,并且用EtOAc(500mL×3)萃取残余物,用H_2O洗涤。将合并的有机层用Na_2SO_4干燥并浓缩,得到粗产物。用石油醚洗涤,得到白色固体状的6-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)己酸(250g,77.4%)。将DPPA(130g,473mmol)和TEA(47.9g,473mmol)加入到在t-BuOH(200mL)中的6-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)己酸(100g,473mmol)溶液中。将混合物在N_2下加热回流8小时。将混合物浓缩,通过硅胶柱层析(PE:EtOAc=3:1)纯化残余物,得到5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基氨基甲酸叔丁酯(13g,10%)。向5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基氨基甲酸叔丁酯(28g,992mmol)的无水EtOAc(30mL)溶液中逐滴加入HCl/EtOAc(50mL)。混合物在室温下搅拌5小时后,过滤,将固体干燥,得到1-(5-氨基戊基)-1H-吡咯-2,5-二酮盐酸盐10a(16g,73.7%)。^1H NMR(400MHz,DMSO-d_6):δ8.02(s,2H),6.99(s,2H),3.37-3.34(m,2H),2.71-2.64(m,2H),1.56-1.43(m,4H),1.23-1.20(m,2H).

[0472] 步骤2:制备(S)-1-(1-(4-(羟甲基)苯基氨基)-1-氧代-5-脲基戊-2-基氨基甲酰基)环丁烷甲酸10b

[0473]



[0474] 向二噁烷和H_2O(50mL/75mL)混合物中的(S)-2-氨基-5-脲基戊酸10g(17.50g,0.10mol)混合物中加入K_2CO_3(34.55g,0.25mol)。在0℃下缓慢加入Fmoc-Cl(30.96g,0.12mol)。将反应混合物历经2小时温热至室温。减压下除去有机溶剂,用6M HCl溶液将水浆体调至pH=3,用EtOAc(100mL×3)萃取。有机层用Na_2SO_4干燥,过滤并减压浓缩,得到(S)-2-((((9H-芴-9-基)甲氧基)羰基)氨基)-5-脲基戊酸10f(38.0g,95.6%)。10f可商购。

[0475] 向DCM和MeOH(100mL/50mL)混合物中的10f(4g,10mmol)溶液中加入(4-氨基苯基)甲醇(1.6g,13mmol,1.3当量)和2-乙氧基-1-乙氧基羰基-1,2-二氢喹啉,即EEDQ、Sigma-Aldrich CAS登记号16357-59-8(32g,13mmol,1.3当量)。混合物在N_2下在室温搅拌16小时后,将其浓缩得到棕色固体。加入MTBE(200mL),在15℃下搅拌2小时。通过过滤收集固体,用MTBE(50mL×2)洗涤,得到(1-((4-(羟甲基)苯基)氨基)-1-氧代-5-脲基戊-2-基)氨基甲酸(S)-(9H-芴-9-基)甲基酯10e,为橙色固体(4.2g,84%)。LCMS(ESI):m/z 503.0[M+1]。

[0476] 在室温下向无水DMF(20ml)中的10e(4.2g,8.3mmol)搅拌溶液中滴加哌啶(1.65mL,17mmol,2当量)。将混合物在室温下搅拌30分钟,形成固体沉淀物。加入无水DCM(50mL),立即使混合物变得透明。将混合物在室温下搅拌另外30分钟,LCMS显示10e被消耗。将其在减压下浓缩至干(确保不存在哌啶),并将残余物在EtOAc和H_2O(50mL/20mL)之间分

配。水相用EtOAc(50mL×2)洗涤并浓缩,得到油状残余物(2.2g,94%)(含少量DMF)的(S)-2-氨基-N-(4-(羟甲基)苯基)-5-脲基戊酰胺10d。

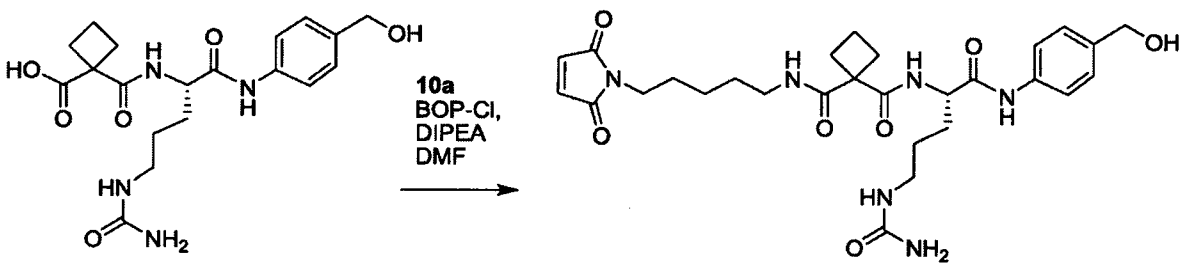
[0477] 将市售的1,1-环丁烷二甲酸,1,1-二乙基酯(CAS登记号3779-29-1)通过碱水溶液的限制皂化转化成半酸/酯1,1-环丁烷二甲酸,1-乙酯(CAS登记号5450-84-9),并用偶联试剂如TBTU(O-(苯并三唑-1-基)-N,N,N',N'-四甲基脲四氟硼酸盐,也称为N,N,N',N'-四甲基-O-(苯并三唑-1-基)脲四氟硼酸盐(CAS No.125700-67-6,Sigma-Aldrich B-2903)和N-羟基琥珀酰亚胺活化为NHS酯,即1-(2,5-二氧代吡咯烷-1-基)1-乙基环丁烷-1,1-二甲酸酯。

[0478] 向DME(50mL)中的1-(2,5-二氧代吡咯烷-1-基)1-乙基环丁烷-1,1-二甲酸酯(8g,29.7mmol)的溶液中加入在水(30mL)中的10d(6.0g,21.4mmol)和$NaHCO_3$(7.48g,89.0mmol)溶液。混合物在室温下搅拌16个小时后,减压浓缩至干,残余物通过柱色谱法(DCM:MeOH=10:1)纯化,得到白色固体(6.4g,68.7%)的(S)-乙基1-((1-(4-(羟甲基)苯基)-2-氧代-6-脲基己烷-3-基)氨基甲酰基)环丁烷甲酸酯10c。LCMS(ESI):m/z 435.0[M+1]

[0479] 在室温下向THF和MeOH(20mL/10mL)混合物中的搅拌的10c(6.4g,14.7mmol)溶液中加入在H_2O(20mL)中的$LiOH \cdot H_2O$(1.2g,28.6mmol)溶液。反应混合物在室温下搅拌16小时后,减压除去溶剂,所得残余物通过制备型HPLC纯化,得到(S)-1-(1-(4-(羟甲基)苯基氨基)-1-氧代-5-脲基戊-2-基氨基甲酰基)环丁烷甲酸10b(3.5g,产率:58.5%)。LCMS(ESI):m/z 406.9[M+1].^1H NMR(400MHz,甲醇-d_4)δ8.86(d,J=8.4Hz,2H),8.51(d,J=8.4Hz,2H),5.88-5.85(m,1H),5.78(s,2H),4.54-4.49(m,3H),4.38-4.32(m,1H),3.86-3.75(m,1H),3.84-3.80(m,2H),3.28-3.21(m,1H),3.30-3.24(m,1H),3.00-2.80(m,1H),2.37-2.28(m,2H)。

[0480] 步骤3:S)-N-(5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基)-N-(1-(4-(羟甲基)苯基氨基)-1-氧代-5-脲基戊-2-基)环丁烷-1,1-二甲酰胺10的制备

[0481]

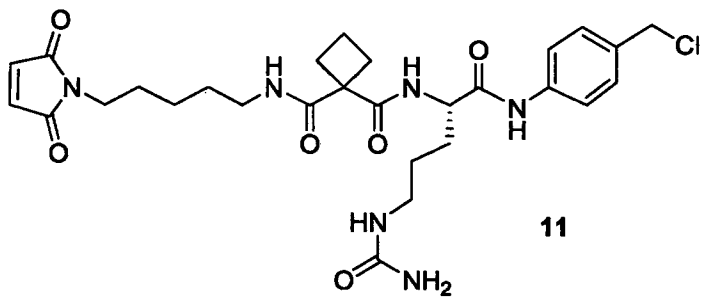


10b 10

[0482] 在0℃将二异丙基乙胺、DIPEA(1.59g,12.3mmol)和双(2-氧代-3-噁唑烷基)次膦酰氯、BOP-Cl(CAS登记号68641-49-6,Sigma-Aldrich,692mg,2.71mmol)加入到DMF(10mL)中的(S)-1-(1-(4-(羟甲基)苯基氨基)-1-氧代-5-脲基戊-2-基氨基甲酰基)环丁烷甲酸10b(1g,2.46mmol)溶液中,然后加入1-(5-氨基戊基)-1H-吡咯-2,5-二酮盐酸盐10a(592mg,2.71mmol)。将混合物在0℃下搅拌0.5小时。将反应混合物用柠檬酸溶液(10mL)淬灭,用DCM/MeOH(10:1)萃取。将有机层干燥并浓缩,残余物通过硅胶柱色谱(DCM:MeOH=10:1)纯化,得到S)-N-(5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基)-N-(1-(4-(羟甲基)苯基氨基)-1-氧代-5-脲基戊-2-基)环丁烷-1,1-二甲酰胺10(1.0g,71%),也称为MC-CBDK-cit-PAB-OH。LCMS(ESI):M+H+=571.28。^1H NMR(400MHz,DMSO-d6):δ10.00(s,1H),7.82-

7.77(m,2H),7.53(d,J=8.4Hz,2H),7.19(d,J=8.4Hz,2H),6.96(s,2H),5.95(t,J=6.4Hz,1H),5.39(s,2H),5.08(t,J=5.6Hz,1H),4.40-4.35(m,3H),4.09(d,J=4.8Hz,1H),3.01(d,J=3.2Hz,2H),3.05-2.72(m,4H),2.68-2.58(m,3H),2.40-2.36(m,4H),1.72-1.70(m,3H),1.44-1.42(m,1H),1.40-1.23(m,6H),1.21-1.16(m,4H)。

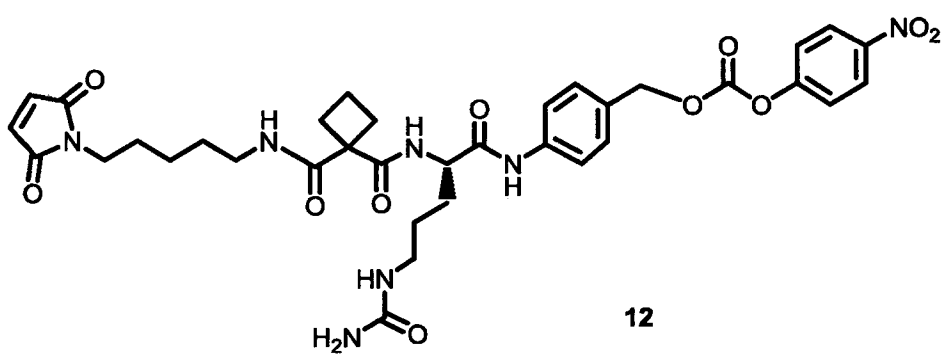
[0483] 实施例10(S)-N-(1-(4-(氯甲基)苯基氨基)-1-氧代-5-脲基戊-2-基)-N-(5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基)环丁烷-1,1-二甲酰胺11

[0484] 

11

[0485] 向在N,N-二甲基甲酰胺、DMF或N-甲基吡咯烷酮NMP(50mL)中的(S)-N-(5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基)-N-(1-(4-(羟甲基)苯基氨基)-1-氧代-5-脲基戊-2-基)环丁烷-1,1-二甲酰胺10(2.0g,3.5mmol)溶液中,在0℃逐渐滴加亚硫酰氯$SOCl_2$(1.25g,10.5mmol)。反应保持黄色。通过LC/MS监测反应,显示>90%转化。将反应混合物在20℃下搅拌30分钟或数小时后,用水(50mL)稀释,用EtOAc(50mL×3)萃取。将有机层干燥,浓缩并通过快速柱(DCM:MeOH=20:1)纯化,形成11,也称为MC-CBDK-cit-PAB-Cl,为灰色固体。LCMS:(5-95,AB,1.5min),0.696min,m/z=589.0$[M+1]^+$。

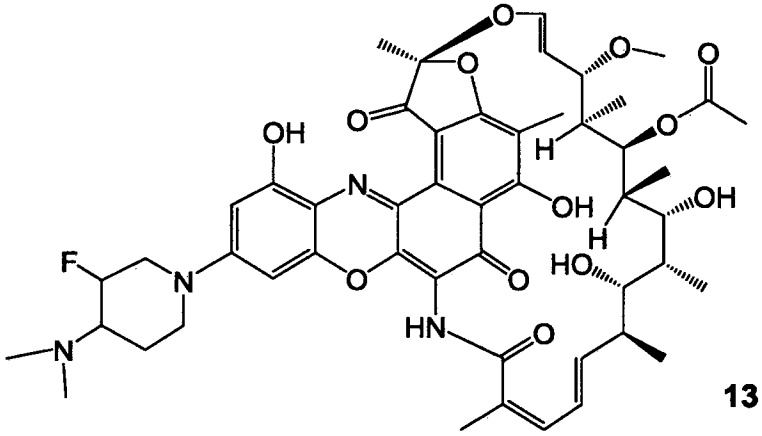
[0486] 实施例11(S)-4-(2-(1-(5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基氨基甲酰基)环丁烷甲酰胺基)-5-脲基戊酰氨基)苄基4-硝基苯基碳酸酯12

[0487] 

12

[0488] 向无水DMF中的(S)-N-(5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基)-N-(1-(4-(羟基甲基)苯基氨基)-1-氧代-5-脲基戊-2-基)环丁烷-1,1-二甲酰胺10的溶液中加入二异丙基乙胺(DIEA),然后加入PNP碳酸酯(碳酸二(4-硝基苯基)酯)。将反应溶液在室温(r.t.)下搅拌4小时,混合物通过制备型HPLC纯化,得到12。LCMS(ESI):$M+H^+$=736.29。

[0489] 实施例12 MC-(CBDK-cit)-PAB-(二甲基,氟代pipBOR)-PLA-1的制备

[0490] 

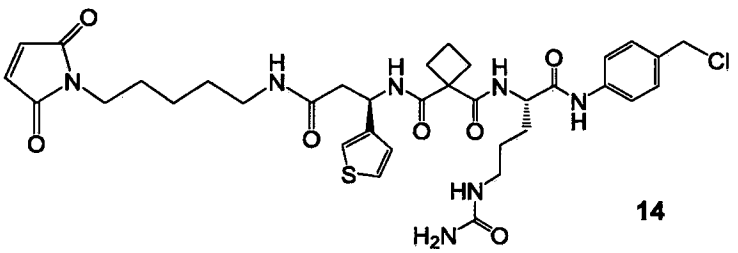
13

[0491] 按照PLA-2的方法，使(S)-N-(1-(4-(氯甲基)苯基氨基)-1-氧代-5-脲基戊-2-基)-N-(5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基)环丁烷-1,1-二甲酰胺11和氟代利福霉素衍生物二甲基氟代pipBOR 13(LCMS(ESI):M+H$^+$=945.43)反应形成MC-(CBDK-cit)-PAB-(二甲基,氟代pipBOR)-PLA-1，表2。LCMS(ESI):M+H$^+$=1499.7

[0492] 实施例13 MC-(CBDK-cit)-PAB-(二甲基pipBOR)-PLA-2的制备

[0493] 将DMF中的(S)-N-(1-(4-(氯甲基)苯基氨基)-1-氧代-5-脲基戊-2-基)-N-(5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基)环丁烷-1,1-二甲酰胺11(0.035mmol)溶液冷却至0℃，加入二甲基pipBOR 6(10mg,0.011mmol)。将混合物用另外0.5mL的DMF稀释。在空气中搅拌30分钟，加入N,N-二异丙基乙胺(DIEA,10μl,0.05mmol)，在空气中将反应物搅拌过夜。通过LC/MS观察到50%的所需产物。加入另外的0.2当量N,N-二异丙基乙胺碱，同时在空气中将反应物再搅拌6小时，直到反应停止进行。反应混合物用DMF稀释，并在HPLC(20-60% ACN/HCOOH在H_2O中)中纯化，得到MC-(CBDK-cit)-PAB-(二甲基pipBOR)-PLA-2，表2。LCMS(ESI):M+H$^+$=1481.8，产率31%。

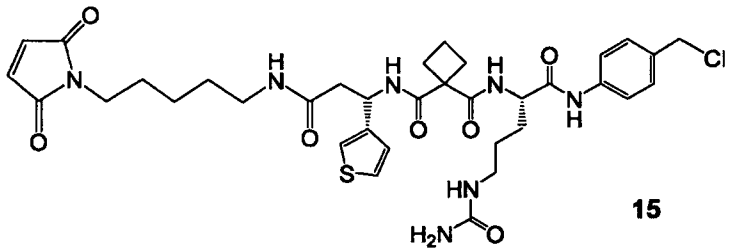
[0494] 实施例14 MC-((R)-噻吩-3-基-CBDK-cit)-PAB-(二甲基pipBOR)(PLA-3)的制备

[0495] 

14

[0496] 按照PLA-2的方法，将(N-((S)-1-(4-(氯甲基)苯基氨基)-1-氧代-5-脲基戊-2-基)-N-((R)-5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基氨基)-3-氧代-1-(噻吩-3-基)丙基)环丁烷-1,1-二甲酰胺14(LCMS(ESI):M+H$^+$=742.3)和二甲基pipBOR 6反应得到MC-((R)-噻吩-3-基-CBDK-cit)-PAB-(二甲基pipBOR)(PLA-3，表2)。LCMS(ESI):M+H$^+$=1633.9

[0497] 实施例15 MC-((S)-噻吩-3-基-CBDK-cit)-PAB-(二甲基pipBOR)(PLA-4)

[0498] 

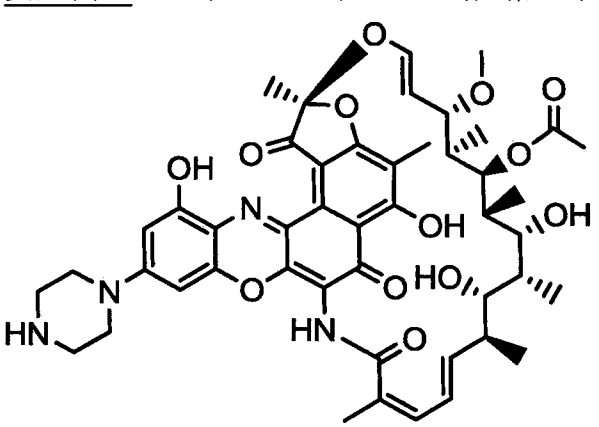
15

[0499] 按照PLA-2的方法，将(N-((R)-1-(4-(氯甲基)苯基氨基)-1-氧代-5-脲基戊-2-基)-N-((R)-5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基氨基)-3-氧代-1-(噻吩-3-基)丙基)环丁烷-1,1-二甲酰胺15(LCMS(ESI):M+H$^+$=742.3)和二甲基pipBOR 6反应得到MC-((R)-噻吩-3-基-CBDK-cit)-PAB-(二甲基pipBOR)(PLA-4,表2)。LCMS(ESI):M+H$^+$=1633.9

[0500] 实施例16 MC-(CBDK-cit)-PABC-(pipBOR)(PLA-5)的制备

[0501] 哌啶基苯并噁嗪并利福霉素(pipBOR)5(15mg,0.0167mmol)、然后(S)-4-(2-(1-(5-(2,5-二氧代-2,5-二氢-1H-吡咯-1-基)戊基氨基甲酰基)环丁烷甲酰氨基)-5-脲基戊酰氨基)苄基4-硝基苯基碳酸酯12(12mg,0.0167mmol)称重后加入小瓶中。加入二甲基甲酰胺DMF(0.3mL)，然后加入二异丙基乙胺DIEA(0.006ml,0.0334mmol)，并将反应物在室温下搅拌2小时。反应溶液通过HPLC(30-70%MeCN/水+1%甲酸)直接纯化，得到MC-(CBDK-cit)-PABC-(pipBOR)(PLA-5,表2)。LCMS(ESI):M+H$^+$=1496.5

[0502] 实施例17 MC-(CBDK-cit)-PABC-(哌嗪BTR)(PLA-6)的制备

[0503] 

16

[0504] 按照PLA-5的步骤，哌啶利福霉素衍生物即哌嗪BOR 16(LCMS(ESI):M+H$^+$=885.4)和(S)-4-(2-(1-(5-2,5-二氢-1H-吡咯-1-基)戊基氨基甲酰基)环丁烷甲酰氨基)-5-脲基戊酰氨基)苄基4-硝基苯基碳酸酯12反应，得到MC-(CBDK-cit)-PABC-(哌嗪BTR)(PLA-6表2)。LCMS(ESI):M+H$^+$=1482.5

[0505] 实施例18 rF1抗体-抗生素缀合物的制备

[0506] 通过将rF1抗体与PML接头-抗生素中间体(包括表2的那些)缀合制备抗体-抗生素缀合物(AAC)表3。在缀合之前，根据WO 2004/010957中描述的方法，使用标准方法用TCEP部分还原rF1抗体，其教导通过引用并入本文。根据例如在Doronina等人(2003)Nat.Biotechnol.21:778-784和US 2005/0238649 A中所述的方法，使用标准方法将部分还原的抗体与接头-抗生素中间体缀合。简言之，将部分还原的抗体与接头-抗生素中间体合并，以使接头-抗生素中间体与抗体的还原半胱氨酸残基缀合。将缀合反应淬灭，并纯化AAC。测定每个AAC的抗生素负荷(每个抗体的抗生素部分的平均数)，并且对于用单个半胱氨酸突变位点改造的rF1抗体而言是约1至2之间。

[0507] 用于缀合的ThioMabs还原/氧化:全长的半胱氨酸工程化改造的单克隆抗体(ThioMabs-Junutula等人,2008b Nature Biotech.,26(8):925-932;Dornan等人(2009) Blood 114(13):2721-2729;US 7521541;US 7723485;WO2009/052249,Shen等人(2012) Nature Biotech.,30(2):184-191;Junutula等人(2008)Jour of Immun.Methods 332:41-52)在CHO细胞中表达,并且用在含有2mM EDTA的50mM Tris pH 7.5中的约20-40倍过量的TCEP(三(2-羧基乙基)膦盐酸盐)或DTT(二硫苏糖醇)在37℃下还原3小时或在室温过夜(Getz等人(1999)Anal.Biochem.Vol 273:73-80;Soltec Ventures,Beverly,MA)。将还原的ThioMab稀释并加载到在10mM乙酸钠pH 5中的HiTrap S柱上,并用含有0.3M氯化钠的PBS洗脱。或者,通过加入1/20体积的10%乙酸将抗体酸化,用10mM琥珀酸盐pH 5稀释,加载到柱中,然后用10个柱体积的琥珀酸缓冲液洗涤。柱用50mM Tris pH7.5(含2mM EDTA)洗脱。

[0508] 用15倍摩尔过量的DHAA(脱氢抗坏血酸)或200nM硫酸铜水溶液(CuSO4)处理洗脱的还原ThioMab。链间二硫键的氧化在约3小时或更长时间完成。环境空气氧化也是有效的。将再氧化的抗体在20mM琥珀酸钠pH5,150mM NaCl,2mM EDTA中透析,并在-20℃冷冻保存。

[0509] ThioMabs与接头-抗生素中间体的缀合:将解封闭的、再氧化的硫代抗体(ThioMab)与6-8倍摩尔过量的表2中的接头-抗生素中间体(来自20mM浓度的DMSO储备液)在50mM Tris,pH8中反应,直到通过反应混合物的LC-MS分析测定反应完成(16-24小时)。

[0510] 然后将粗抗体-抗生素缀合物(AAC)在用20mM琥珀酸钠pH 5稀释后,施加到阳离子交换柱上。用至少10个柱体积的20mM琥珀酸钠pH 5洗涤该柱,并且用PBS洗脱抗体。使用凝胶过滤柱将AAC配制到含有240mM蔗糖的20mM His/乙酸盐pH5中。通过紫外光谱法鉴定AAC来测定蛋白质浓度,在用赖氨酸C内肽酶处理之前和之后通过分析型SEC(尺寸排阻色谱)进行聚集分析和LC-MS分析。

[0511] 使用Shodex KW802.5柱,在含0.25mM氯化钾和15%IPA的0.2M磷酸钾pH 6.2中以0.75ml/min的流速进行尺寸排阻色谱法。AAC的聚集状态通过280nm处的洗脱峰面积吸光度的积分来确定。

[0512] 使用Agilent QTOF 6520ESI仪器进行LC-MS分析。例如,使用该化学产生的AAC在Tris pH7.5中用1:500w/w胞内蛋白酶Lys C(Promega)处理,在37℃下处理30分钟。将所得切割片段加载到加热至80℃的1000A、8um PLRP-S柱上,并在5分钟内以30%B至40%B的梯度洗脱。流动相A:含0.05%TFA的H_2O。流动相B:含0.04%TFA的乙腈。流速:0.5ml/min。在电喷雾电离和MS分析之前,通过280nm处的紫外吸收检测来监测蛋白质洗脱。通常获得未结合的Fc片段、残留的非缀合Fab和抗生素-Fab的色谱分辨率。使用Mass Hunter ™软件(Agilent Technologies)将获得的m/z光谱解卷积以计算抗体片段的质量。

[0513] 使用含有工程改造的Cys205的SEQ ID NO.9的rF1L链,以及含有SEQ ID NO:10的rF1H链制备AAC 103(AAR=1.9)硫代-rF1-HC-121C,LC-V205C-MC-(CBDK-cit)-PAB-(二甲基pipBOR)。使用在前述含有工程改造的Cys205的SEQ ID NO.9的rF1L链,以及含有包含工程改造的Cys114(Kabat编号114与Eu编号118和序列编号121相同)的SEQ ID NO:12的rF1H链制备AAC 102(AAR=3.9)硫代-rF1-HC-121C,LC-V205C-MC-(CBDK-cit)-PAB-(二甲基pipBOR)。将Cys工程化改造的L和/或H链与如表2所示的PML接头和利福霉素型抗生素缀合。

[0514] 实施例19 rF1-AAC的体外功效

[0515] 将金黄色葡萄球菌(USA300NRS384菌株)与各种剂量(100μg/mL、10μg/mL、1μg/mL

或0.1μg/mL)的以下的抗金黄色葡萄菌的非缀合抗体孵育1小时:每个抗体负载1.9个平均抗生素分子(AAR2)的103AAC,或者每个抗体负载有3.9个平均抗生素分子(AAR4)的102AAC,以使抗体与细菌结合。将所得的调理细菌喂养小鼠巨噬细胞,并在37℃下孵育以允许吞噬(体外巨噬细胞测定)。2小时后,将感染混合物除去,并用补充有50μg/mL庆大霉素的正常生长培养基代替,以杀死任何剩余的细胞外细菌。2天后,通过在胰酶解大豆琼脂平板上将巨噬细胞裂解物的连续稀释液进行接种,测定存活的细胞内细菌总数。

[0516] 结果如图10所示。测试的AAC(AAR2与AAR4)均显示相似的剂量效应,并以10μg/mL或更高的剂量产生最大的杀死,在1μg/mL及更低剂量下部分杀死至不杀死,表明AAC的剂量效应受到细菌上抗体结合位点数目的限制。通过每个抗体加载4个抗生素分子,AAR4 AAC在所有测试剂量下对于AAC的细菌杀死和细菌的总体杀死都更好。在测试的最高剂量下,2DAR AAC将细菌负荷降低了350倍,而4AAR AAC将细菌负荷降低了超过4000倍。(虚线表示所示分析的检测限)。

[0517] 该实施例证明,表3的rF1-AAC 102(AAR=3.9)和103(AAR=1.9),即硫代-rF1-HC-121C,LC-V205C-MC-(CBDK-cit)-PAB-(二甲基pipBOR)在体外巨噬细胞测定中杀死了细胞内MRSA。结果如图10所示。

[0518] 实施例20 rF1-AAC的体内功效

[0519] 该实施例证明,在鼠静脉感染模型中,rF1-AAC可大大减少或消除细胞内金黄色葡萄球菌感染。

[0520] 腹膜炎模型。7周龄的雌性A/J小鼠(Jackson Laboratories)用5×10^7CFU的USA300通过腹膜注射感染。在感染后2天处死小鼠,并用5ml冷磷酸盐缓冲盐水溶液(PBS)冲洗腹膜。如下文所述,将肾脏在5mL PBS中匀浆用于静脉内感染模型。在台式离心机中,腹膜洗涤液在4℃以1,000rpm离心5分钟。收集上清液作为细胞外细菌,并且收集含有腹膜细胞的细胞沉淀作为细胞内部分。细胞在37℃下用50μg/mL的溶葡萄球菌素处理20分钟以杀死污染的细胞外细菌。将腹膜细胞在冰冷的PBS中洗涤3次,以在分析前除去溶葡萄球菌素。为了计数细胞内CFU的数量,将腹膜细胞在含有0.1%Triton-X的HB(补充有10mM HEPES和0.1%牛血清白蛋白的Hanks平衡盐溶液)中溶解,将裂解物在含有0.05%吐温-20的PBS中连续稀释。

[0521] 鼠静脉感染模型。对于涉及竞争性人IgG(SCID IVIG模型)的研究,CB17.SCID小鼠(Charles River Laboratories,Hollister,CA)用GammaGard S/D IGIV免疫球蛋白(ASD Healthcare,Brooks KY)重建,使用优化的给药方案以在血清中达到至少10mg/mL人IgG的恒定血清水平。给予IGIV,以每只小鼠30mg的初始静脉内剂量,然后在6小时后通过腹膜内注射15mg/小鼠的第二剂量,然后通过腹膜内注射来每日给予每只小鼠15mg剂量,连续3天。

[0522] 在第一剂量IGIV后4小时,通过静脉内注射用磷酸盐缓冲盐水稀释的1×10^7CFU MRSA(USA300 NRS384菌株)感染小鼠(对于每个抗体或AAC,n=8)。感染的小鼠用50mg/kg的rF1裸抗体、103 AAC DAR2或102 AAC DAR4处理。通过静脉注射在感染26h后给小鼠单次剂量AAC,在感染后第4天处死,并在5mL磷酸盐缓冲盐水中获取肾脏和心脏。使用GentleMACS Dissociator™(Miltenyi Biotec,Auburn,CA)将组织样品匀浆。通过将组织匀浆液在PBS 0.05%吐温中的连续稀释液在含有5%去纤维蛋白的羊血的胰酶解大豆琼脂平板上接种来测定每个器官回收的细菌总数。

[0523] 图11A显示在体内用AAC治疗对感染小鼠肾脏细菌负荷的结果。用含有每个抗体(DAR2)2个抗生素分子的AAC治疗将细菌负荷降低约30倍，用含有每个抗体(AAR4)4个抗生素分子的AAC治疗将细菌负荷降低了30,000倍以上。

[0524] 图11B显示在体内用AAC治疗对心脏内细菌计数的结果。使用AAC AAR2治疗使细菌负荷降低了约70倍，8只小鼠中有6只在心脏中具有不可检测的细菌水平；用AAC DAR4治疗彻底根除了心脏中的感染，导致8只小鼠中全部8只具有不可检测的细菌水平。

[0525] 虽然为了清楚理解的目的，已经通过说明和示例的方式对前述发明进行了详细描述，但这些描述和实施例不应被解释为限制本发明的范围。在整个说明书中引用的所有专利、专利申请和参考文献通过引用明确地并入本文。

序列表

<110> 基因技术公司等

<120> 抗金黄色葡萄球菌抗体利福霉素缀合物及其用途

<130> P32350-WO

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[0001]

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20 25 30

Leu Ala Trp Tyr Arg Gln Lys Pro Gly Lys Ala Pro Asn Leu Leu Ile
35 40 45

Tyr Lys Thr Ser Ile Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Asp Asp Phe Ala Thr Tyr Tyr Cys Gln His Tyr Met Arg Phe Pro Tyr
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
100 105 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
115 120 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
130 135 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln

145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
165 170 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
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1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Leu Ser Arg Phe
20 25 30

Ala Met Ser Trp Val Arg Gln Ala Pro Gly Arg Gly Leu Glu Trp Val
35 40 45

Ala Ser Ile Asn Ser Gly Asn Asn Pro Tyr Tyr Ala Arg Ser Val Gln
50 55 60

[0003]

Tyr Arg Phe Thr Val Ser Arg Asp Val Ser Gln Asn Thr Val Ser Leu
65 70 75 80

Gln Met Asn Asn Leu Arg Ala Glu Asp Ser Ala Thr Tyr Phe Cys Ala
85 90 95

Lys Asp His Pro Ser Ser Gly Trp Pro Thr Phe Asp Ser Trp Gly Pro
100 105 110

Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
115 120 125

Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala
130 135 140

Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
145 150 155 160

Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
165 170 175

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
180 185 190

Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys
195 200 205

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
210 215 220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile

245 250 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
260 265 270

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
275 280 285

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
290 295 300

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
305 310 315 320

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
325 330 335

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Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu
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Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
370 375 380

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
385 390 395 400

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
405 410 415

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
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[0004]

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
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<213> 人工序列

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Asp Ile Gln Leu Thr Gln Ser Pro Ser Ala Leu Pro Ala Ser Val Gly
1 5 10 15

Asp Arg Val Ser Ile Thr Cys Arg Ala Ser Glu Asn Val Gly Asp Trp
20 25 30

Leu Ala Trp Tyr Arg Gln Lys Pro Gly Lys Ala Pro Asn Leu Leu Ile
35 40 45

Tyr Lys Thr Ser Ile Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Asp Asp Phe Ala Thr Tyr Tyr Cys Gln His Tyr Ile Arg Phe Pro Tyr
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala

100 105 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
115 120 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
130 135 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145 150 155 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
165 170 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
180 185 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Cys Thr Lys Ser
195 200 205

Phe Asn Arg Gly Glu Cys
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1 5 10 15

[0005]

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Leu Ser Arg Phe
20 25 30

Ala Met Ser Trp Val Arg Gln Ala Pro Gly Arg Gly Leu Glu Trp Val
35 40 45

Ala Ser Ile Asn Ser Gly Asn Asn Pro Tyr Tyr Ala Arg Ser Val Gln
50 55 60

Tyr Arg Phe Thr Val Ser Arg Asp Val Ser Gln Asn Thr Val Ser Leu
65 70 75 80

Gln Met Asn Asn Leu Arg Ala Glu Asp Ser Ala Thr Tyr Phe Cys Ala
85 90 95

Lys Asp His Pro Ser Ser Gly Trp Pro Thr Phe Asp Ser Trp Gly Pro
100 105 110

Gly Thr Leu Val Thr Val Ser Ser Cys Ser Thr Lys Gly Pro Ser Val
115 120 125

Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala
130 135 140

Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser
145 150 155 160

Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
165 170 175

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro
180 185 190

Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys

195 200 205

Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp
210 215 220

Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly
225 230 235 240

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
245 250 255

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu
260 265 270

Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
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Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg
290 295 300

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
305 310 315 320

Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu
325 330 335

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
340 345 350

Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu
355 360 365

Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
370 375 380

[0006]

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
385 390 395 400

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
405 410 415

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His
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Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro
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Gly

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1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Leu Ser Arg Phe
20 25 30

Ala Met Ser Trp Val Arg Gln Ala Pro Gly Arg Gly Leu Glu Trp Val
35 40 45

Ala Ser Ile Asn Ser Gly Asn Asn Pro Tyr Tyr Ala Arg Ser Val Gln

50 55 60

Tyr Arg Phe Thr Val Ser Arg Asp Val Ser Gln Asn Thr Val Ser Leu
65 70 75 80

Gln Met Asn Asn Leu Arg Ala Glu Asp Ser Ala Thr Tyr Phe Cys Ala
85 90 95

Lys Asp His Pro Ser Ser Gly Trp Pro Thr Phe Asp Ser Trp Gly Pro
100 105 110

Gly Thr Leu Val Thr Val Ser Ser
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1 5 10 15

Asp Arg Val Ser Ile Thr Cys Arg Ala Ser Glu Asn Val Gly Asp Trp
20 25 30

Leu Ala Trp Tyr Arg Gln Lys Pro Gly Lys Ala Pro Asn Leu Leu Ile
35 40 45

Tyr Lys Thr Ser Ile Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

[0007]

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Asp Asp Phe Ala Thr Tyr Tyr Cys Gln His Tyr Met Arg Phe Pro Tyr
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val
100 105 110

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<211> 110
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<213> 人工序列

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1 5 10 15

Asp Arg Val Ser Ile Thr Cys Arg Ala Ser Glu Asn Val Gly Asp Trp
20 25 30

Leu Ala Trp Tyr Arg Gln Lys Pro Gly Lys Ala Pro Asn Leu Leu Ile
35 40 45

Tyr Lys Thr Ser Ile Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Asp Asp Phe Ala Thr Tyr Tyr Cys Gln His Tyr Ile Arg Phe Pro Tyr
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val
100 105 110

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1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Leu Ser
20 25 30

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[0008]

<220>
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Met Asn Asn Leu Arg Ala Glu Asp Ser Ala Thr Tyr Phe Cys Ala Lys
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Asp Arg Val Ser Ile Thr Cys
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<210> 21
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<210> 23
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[0009]

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Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
20 25 30

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
35 40 45

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
50 55 60

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
65 70 75 80

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
85 90 95

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
100 105 110

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
115 120 125

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
130 135 140

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
145 150 155 160

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
165 170 175

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
180 185 190

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
195 200 205

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Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
225 230 235 240

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245 250 255

[0010] Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
260 265 270

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
275 280 285

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
290 295 300

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
305 310 315 320

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
325 330 335

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
340 345 350

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
355 360 365

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
370 375 380

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
385 390 395 400

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
405 410 415

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
420 425 430

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp

435 440 445

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
450 455 460

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
465 470 475 480

Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
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Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp Ser Asp
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Ser Asp Ser Asp Ser Asp
545 550

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<210> 27
<211> 8
<212> PRT
<213> 葡萄球菌物种

<400> 27
Ser Asp Ser Asp Ser Asp Ser Asp
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<210> 28
<211> 6
<212> PRT
<213> 葡萄球菌物种.

<400> 28

[0012] Ser Asp Ser Asp Ser Asp
1 5

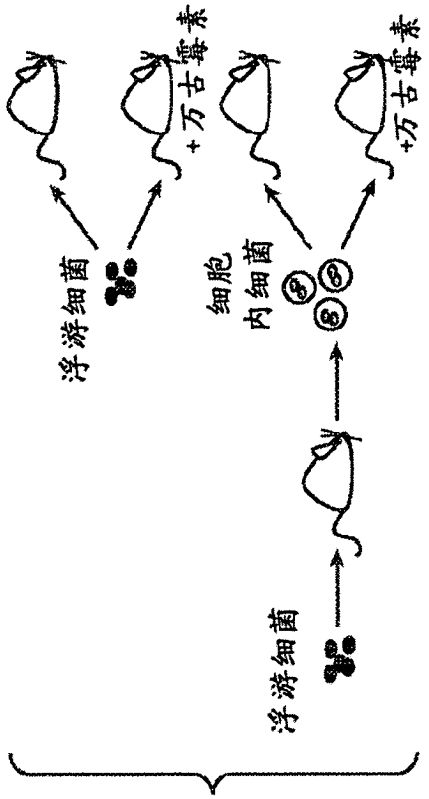
浮游细菌
+万古霉素
细胞
内细菌
浮游细菌
+万古霉素

图1A

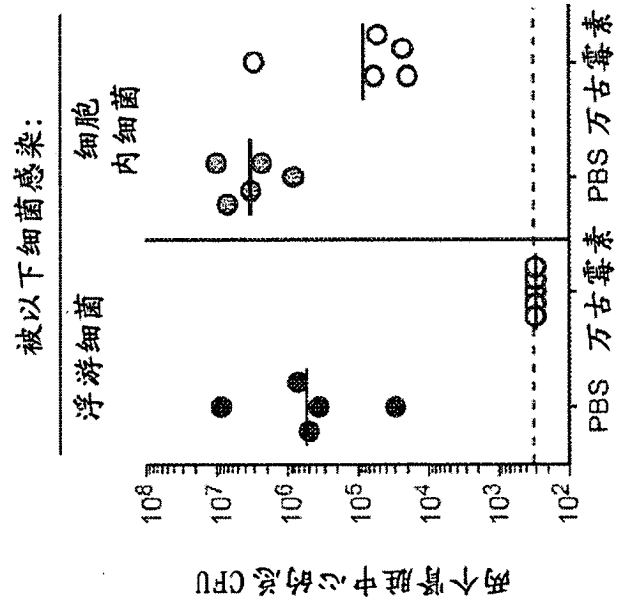
被以下细菌感染:
浮游细菌
细胞
内细菌
PBS
万古霉素
PBS
万古霉素
10^8
10^7
10^6
10^5
10^4
10^3
10^2

图1B

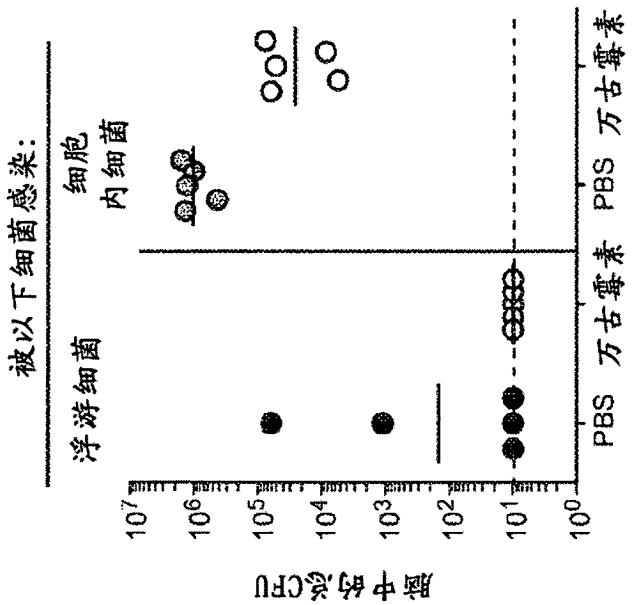
被以下细菌感染:
浮游细菌
细胞
内细菌
肺中的cfu
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
PBS
万古霉素
PBS
万古霉素

图1C

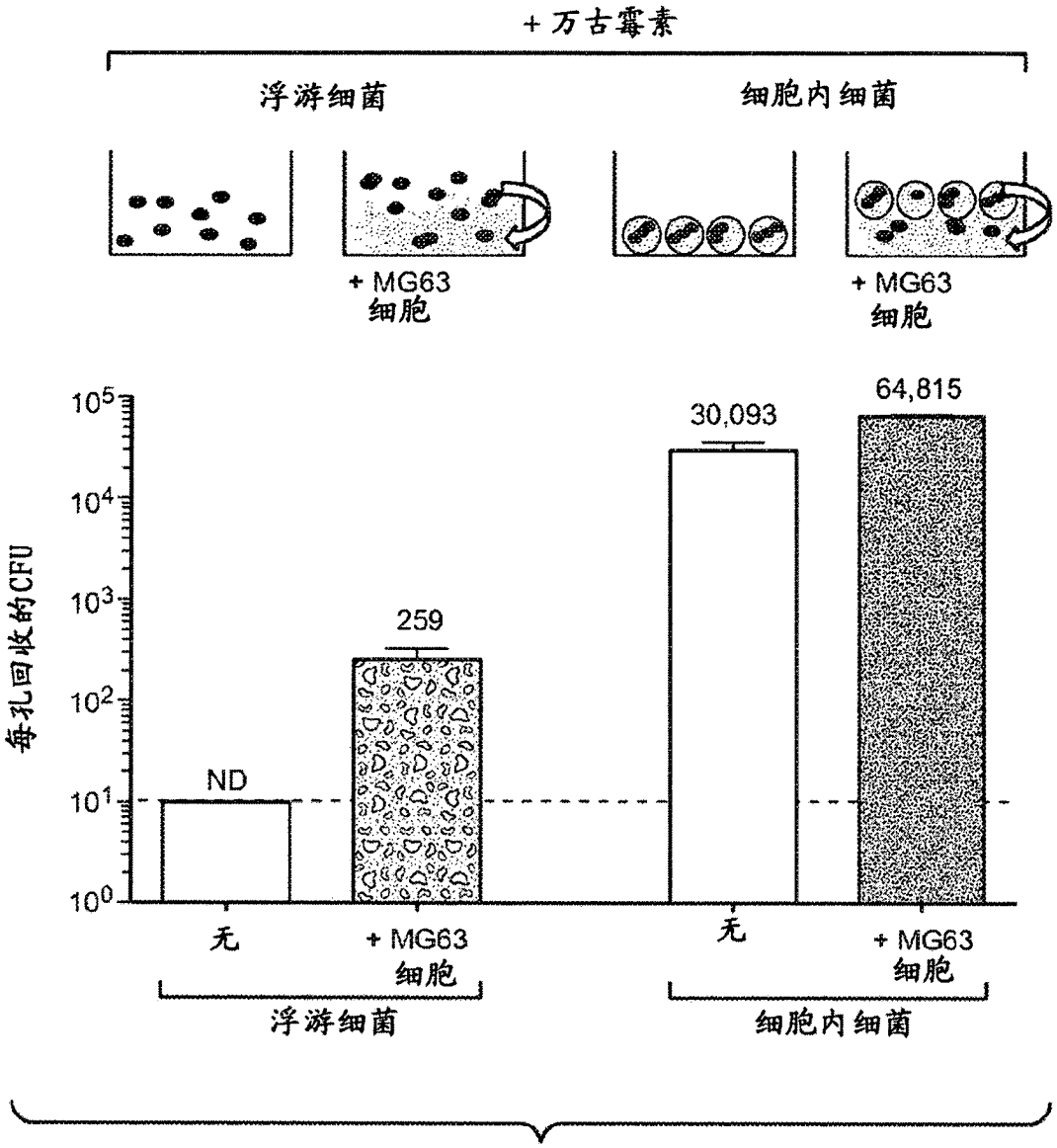
+万古霉素
浮游细菌
细胞内细菌
+ MG63 细胞
+ MG63 细胞
每孔回收的CFU
10^5
10^4
10^3
10^2
10^1
10^0
ND
259
30,093
64,815
无
+ MG63 细胞
无
+ MG63 细胞
浮游细菌
细胞内细菌

图1D

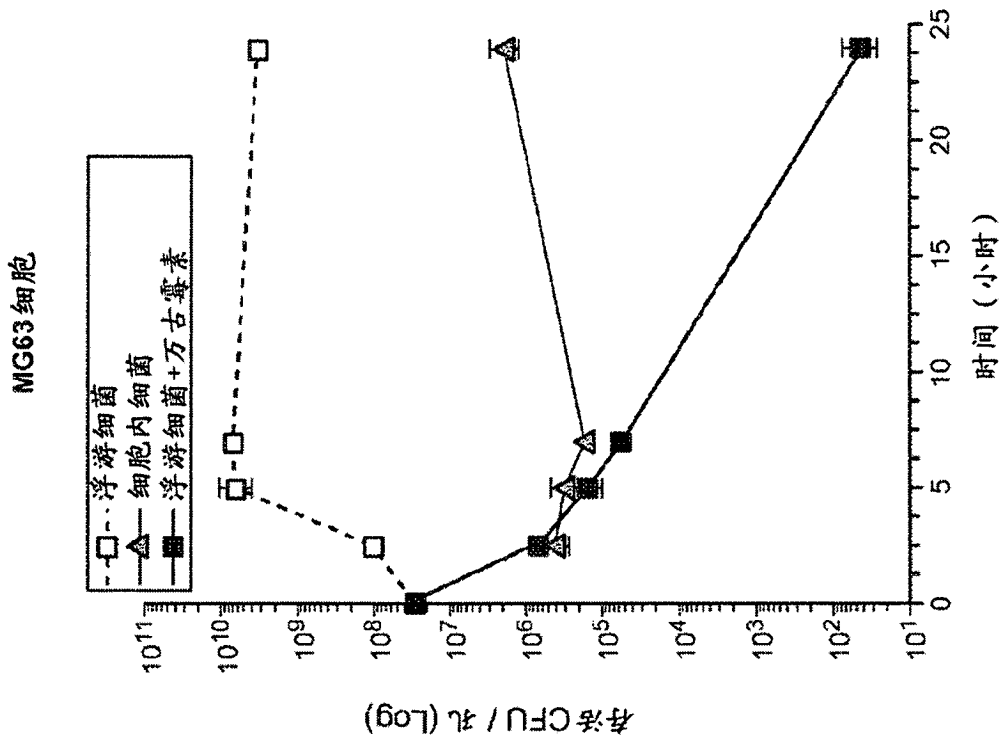
MG63细胞
浮游细菌
细胞内细菌
浮游细菌+万古霉素
存活CFU / 孔 (Log)
时间（小时）

图1E

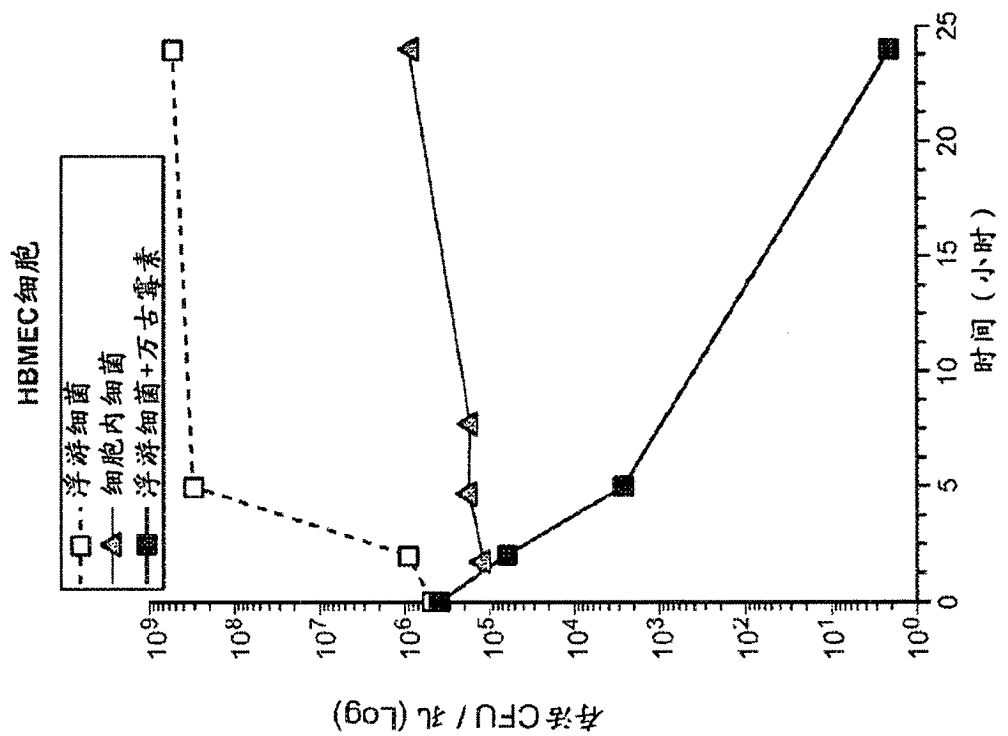
HBMEC细胞
浮游细菌
细胞内细菌
浮游细菌+万古霉素
存活CFU / 孔 (Log)
时间（小时）

图1F

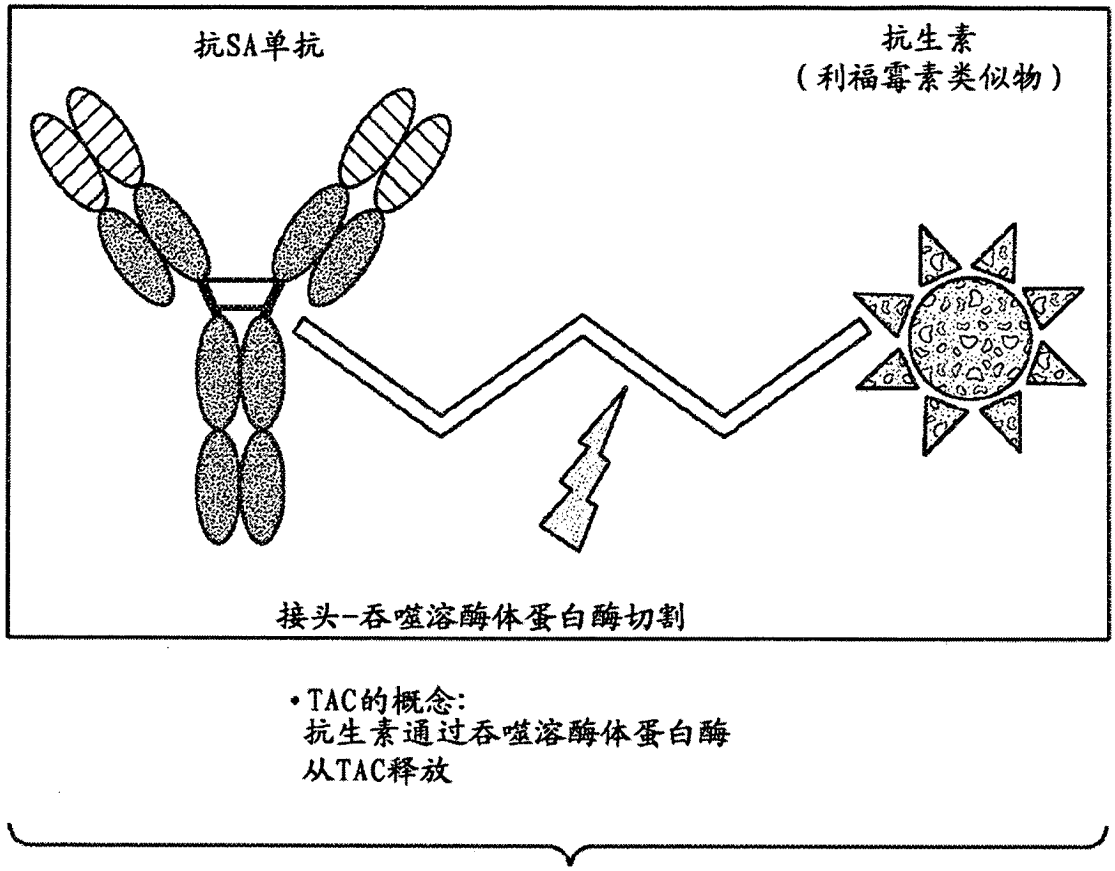
抗SA单抗
抗生素
（利福霉素类似物）
接头-吞噬溶酶体蛋白酶切割
•TAC的概念:
抗生素通过吞噬溶酶体蛋白酶
从TAC释放

图2

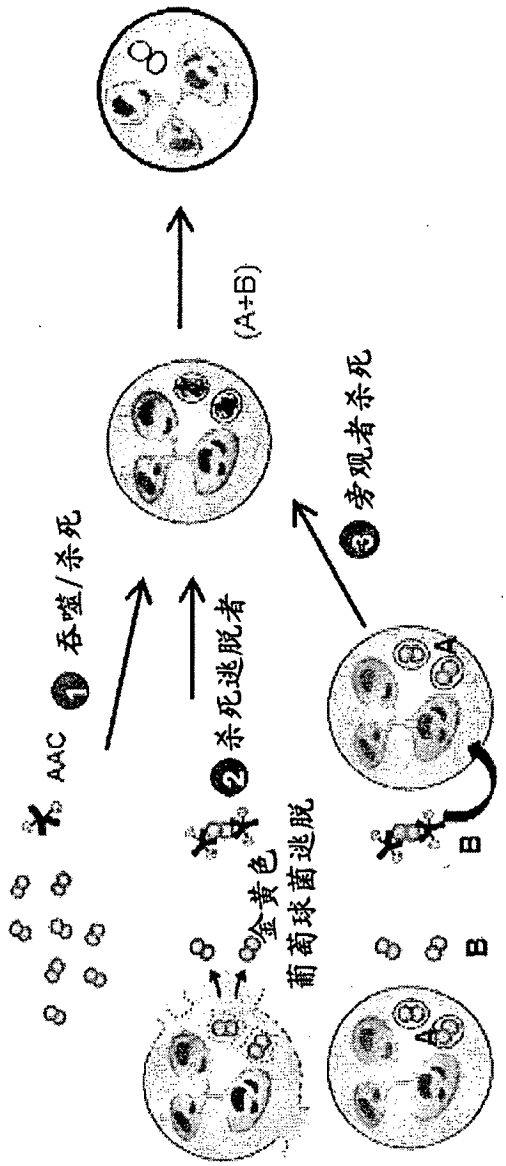

图3

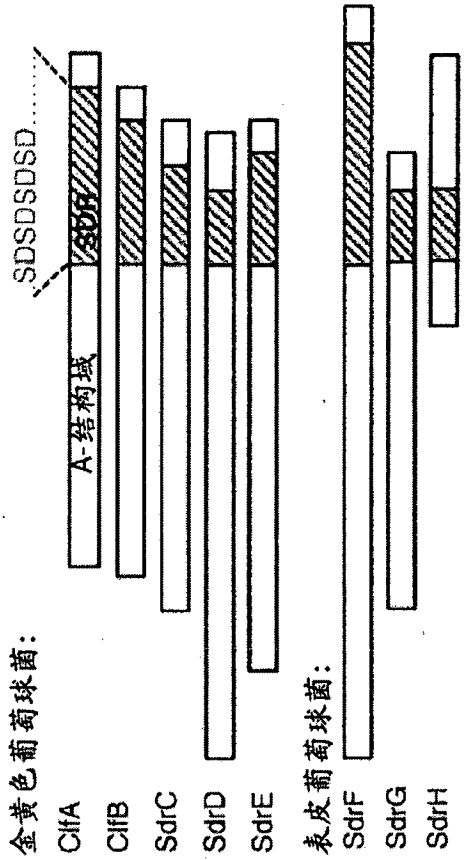
SDSDSDSD
SDR
A-结构域
金黄色葡萄球菌:
ClfA
ClfB
SdrC
SdrD
SdrE
表皮葡萄球菌:
SdrF
SdrG
SdrH

图4A

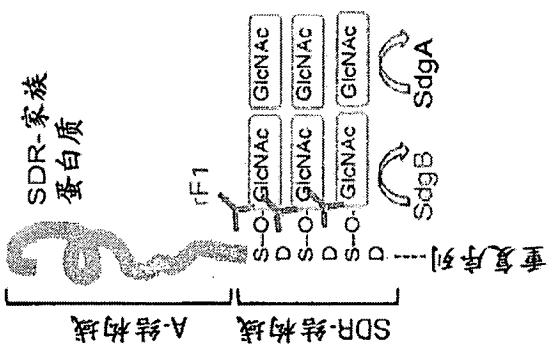
SDR-家族
蛋白质
rF1
GlcNAc
SdgA
SdgB
S-O
D
重复单元
A-结构域
SDR-结构域

图4B

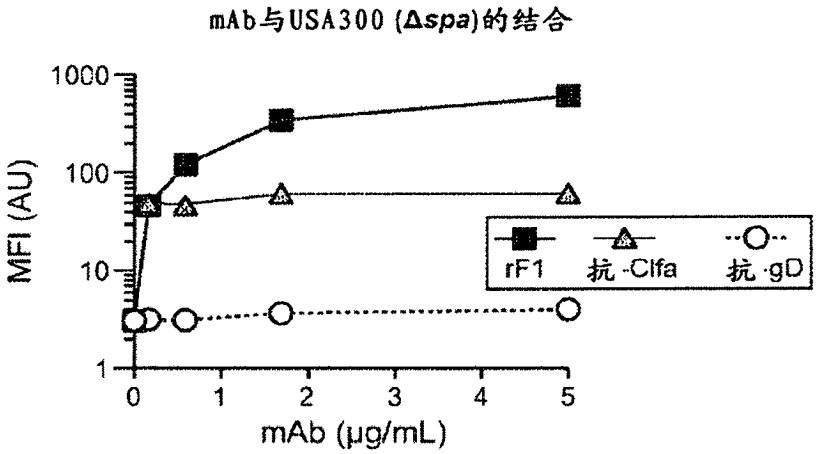
mAb与USA300 (Δspa)的结合
MFI (AU)
1000
100
10
1
0
1
2
3
4
5
mAb (μg/mL)
rF1
抗-Clfa
抗-gD

图5A

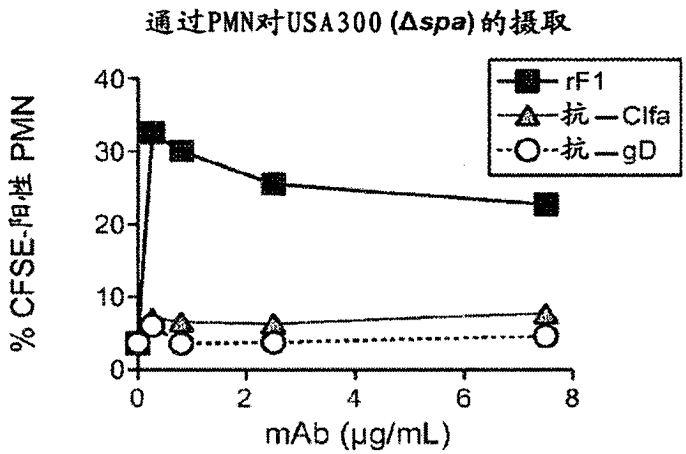
通过PMN对USA300 (Δspa)的摄取
% CFSE-阳性 PMN
40
30
20
10
0
0
2
4
6
8
mAb (μg/mL)
rF1
抗—Clfa
抗—gD

图5B

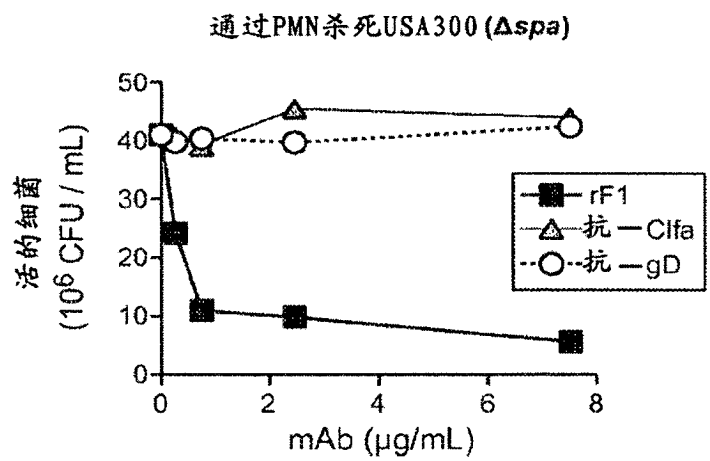
通过PMN杀死USA300 (Δspa)
活的细菌
(10^6 CFU / mL)
50
40
30
20
10
0
0
2
4
6
8
mAb (μg/mL)
rF1
抗—Clfa
抗—gD

图5C

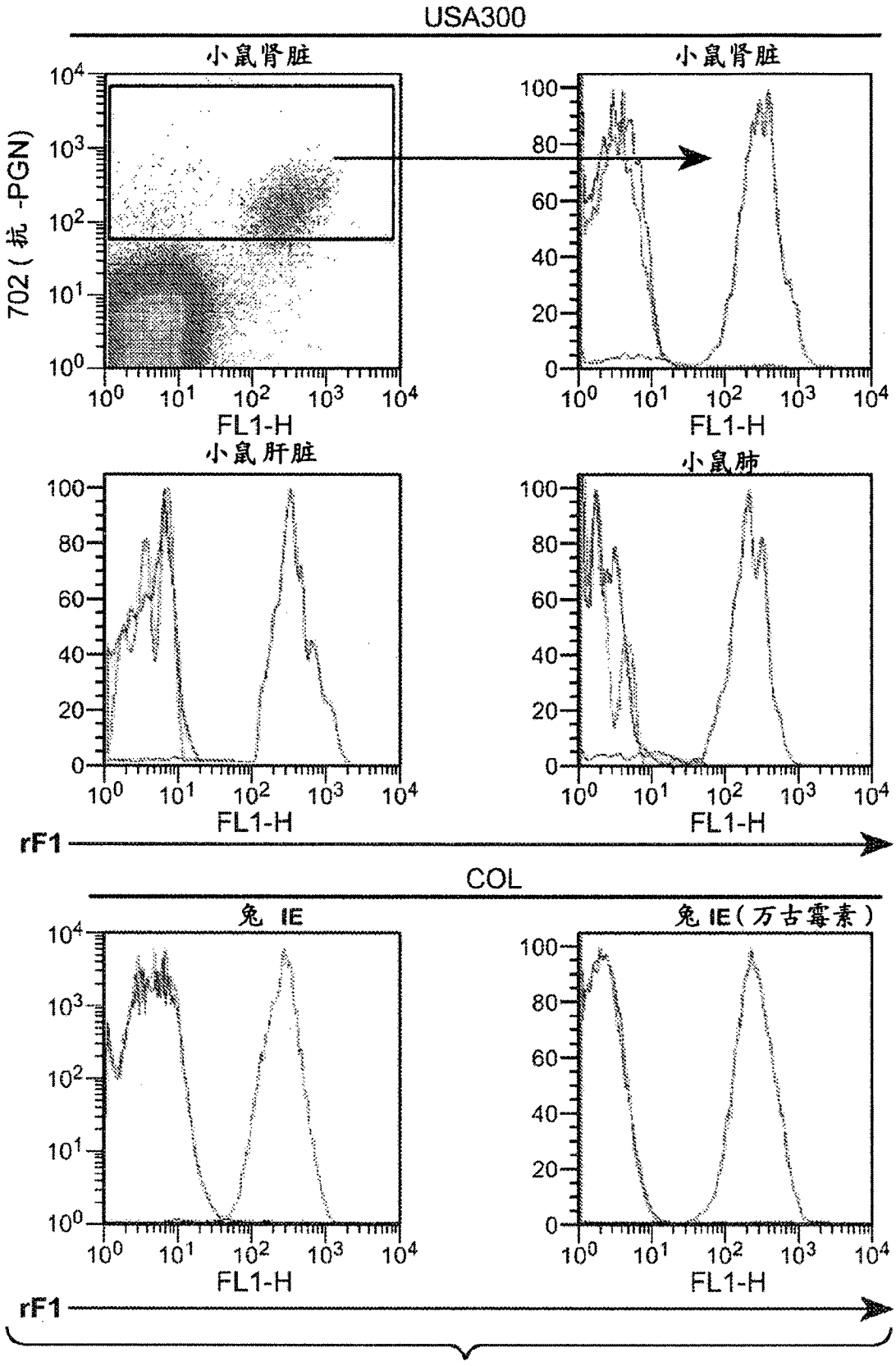
USA300
小鼠肾脏
小鼠肾脏
702(抗-PGN)
FL1-H
FL1-H
小鼠肝脏
小鼠肺
FL1-H
FL1-H
rF1
COL
兔 IE
兔 IE(万古霉素)
FL1-H
FL1-H
rF1

图6

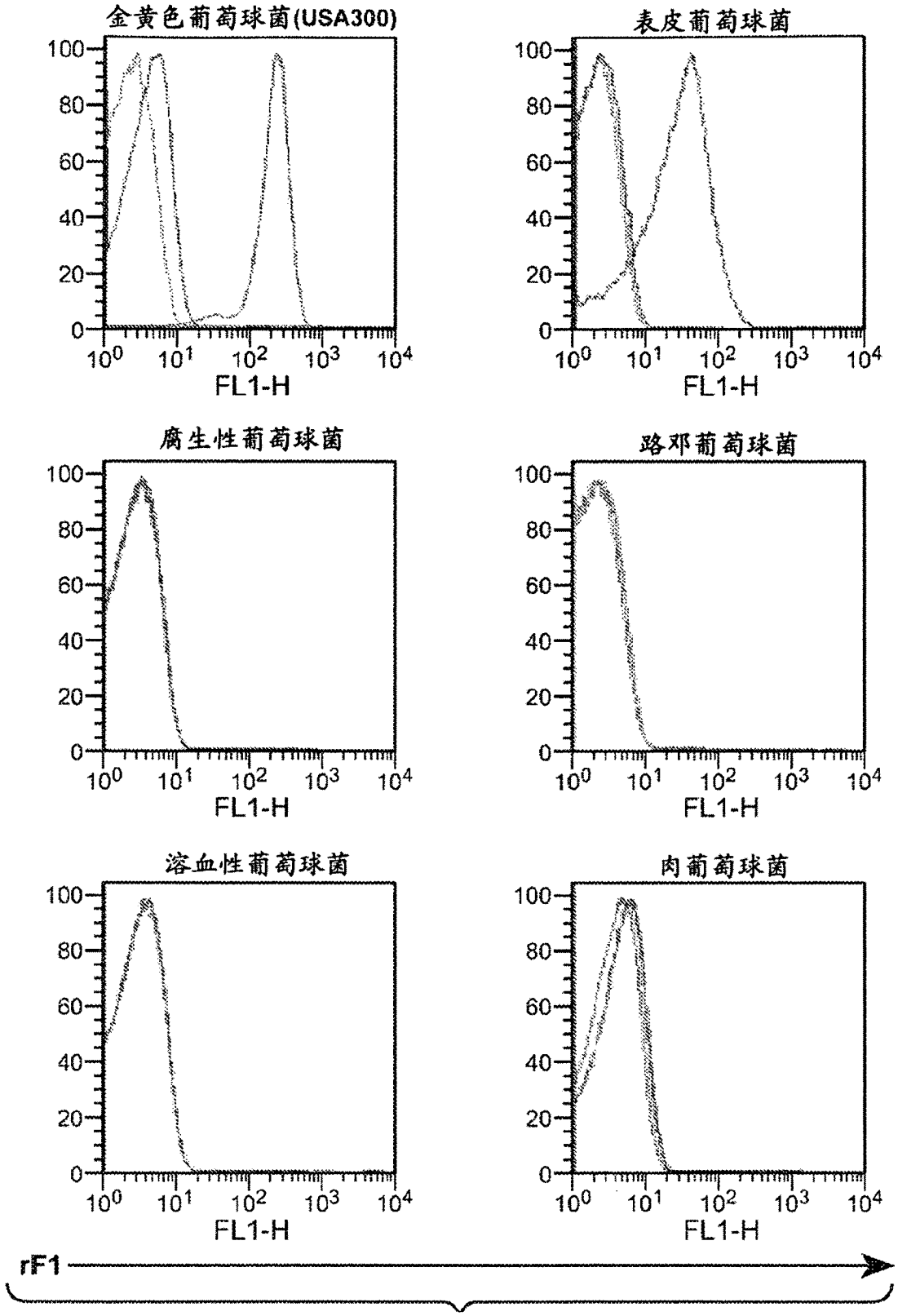
金黄色葡萄球菌(USA300)
表皮葡萄球菌
腐生性葡萄球菌
路邓葡萄球菌
溶血性葡萄球菌
肉葡萄球菌
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4
FL1-H
rF1

图7A

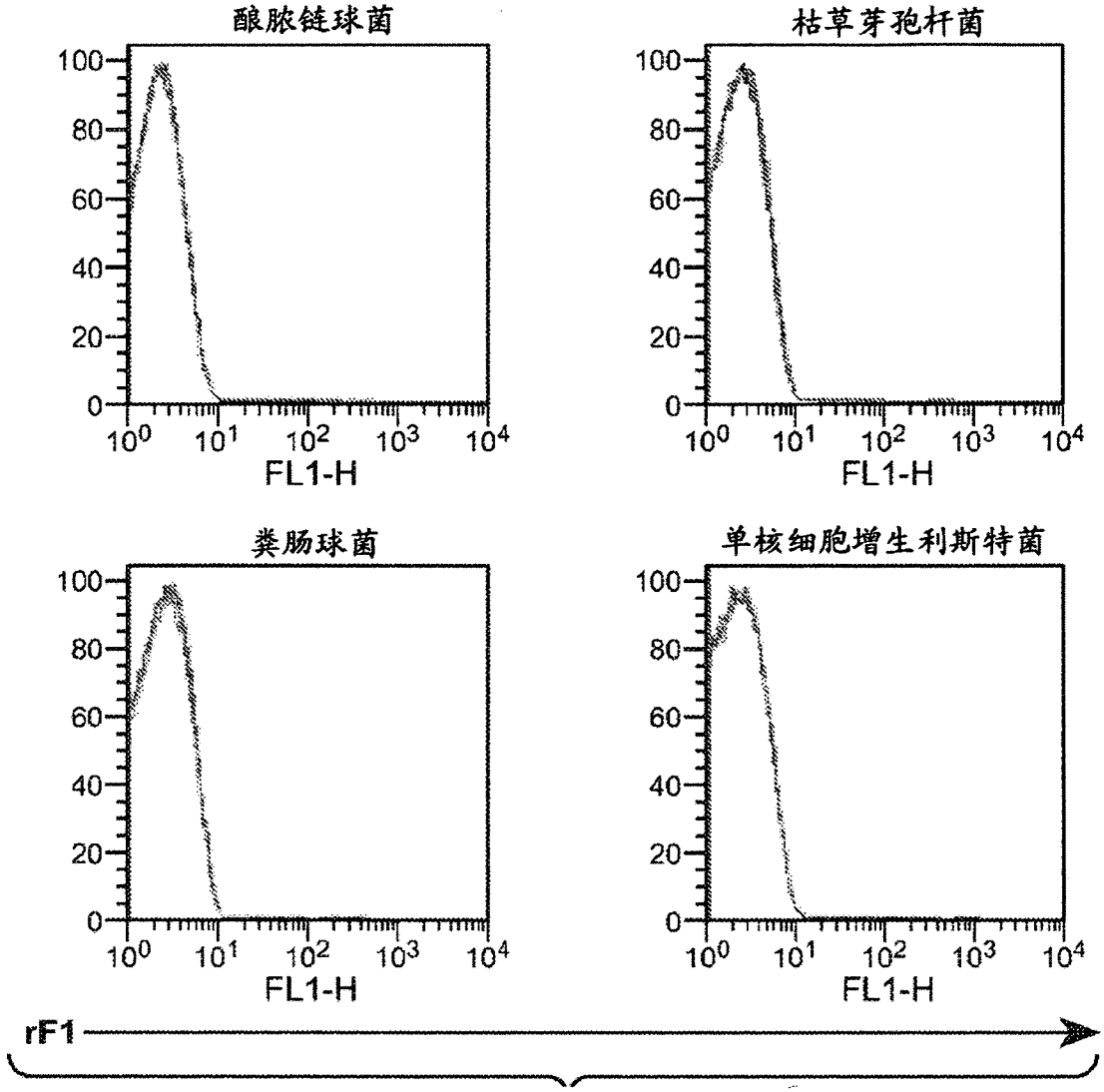
酿脓链球菌
枯草芽孢杆菌
粪肠球菌
单核细胞增生利斯特菌
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4
FL1-H
rF1

图7B

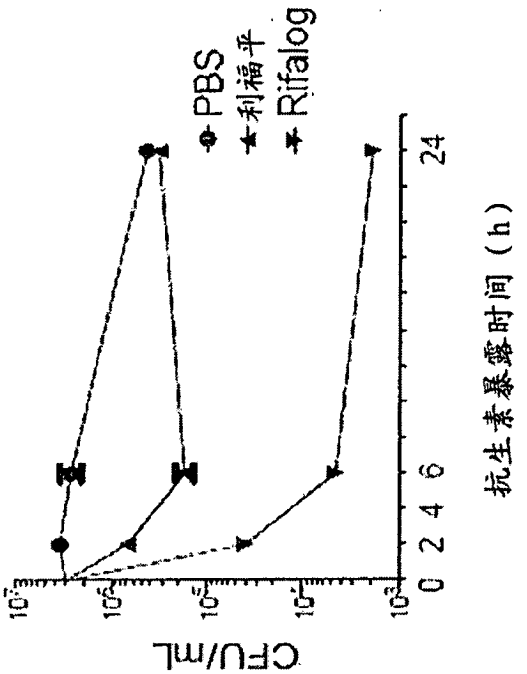
PBS
利福平
Rifalog
CFU/mL
抗生素暴露时间（h）
0 2 4 6 24

图8

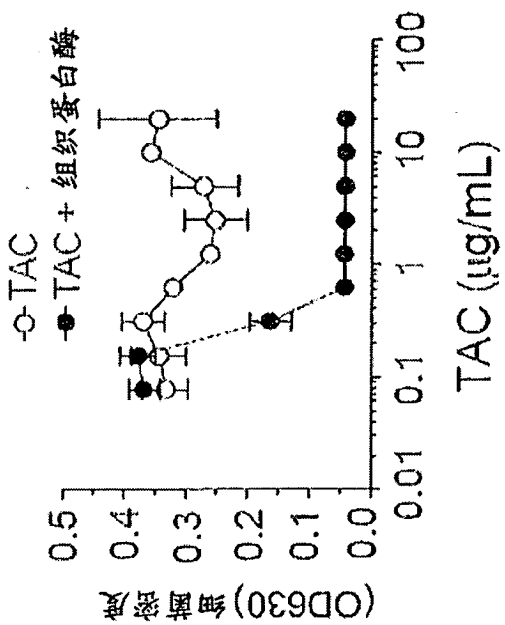
TAC
TAC + 组织蛋白酶
细菌密度 (OD630)
TAC (μg/mL)
0.0 0.1 0.2 0.3 0.4 0.5
0.01 0.1 1 10 100

图9

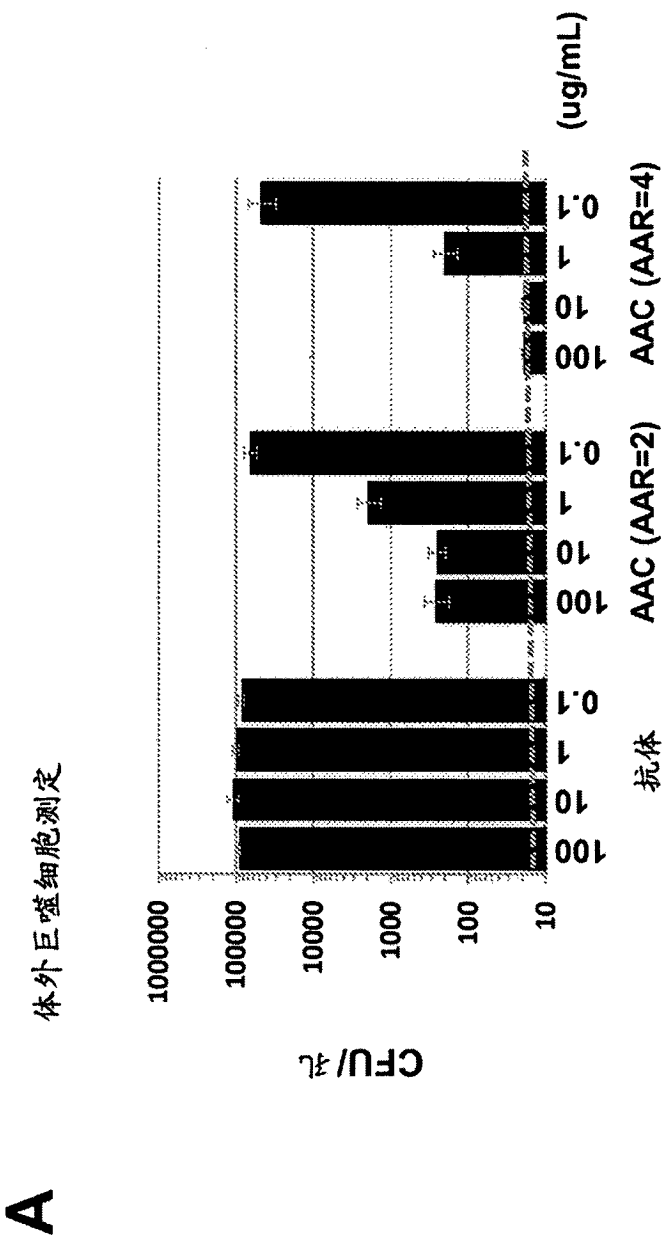
A
体外巨噬细胞测定
CFU/孔
1000000
100000
10000
1000
100
10
抗体
AAC (AAR=2)
AAC (AAR=4)
0.1
1
10
100
(ug/mL)

图10

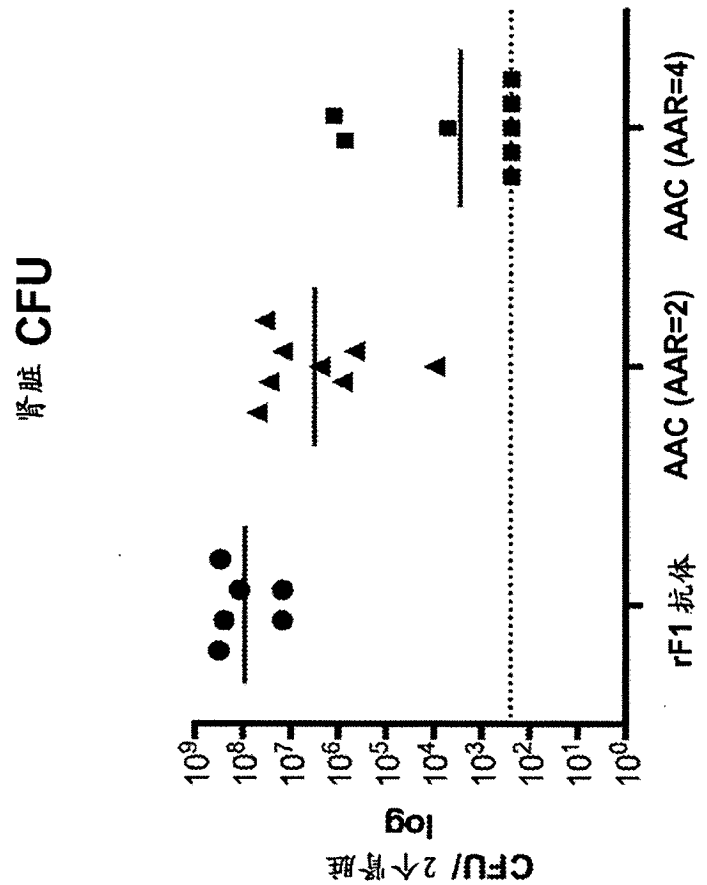
肾脏 CFU
CFU/ 2个肾脏
log
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
rF1 抗体
AAC (AAR=2)
AAC (AAR=4)

图11A

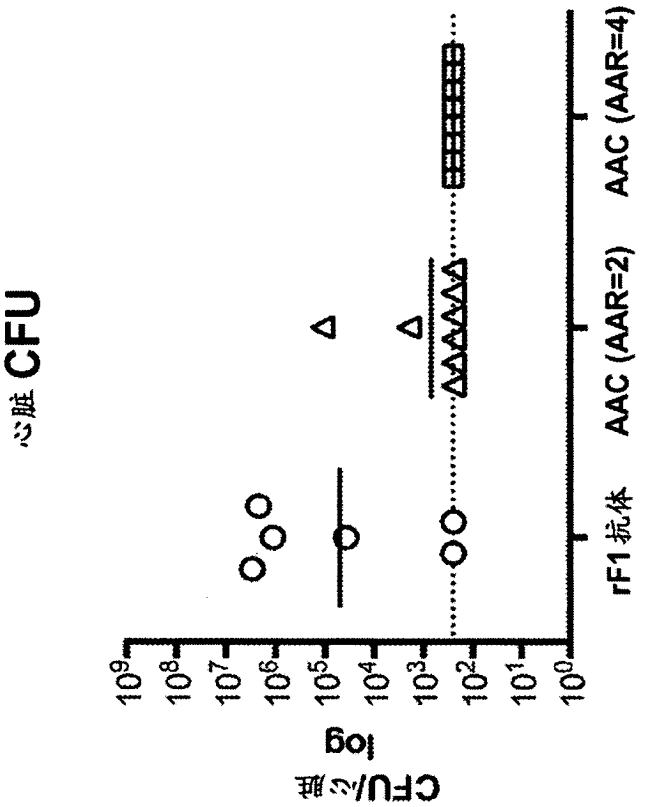
CFU/心脏
log
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
rF1抗体
AAC (AAR=2)
AAC (AAR=4)
心脏 CFU

图11B

Abstract

The invention provides rF1 antibody antibiotic conjugates and methods of using same.