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(54) Title: METHODS FOR SCREENING FOR ANTIBIOTIC COMPOUNDS

(57) Abstract: The present invention is a method for screening drugs for antibiotic activity by screening a drug for activity to dis-
rupt a toxin-antitoxin complex in a bacterial cell.



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METHODS FOR SCREENING FOR ANTIBIOTIC COMPOUNDS**Introduction**

[0001] This application claims benefit of priority to U.S. Provisional Application Serial No. 61/222,304, filed July 1, 2009, the content of which is incorporated herein by reference in its entirety.

Background of the Invention

[0002] Toxin and antitoxin (TA) systems are commonly found in prokaryotes. These systems function to allow the organisms to rapidly adjust rates of protein and DNA synthesis in order to respond to external stimuli and/or stress (Gerdes et al. 2005. *Nature Rev. Microbiol.* 3:371-382). Under normal circumstances, TA genes are co-transcribed and co-translated as part of an operon so that both antitoxin and toxin are produced together within the cytosol to form an inert complex. Under specific stress, transcription of the TA promoter will be repressed, disrupting transcription and subsequently translation. As toxins are stable compounds while the antitoxins compounds are more labile and prone to proteolytic attack by bacterial Lon/ClpP proteases, disruption of transcription from the TA promoter will result in excess toxin and activity of the toxin in the cell. The target of such toxins may be mRNA, DNA gyrase or DNA helicase, where interaction of toxin with these targets leads to disruption of transcription and translation of genes responsible for important cellular processes.

[0003] Based on sequence homology and cellular targets, there are eight major TA systems that have been identified in prokaryotes (Gerdes et al. 2005. *Nature Rev. Microbiol.* 3:371-382; Kamphius et al. 2007. *Protein Peptide Lett.*

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14:113-124). Among these TA systems is the MazEF system, which includes the toxin MazF and the antitoxin MazE. These two proteins form a linear heterohexamer made up of alternating toxin and antitoxin homodimers (Kamada et al. 2003. *Mol. Cell.* 11:875-884). The MazEF TA complex in *E. coli* has been shown to autoregulate by binding of the DNA by the N-terminal domain of MazE. The MazF toxin has been shown to cleave translated mRNAs and through this mechanism to block protein synthesis within prokaryotic cells (Christensen et al. 2003. *J. Mol. Biol.* 332:809-819). The cleavage of mRNAs in *E. coli* is at ACA sites (Zhang et al. 2003. *Mol. Cell.* 12:913-923). A variety of conditions have been shown to trigger the activity of MazF in prokaryotic cells including, for example, stress linked to high temperatures, oxidative stress, DNA damage by thymidine starvation, UV irradiation, and contact with protein-inhibiting antibiotics (Kamphius et al. 2007. *Protein Peptide Lett.* 14:113-124). Although MazEF clearly functions as a bacteriostatic system (Gerdes et al. 2005. *Nature Rev. Microbiol.* 3:371-382) within prokaryotic cells, it is not clear whether MazEF also functions within cells as a system for programmed cell death.

[0004] Sequence analysis has revealed that the MazF toxin is more conserved among different bacteria than is the antitoxin MazE. This finding is consistent with the finding that the activity of similar TA systems in different bacteria is dependent on the specificity of the antitoxin. In fact, it has been found that *Staphylococcus aureus* MazEF homologs are quite different from *E. coli* MazEF homologs (Fu et al. 2007. *J. Bacteriol.* 189:8871-8879; Fu et al. 2009. *J. Bacteriol.* 191:2051-2059; Niles et al. 2009. *J. Bacteriol.* 191:2795-2805). It has been found through

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transcriptional analysis that the mRNA target of the toxin MazF in *S. aureus* is selective, sparing important transcripts such as *gyrA* and *recA* (Niles et al. 2009. *J. Bacteriol.* 191:2795-2805). Therefore, in *S. aureus* MazF has features of a bacteriostatic effect rather than a bacteriocidal effect. The toxic effect of MazF can be rescued by the antitoxin within a fixed time window. However, the effect of MazF, if prolonged, can lead to nonviable cells.

[0005] Based on the importance of TA systems within cells, including the MazEF system, interest has grown in the use of these systems in the development of new antibiotic compounds. To date, the only organisms not identified as having MazEF systems are *Mycobacterium leprae*, *Chlymidia*, *Rickettsia*, and *Mycoplasma*. MazEF has been found to be an important TA system within a variety of prokaryotes including *E. coli*, *S. aureus*, and *S. pneumonia*. There remains a need for methods to identify new antibiotic compounds active against clinically important bacteria.

Summary of the Invention

[0006] The present invention is a method of screening a drug for activity to disrupt a toxin-antitoxin complex in a bacterial cell by contacting a TA complex with a test drug and determining whether the test drug increases the amount of cleavage of RNA by the toxin, wherein an increase in the amount of cleavage of RNA by the toxin is indicative of activity to disrupt a toxin-antitoxin complex in the bacterial cell. In some embodiments, the TA complex is a MazEF complex, e.g., from a *S. aureus* cell. In other embodiments, cleavage of the RNA by the toxin is determined by measuring cleavage of a synthetic RNA substrate, e.g.,

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containing a MazF recognition site (AUUC), wherein said RNA is flanked on each end by at least four deoxyribonucleotides. In further embodiments, the synthetic RNA substrate includes a fluorescent marker-quencher pair. In particular embodiments, cleavage of RNA by the toxin of a TA complex is determined by measuring cleavage of a synthetic RNA-DNA substrate comprising the 4-base MazF recognition site (AUUC) flanked on each side by at least four deoxyribonucleotides, wherein said substrate comprises a fluorescence marker at one end and a quencher at the other end. A synthetic substrate of use in the instant method is also provided.

Detailed Description of the Invention

[0007] The present invention is a screening assay for identifying compounds that disrupt a TA complex within a prokaryotic organism. Disruption of the TA complex within a bacterial cell leads to either cell death or a bacteriostatic effect, depending on the activity of the TA system within the cell. The MazEF complex is found in a variety of bacteria and as such has now been found to be a useful target TA system for developing a screening assay. It has been found that the MazEF complex within *S. aureus* is a particularly useful target system.

[0008] The present invention was developed based on experiments to identify the cleavage site on mRNA that is attacked by MazF in *S. aureus*. In the first experiments, the cleavage site of the MazF toxin of *S. aureus* on mRNA was found to be VUUV' where V and V' are adenine (A), cytosine (C) or guanine (G), but not uracil (U). As RNA is unstable and prone to RNase degradation from the environment, it would be preferable to use an RNA-hybrid

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template to screen for compounds that disrupt the MazEF complex in order to detect toxin activity. Therefore, a hybrid RNA-DNA hybrid molecule, which is less amenable to RNase-mediated cleavage, was developed. The hybrid molecule was synthesized that contained 12 bases. The hybrid contained the 4-base recognition site (AUUC) of MazF from *S. aureus*, and flanked the four bases on each side with four DNA bases to protect the hybrid from RNase-mediated cleavage. On one end of the hybrid molecule, the fluorescence marker FAM-6 was attached while at the other end the quencher BHG-1 was attached. The resulting RNA-DNA hybrid molecule did not yield significant fluorescence unless the RNA target site was cleaved by the MazF toxin to separate FAM-6 from BHG-1. Moreover, exposure of the 12-base hybrid molecule to the MazF toxin yielded fluorescence comparable to and even at levels lower than RNase A, which has been shown to efficiently cleave single-stranded RNA. This hybrid molecule was then used as the substrate for development of an antibiotic screening assay.

[0009] Thus, a screening assay was developed wherein MazEF complex of *S. aureus* was incubated with the hybrid molecule substrate and a compound to be tested for activity to disrupt the MazEF complex. In accordance with this assay, compounds that disrupt the complex will cleave the hybrid fluorescence substrate and yield detectable fluorescence. In one embodiment, multi-well plates are loaded with microliter amounts of the hybrid fluorescent substrate, the test compound and the MazEF complex. Control samples contain no MazEF complex. Results have been collected with *S. aureus* and the assay has been validated for use with *S. aureus* and activity of the MazEF complex.

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[0010] As one of skill will appreciate, this screening assay can be modified to accommodate detection of disruption of the TA complex from any organism as long as the cleavage site of the toxin on RNA is identified.

[0011] Thus, the present invention is a method of screening drugs for activity to disrupt a toxin-antitoxin complex in a bacterial cell by contacting a TA complex with a test drug and determining whether the test drug increases the amount of cleavage of RNA by the toxin, wherein an increase in the amount of cleavage of RNA by the toxin is indicative of activity to disrupt a toxin-antitoxin complex in the bacterial cell. As described herein, there are a number of well-known toxin-antitoxin complexes that can be used in the assay of this invention. These include, but are not limited to, the MazEF module of, e.g., *E. coli*, *S. aureus*, *S. pneumonia* or *M. tuberculosis*; the relBE module of *M. tuberculosis*; and the *higBA* and *yoeB/yefm* modules. See Buts et al. 2005. *Trends Biochem. Sci.* 30:672-9; Gerdes et al. 2005. *Nature Rev. Microbiol.* 3:371-382; and Condon 2006. *Mol. Microbiol.* 61:573-583. In one embodiment, the toxin-antitoxin complex is a MazEF complex. In a particular embodiment the toxin-antitoxin complex is a MazEF complex from *S. aureus*.

[0012] As demonstrated herein, release of toxin from the TA complex allows for cleavage of its RNA substrate, an activity which can be detected using fluorescent substrates. In this respect, certain embodiments of this invention include the use of a synthetic RNA fluorescent substrate. In a more preferred embodiment, the synthetic RNA fluorescent substrate is a hybrid molecule composed of 12 bases of RNA and DNA. In specific embodiments, the RNA component of the synthetic RNA fluorescent substrate

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contains the 4-base recognition site (AUUC) of MazF from *S. aureus*, which is flanked on both sides by at least four deoxyribonucleotides (*i.e.*, dNdNdNdN-AUUC-dNdNdNdN; SEQ ID NO:1). However, as the skilled artisan will appreciate, the synthetic hybrid substrate is flexible and can accommodate longer RNA-DNA hybrids, *e.g.*, from 4 to 10 ribonucleotides (including the sequence AUUC) flanked by 4 to 10 deoxyribonucleotides on either side. The synthetic hybrid substrate can be produced by chemical synthesis, recombinant techniques or a combination thereof routinely practiced in the art.

[0013] To facilitate detection of cleavage of the synthetic hybrid substrate by the toxin of the TA system, the present construct also features a fluorescent marker on one end of the substrate and a quencher on the other end of the substrate (*i.e.*, fluorescent marker-DNA-RNA-DNA-quencher). Fluorescent marker-quencher pairs are well-known in the art and routinely used in fluorescent hybridization reactions (Marras 2006. *Meth. Mol. Biol.* 335:3-16). Examples of suitable fluorescent markers of use in the instant construct include, but are not limited to, FAM, TET, HEX, Cy3, TMR, ROX, TEXAS RED, and Cy5 fluorophores. Fluorophores with an emission maximum between 500 and 550 nm, such as FAM, TET and HEX, are best quenched by quenchers with absorption maxima between 450 and 550 nm, such as dabcyI and BHQ-1. Fluorophores with an emission maximum above 550 nm, such as rhodamines (including TMR, ROX and TEXAS RED) and Cy dyes (including Cy3 and Cy5) are best quenched by quenchers with absorption maxima above 550 nm (including BHQ-2). Other quenchers include DDQ-I (absorption maximum of 430 nm), QSY-7 (absorption maximum

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of 571 nm), DDQ-II (absorption maximum of 630 nm), and QSY-21 (absorption maximum of 660 nm).

[0014] Agents, compounds or test drugs which can be screened in accordance with the method of the present invention are generally derived from libraries of agents or compounds. Such libraries can contain either collections of pure agents or collections of agent mixtures. Examples of pure agents include, but are not limited to, proteins, polypeptides, peptides, nucleic acids, oligonucleotides, carbohydrates, lipids, synthetic or semi-synthetic chemicals, and purified natural products. Examples of agent mixtures include, but are not limited to, extracts of prokaryotic or eukaryotic cells and tissues, as well as fermentation broths and cell or tissue culture supernates. Databases of chemical structures are also available from a number of sources including Cambridge Crystallographic Data Centre (Cambridge, UK) and Chemical Abstracts Service (Columbus, OH). *De novo* design programs include Ludi (Biosym Technologies Inc., San Diego, CA), Sybyl (Tripos Associates) and Aladdin (Daylight Chemical Information Systems, Irvine, CA).

[0015] Once a drug has been shown to be effective at cleaving RNA in this *in vitro* assay, the effect of the drug will be confirmed by cleavage of *in vitro* synthesized mRNA and mRNA synthesized by the bacteria *in vivo* as described in our publications. The next step will be to test the drugs for activity *in vitro* to inhibit growth of bacterial cells and determination of minimum inhibitory concentration (MIC) doses for the drugs. Determining MIC doses is a standard assay in development of antibiotic drugs and methods are well known in the art. For example, MIC can be

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determined according to the Clinical and Laboratory Standards Institute guidelines.

[0016] Once the efficacy of a drug or a combination of drugs has been shown based on the use of the *in vitro* screening method of the present invention, there are many different *in vivo* model systems that can be used by one of skill in the art to further demonstrate efficacy against MSSA, MRSA and CA-MRSA and aid in identification of doses that will be both safe and effective in humans. Such animal model systems are well-accepted and used during development of new human pharmaceuticals that will undergo scrutiny by various regulatory bodies worldwide and approved for use in humans. Examples of such model systems include but are not limited to a guinea pig model of *S. aureus* wound infection (Kernodle, D.S. and A.B. Kaiser. 1994. *Antimicrob. Agents Chemother.* 38:1325-1330); a rabbit model of *S. aureus* abscess in rabbits (Fernandez et al. 1999. *Antimicrob. Agent Chemother.* 43:667-671); a mouse model of *S. aureus* skin infection (Gisby, J. and J. Bryant. 2000. *Antimicrob. Agents Chemother.* 44:255-260); a mouse model of deep dermal *S. aureus* infection (Godin et al. 2005. *J. Antimicrob. Chemother.* 55:989-994); and a mouse intraperitoneal infection model (Patel et al. 2004. *Antimicrob. Agents Chemother.* 48:4754-4761). In such models, drugs can be tested against infections where the infection established is from inoculation of the animal with various strains of *S. aureus*. Demonstration of efficacy in such models is measured in many ways and would include but not be limited to a reduction in mortality rate, a reduction in bacterial cell counts determined by microscopic examination of tissue or blood samples taken from the animals, or even assessment of wound healing in the animals.

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[0017] The efficacy of a drug that has been screened *in vitro* and shown to have activity to inhibit growth of *S. aureus* including methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA) and community-acquired methicillin-resistant *S. aureus* (CA-MRSA) can be further examined using the model described by Patel et al. (2004. *Antimicrob. Agents Chemother.* 48:4754-4761). Briefly, Swiss mice (6 mice per dose group, 4 weeks of age) will be inoculated intraperitoneally (i.p.) with 0.5 ml of bacterial suspension so that each mouse will receive from 2×10^8 to 3×10^8 CFU of isolate. The drug to be tested, or the combination of drugs to be tested, is then at a dose shown to be effective *in vitro* but also known to be safe in animals. The doses to be tested are routinely chosen by those of skill in the art by using clinical judgment based on results of *in vitro* pharmacological assays. For example, doses can be ones that are equivalent to an ED₁₀, an ED₂₅, an ED₅₀, and an ED₇₅ for inhibiting bacterial growth *in vitro*. The drug will be administered at 1 and 4 hours after i.p. inoculation of mice with isolates. The drug to be tested can be administered subcutaneously, intravenously, or orally. A vehicle control group will be used. All mice are observed for survival up to 7 days. Efficacy of the test drug will be measured as an increased survival rate as compared to control animals (untreated) and as compared to survival in a group of animals administered a positive control agent (e.g., an antibiotic known to have efficacy to treat *S. aureus*).

[0018] A mouse model of *S. aureus* skin infection (e.g. Godin et al. 2005. *J. Antimicrob. Chemother.* 55:989-994) will be used to examine the efficacy of a drug that has been screened *in vitro* and shown to have activity to

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inhibit growth of isolates. Briefly, 4 to 5 week old immunocompetent ICR male mice will be used. Three groups of 18 mice each will be inoculated intracutaneously with isolates. The intracutaneous injections will be applied to the back of each animal that will have been previously shaved with clippers. Six mice from each group will be inoculated with 0.1 ml of saline containing 10^7 , 10^8 or 10^9 CFU/ml of isolate. The mice are then examined daily for development of deep dermal abscesses, inflammatory reaction in the inoculated area and wound size for a total of 3 weeks. The drug to be tested for antibiotic activity can be given orally, by intravenous injection or dermally. If dermal administration is to be tested, the drug will be spread over the area of the abscess. The dose of test drug to be administered will be chosen based on the results of *in vitro* studies of inhibition of bacterial growth. As discussed above, doses can be chosen based on the percentage of growth inhibition seen *in vitro*. The test drug will be administered 72 hours after intracutaneous injection with MSSA, MRSA or CA-MRSA inoculates and can last for 7 days or longer depending on the response of the animals to the treatment. At the end of 7 days treatment, animals will be sacrificed and the skin area corresponding to the infection site and underlying tissues can be processed for bacterial count and histopathological examination. Alternatively, mice can be sacrificed at various times, at least 3 mice per time period, such as 1, 3, and 7 days in order to monitor the progression of infection in response to the test drug.

[0019] It is contemplated that one of skill in the art will choose the most appropriate MSSA, MRSA and CA-MRSA strains and the most relevant *in vivo* model system depending on the

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type of drug product being developed. Some *in vivo* models are more amenable to oral or intravenous injection while others are more desirable for dermal application methods. The medical literature provides detailed disclosure on the advantages and uses of a wide variety of such models.

[0020] Once a test drug or a combination of drugs has shown to be effective *in vivo* in animals, the frequency in the emergence of resistance *in vivo* can be assessed. If the rate of emergence of resistance is low, clinical studies can be designed based on the doses shown to be safe and effective in animals. One of skill in the art will design such clinical studies using standard protocols as described in textbooks such as Spilker (2000. *Guide to Clinical Trials*. Lippincott Williams & Wilkins: Philadelphia).

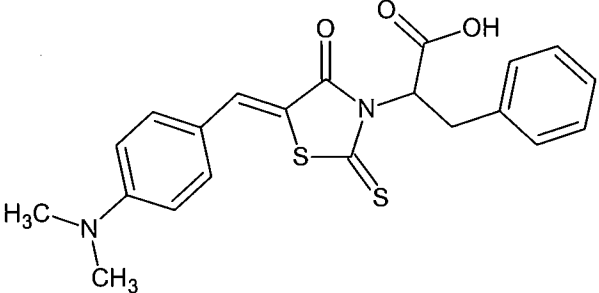
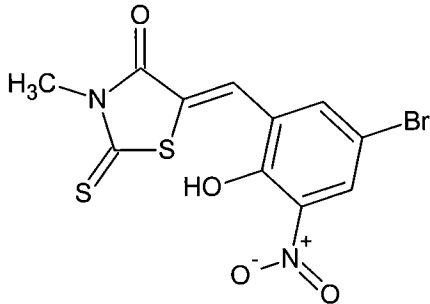
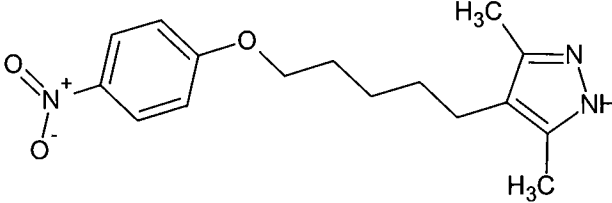
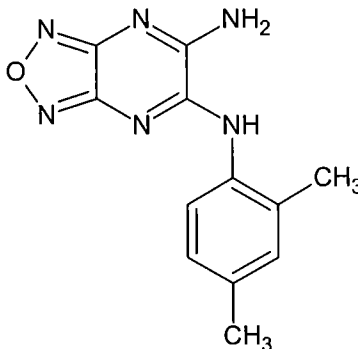
Example 1: Library Screen

[0021] To identify compounds as antibiotic compounds with potential activity against *S. aureus*, experiments were performed wherein MazEF complex of *S. aureus* was incubated with the hybrid molecule substrate and the compounds, which were to be tested for activity to disrupt the MazEF complex. Compounds that disrupted the complex cleaved the hybrid fluorescence substrate and yielded detectable fluorescence. The results of this screening assay identified the compounds listed in Table 1.

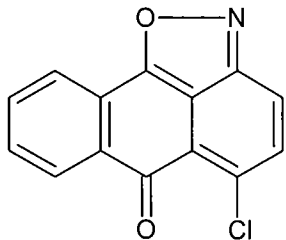
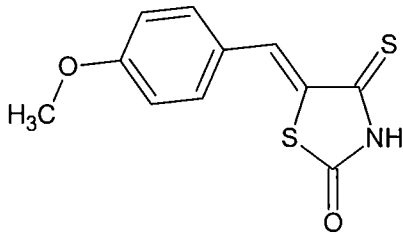
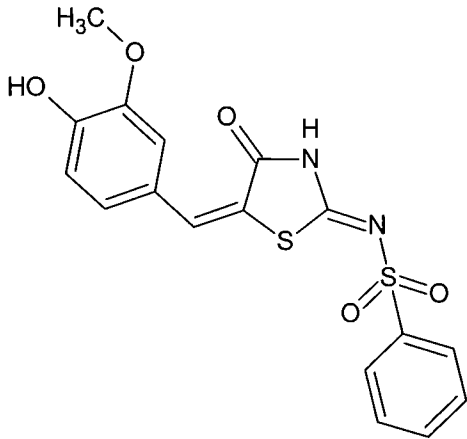
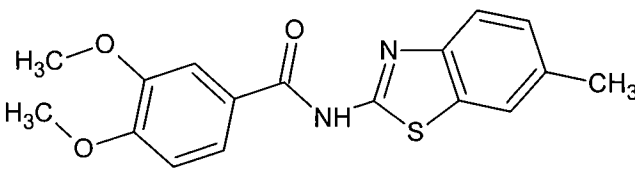
TABLE 1

Compound #	Compound Structure and Name	ChemBridge ID #	MIC ($\mu\text{g/ml}$)
1		6048022	12.5

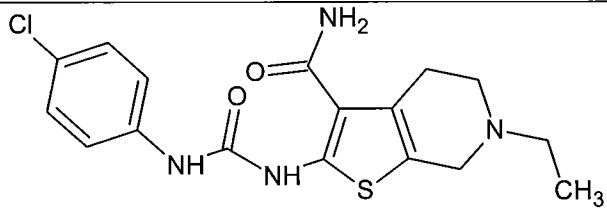
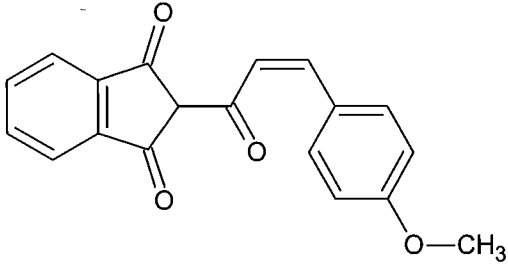
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	 2-{5-[4-(dimethylamino)benzylidene]-4-oxo-2-thioxo-1,3-thiazolidin-3-yl}-3-phenylpropanoic acid		
2	 5-(5-bromo-2-hydroxy-3-nitrobenzylidene)-3-methyl-2-thioxo-1,3-thiazolidin-4-one	5902920	12.5
3	 3,5-dimethyl-4-[5-(4-nitrophenoxy)pentyl]-1H-pyrazole	5362508	ND
4	 N-(2,4-dimethylphenyl)[1,2,5]oxadiazolo[3,4-b]pyrazine-5,6-diamine	5380590	12.5

-14-

5	 5-chloro-6H-anthra[1,9-cd]isoxazol-6-one	5468117	6.25
6	 5-(4-methoxybenzylidene)-4-thioxo-1,3-thiazolidin-2-one	5761926	0.78
7	 N-[5-(4-hydroxy-3-methoxybenzylidene)-4-oxo-1,3-thiazolidin-2-ylidene]benzenesulfonamide	5957303	100
8	 3,4-dimethoxy-N-(6-methyl-1,3-benzothiazol-2-yl)benzamide	5564414	ND
9		6131237	100

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	 2-((4-chlorophenyl)amino)carbonyl)-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxamide		
10		5215283	100

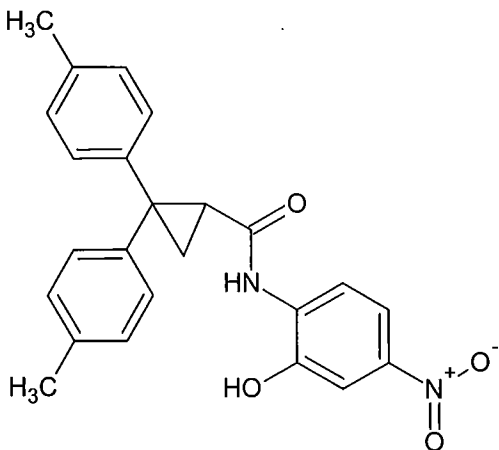
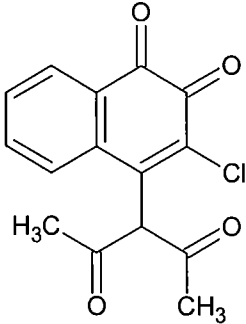
[0022] Once the compounds of the present invention were identified through the MazEF screening assay described above, the activity of the compounds to inhibit growth of *S. aureus* *in vitro* was tested using the standard method of determining Minimum Inhibitory Concentrations (MICs). Such methods are well known to those of skill in the art since determining MIC doses is a standard assay in development of antibiotic drugs. For example, MIC values can be determined according to the Clinical and Laboratory Standards Institute guidelines. In Table 1, the MIC values are presented for each of the ten compounds identified through the MazEF screening assay as having potential for antibiotic activity in *S. aureus*.

[0023] In addition to the above screen, an additional screen was employed. In this second assay, compounds from a compound library (natural or synthetic compounds) were incubated with either wild-type bacteria (with the MazEF

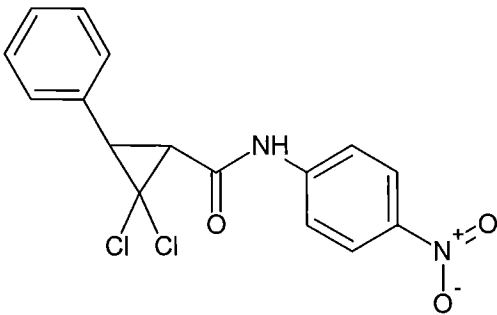
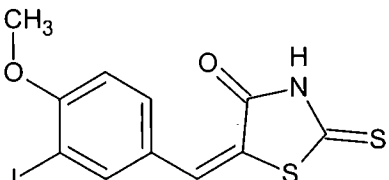
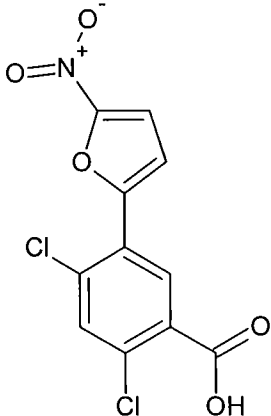
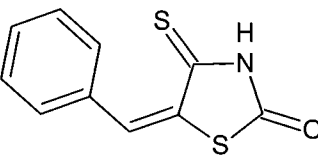
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complex) or a *mazEF* mutant of *S. aureus*. When the compound of interest disrupts the MazEF complex of *S. aureus* to free up the toxin (MazF), the mRNA-cleavage toxicity of the toxin is available to kill the parental strain; however, the isogenic *mazEF* mutant will not be killed. Using this assay, the compounds listed in Table 2 were identified as inhibiting the growth of wild-type strain Newman but not the isogenic *mazEF* mutants.

TABLE 2

Compound #	Structure	ChemBridge ID #
11	 N-(2-hydroxy-4-nitrophenyl)-2,2-bis(4-methylphenyl)cyclopropanecarboxamide	5634518
12	 4-(1-acetyl-2-oxopropyl)-3-chloro-1,2-naphthalenedione	5286499
13		6079510

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	 2,2-dichloro-N-(4-nitrophenyl)-3-phenylcyclopropanecarboxamide	
14	 5-(3-iodo-4-methoxybenzylidene)-2-thioxo-1,3-thiazolidin-4-one	5486272
15	 2,4-dichloro-5-(5-nitro-2-furyl)benzoic acid	6047950
16	 5-benzylidene-4-thioxo-1,3-thiazolidin-2-one	5765232

[0024] These results demonstrate the effectiveness of the using the instant assay in identifying compounds of use in

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inhibiting the growth of *S. aureus* and in the prevention and/or treatment of an *S. aureus* infection.

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What is claimed is:

1. A method of screening a drug for activity to disrupt a toxin-antitoxin (TA) complex in a bacterial cell comprising contacting a TA complex with a test drug and determining whether the test drug increases cleavage of RNA by the toxin, wherein an increase in cleavage of RNA by the toxin is indicative of activity to disrupt a TA complex in the bacterial cell.

2. The method of claim 1, wherein the TA complex is a MazEF complex.

3. The method of claim 2, wherein the MazEF complex is from a *S. aureus* cell.

4. The method of claim 1, wherein the cleavage of the RNA by the toxin is determined by measuring cleavage of a synthetic RNA substrate.

5. The method of claim 4, wherein the synthetic RNA substrate comprises a MazF recognition site (AUUC).

6. The method of claim 4, wherein the RNA of the substrate is flanked on each end by at least four deoxyribonucleotides.

7. The method of claim 4, wherein the synthetic RNA substrate comprises a fluorescent marker-quencher pair.

8. The method of claim 1, wherein cleavage of the RNA by the toxin is determined by measuring cleavage of a

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synthetic RNA-DNA substrate comprising the 4-base MazF recognition site (AUUC) flanked on each side by at least four deoxyribonucleotides, wherein said substrate comprises a fluorescence marker at one end and a quencher at the other end.

9. A synthetic substrate comprising a MazF recognition site (AUUC) flanked on either side by at least four deoxyribonucleotides, wherein said substrate comprises a fluorescence marker at one end and a quencher at the other end.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/40695

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68 (2010.01)

USPC - 435/6

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)
USPC- 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/69.1; 514/44A; 435/91.1

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

PubWEST (PGPB,USPT,USOC,EPAB,JPAB), Google Scholar: mazEF fluorescent marker quencher, mazEF, mazE, mazF, ChpAK
ChpAI, ChpAK, PemK, PemI, fluorophore

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2008/0058275 A1 (INOUE et al.) 6 March 2008 (06.03.2008) para [0003], [0009], [0034], [0039], [0093], [0205]	1-3 ----- 4-8
Y	FU et al. Characterization of MazFSa, an Endoribonuclease from Staphylococcus aureus. JOURNAL OF BACTERIOLOGY 2007, 189(24):8871-8879; abstract; pg 8872, Cleavage of synthetic RNAs by MazFSa	4-9
Y	WANG et al. A Continuous Fluorometric Assay for the Assessment of MazF Ribonuclease Activity. Anal Biochem 2007, 371(2):173-183; pg 2, para 4; pg 6, Substrate design	6-9

☐ Further documents are listed in the continuation of Box C.


* 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

2 August 2010 (02.08.2010)

Date of mailing of the international search report

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