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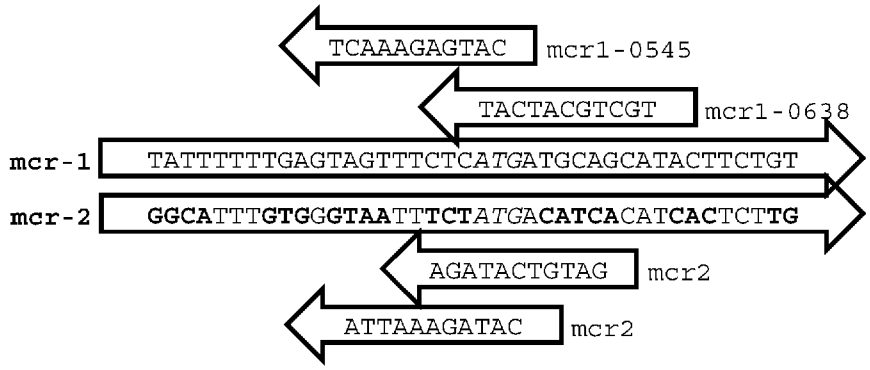

FIG. 2

(57) Abstract: Provided are antisense morpholino oligomers targeted against bacterial virulence factors such as genes that contribute to antibiotic resistance or biofilm formation, or essential genes, and related compositions and methods of using the oligomers and compositions, for instance, in the treatment of an infected mammalian subject.

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ANTISENSE ANTIBACTERIAL COMPOUNDS AND METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 62/466,852, filed March 3, 2017, which is incorporated by reference in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is SATH_009_01WO_ST25.txt. The text file is about 6 KB, was created on March 2, 2018 and is being submitted electronically via EFS-Web.

BACKGROUND

Technical Field

The present disclosure relates to antisense morpholino oligomers targeted against bacterial virulence factors such as genes that contribute to antibiotic resistance, biofilm formation or essential processes, and related compositions and methods of using the oligomers and compositions, for instance, in the treatment of an infected mammalian subject.

Description of the Related Art

Currently, there are several types of antibiotic compounds in use against bacterial pathogens and these compounds act through a variety of anti-bacterial mechanisms. For example, beta-lactam antibiotics, such as penicillin and cephalosporin, act to inhibit the final step in peptidoglycan synthesis. Glycopeptide antibiotics, including vancomycin and teichoplanin, inhibit both transglycosylation and transpeptidation of muramyl-pentapeptide, again interfering with peptidoglycan synthesis. Other well-known antibiotics include the quinolones, which inhibit bacterial DNA replication, inhibitors of bacterial RNA polymerase, such as rifampin, and inhibitors of enzymes in the pathway for production of tetrahydrofolate, including the sulfonamides.

Some classes of antibiotics act at the level of protein synthesis. Notable among these are the aminoglycosides, such as kanamycin and gentamicin. This class of compounds targets the bacterial 30S ribosome subunit, preventing the association with the 50S subunit to form functional ribosomes. Tetracyclines, another important class of antibiotics, also target the 30S ribosome subunit, acting by preventing alignment of aminoacylated tRNA's with the corresponding mRNA codon. Macrolides and

lincosamides, another class of antibiotics, inhibit bacterial synthesis by binding to the 50S ribosome subunit, and inhibiting peptide elongation or preventing ribosome translocation.

Despite impressive successes in controlling or eliminating bacterial infections by antibiotics, the widespread use of antibiotics both in human medicine and as a feed supplement in poultry and livestock production has led to drug resistance in many pathogenic bacteria. Antibiotic resistance mechanisms can take a variety of forms. One of the major mechanisms of resistance to beta lactams, particularly in Gram-negative bacteria, is the enzyme beta-lactamase, which renders the antibiotic inactive by cleaving the lactam ring. Likewise, resistance to aminoglycosides often involves an enzyme capable of inactivating the antibiotic, in this case by adding a phosphoryl, adenyl, or acetyl group. Active efflux of antibiotics is another way that many bacteria develop resistance. Genes encoding efflux proteins, such as the *tetA*, *tetG*, *tetL*, and *tetK* genes for tetracycline efflux, have been identified. A bacterial target may develop resistance by altering the target of the drug. For example, the so-called penicillin binding proteins (PBPs) in many beta-lactam resistant bacteria are altered to inhibit the critical antibiotic binding to the target protein. Resistance to tetracycline may involve, in addition to enhanced efflux, the appearance of cytoplasmic proteins capable of competing with ribosomes for binding to the antibiotic. For those antibiotics that act by inhibiting a bacterial enzyme, such as for sulfonamides, point mutations in the target enzyme may confer resistance.

Biofilm formation can also lead to antibiotic resistance, among other clinical difficulties. Typically, in situations where bacteria form a biofilm within an infected host, the infection turns out to be untreatable and can develop into a chronic state. Hallmarks of chronic biofilm-based infections not only include resistance to antibiotic treatments and many other conventional antimicrobial agents but also a capacity for evading host defenses. Therefore, strategies that prevent or breakdown biofilm would be of therapeutic interest and benefit.

The appearance of antibiotic resistance in many pathogenic bacteria, including cases involving multi-drug resistance (MDR), raises the fear of a post-antibiotic era in which many bacterial pathogens were simply untreatable by medical intervention.

Liu *et al.* (Liu, Y-Y et al. (2016) Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study, Lancet Infect Dis 16: 161-168) reported the first example of a transferrable polymyxin resistance mechanism, mcr-1, in gram-negative pathogens. Since that report mcr-1 has been found in many major gram-negative pathogens, including those already harboring high-level resistance mechanisms. The mechanism of mcr-1 was also rapidly described; an ethanolamine is attached to lipid A phosphate groups, rendering the membrane more electropositive, repelling positively-charge

polymyxins. Acquisition of mcr-1 is clinically frightening because polymyxins are last-line antibiotics used to treat extensively resistant organisms, so acquisition would lead to pan-resistance. Therefore, the ability to inhibit mcr-1 and restore polymyxin sensitivity would be clinically significant.

Thus, there is a need for antimicrobial agents that (i) are not subject to the principal types of antibiotic resistance, such as polymyxin resistance including, for example, polymyxin resistance mechanism mcr-1, currently hampering antibiotic treatment of bacterial infection, (ii) can be developed rapidly and with some reasonable degree of predictability as to target-bacteria specificity, (iii) are effective at low doses, and (iv) show few side effects.

BRIEF SUMMARY

Embodiments of the present disclosure relate, in part, to the discovery that the antisense targeting of bacterial virulence factors can, inter alia, increase the antibiotic susceptibility of otherwise antibiotic-resistant pathogenic bacteria, and reduce the ability of certain pathogenic bacteria to form and maintain difficult-to-treat biofilms. For example, the antisense targeting of antibiotic resistance genes such as carbapenemases and efflux pumps was shown to increase the susceptibility of antibiotic resistant (e.g., multi-drug resistant) bacteria to many commonly used antibiotics, and could thus find utility in the treatment of such bacteria, for instance, in combination with antibiotics. Also, the antisense targeting of genes associated with biofilm formation was shown to break down existing biofilms and reduce the production of new biofilms. Such antisense targeting could find utility in standalone therapies against biofilm-forming bacteria, and as combination therapies, for example, to increase the susceptibility of biofilm-forming bacteria to antibiotics.

Embodiments of the present disclosure therefore include a substantially uncharged antisense morpholino oligomer, composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit, and having (a) about 10-40 nucleotide bases, and (b) a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor; where the oligomer is conjugated to a cell-penetrating peptide (CPP).

In certain embodiments, the target sequence comprises a translational start codon of the bacterial mRNA and/or a sequence within about 30 bases upstream or downstream of the translational start codon of the bacterial mRNA.

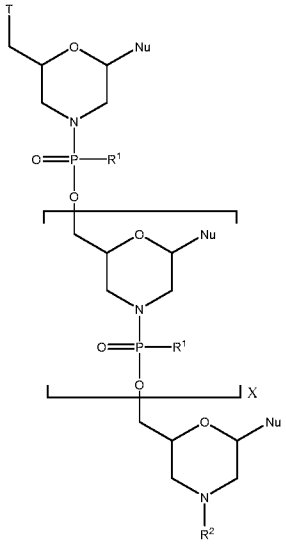
In some embodiments, the virulence factor is an antibiotic resistance protein or a biofilm formation protein. In certain embodiments, the antibiotic resistance protein is selected from one or more of beta-lactamase and polymyxin resistance protein. In some embodiments, the polymyxin

resistance is plasmid-mediated. In further embodiments, the polymyxin resistance is transmissible from one bacterium to another. In some embodiments, the virulence factor is a protein associated with one or more essential biochemical pathways and/or cellular processes.

In certain embodiments, the antibiotic resistance protein is selected from one or more of beta-lactamase TEM-1 and CTX-M-1-ATG. In certain embodiments, the antibiotic resistance protein is selected from one or more of polymyxin resistance proteins (e.g., MCR). In some embodiments, the polymyxin resistance protein is encoded by *mcr-1*. In some embodiments, the polymyxin resistance protein is encoded by *mcr-2*. In specific embodiments, the target sequence is selected from **Table 1A**. Some antisense oligomers comprise, consist, or consist essentially of a targeting sequence set forth in **Table 2A**, a fragment of at least 10 contiguous nucleotides of a targeting sequence in **Table 2A**, or variant having at least 80% sequence identity to a targeting sequence in **Table 2A**.

In some embodiments, the protein associated with one or more essential biochemical pathways and/or cellular processes is encoded by one or more of *acpP* and *murA*. In particular embodiments, the target sequence is selected from **Table 1B**. Some antisense oligomers comprise, consist, or consist essentially of a targeting sequence set forth in **Table 2B**, a fragment of at least 10 contiguous nucleotides of a targeting sequence in **Table 2B**, or variant having at least 80% sequence identity to a targeting sequence in **Table 2B**.

In certain embodiments, an antisense morpholino oligomer of the disclosure may be of formula (I):



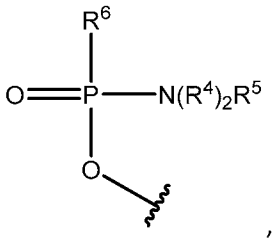
(I)

or a pharmaceutically acceptable salt thereof,

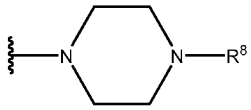
where each Nu is a nucleobase which taken together forms a targeting sequence;

X is an integer from 9 to 38;

T is selected from OH and a moiety of the formula:



where each R^4 is independently C_1-C_6 alkyl, and R^5 is selected from an electron pair and H, and R^6 is selected from OH, $–N(R^7)CH_2C(O)NH_2$, and a moiety of the formula:



where:

R^7 is selected from H and C_1-C_6 alkyl, and

R^8 is selected from G, -C(O)-R^9OH, acyl, trityl, and 4-methoxytrityl, where:

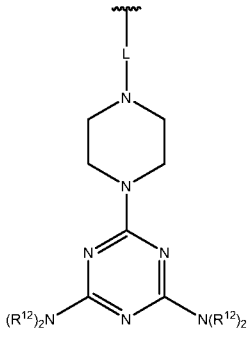
R^9 is of the formula -(O-alkyl)$_y$- wherein y is an integer from 3 to 10 and each of

the y alkyl groups is independently selected from C_2-C_6 alkyl;

each instance of R^1 is $–N(R^{10})_2R^{11}$ wherein each R^{10} is independently C_1-C_6 alkyl, and R^{11} is

selected from an electron pair and H;

R^2 is selected from H, G, acyl, trityl, 4-methoxytrityl, benzoyl, stearoyl, and a moiety of the formula:

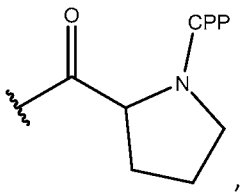


where L is selected from $–C(O)(CH_2)_6C(O)–$ and $-C(O)(CH_2)_2S_2(CH_2)_2C(O)–$, and each R^{12} is of the formula $–(CH_2)_2OC(O)N(R^{14})_2$ wherein each R^{14} is of the formula $-(CH_2)_6NHC(=NH)NH_2$; and

wherein G is a cell penetrating peptide (“CPP”) and linker moiety selected from

$-C(O)(CH_2)_5$NH-CPP, $-C(O)(CH_2)_2$NH-CPP, $-C(O)(CH_2)_2NHC(O)(CH_2)_5$NH-CPP,

and -C(O)CH_2NH-CPP, or G is of the formula:



,

wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus, with the proviso that only one instance of G is present,

wherein the targeting sequence specifically hybridizes to a bacterial mRNA target sequence that encodes a virulence factor.

In certain embodiments, the CPP is an arginine-rich peptide. In certain embodiments, the CPP is selected from **Table C1**.

Also included are methods of reducing expression and activity of a virulence factor in an mcr-positive bacteria or bacterium or methods of treating an mcr-positive bacterial infection, comprising contacting the bacteria or bacterium with an antisense oligomer described herein. In certain embodiments, the bacterium is mcr-1 positive.

In some embodiments, the bacterium is in a subject, and the method comprises administering the antisense oligomer to the subject.

In some embodiments, the bacterium is mcr-positive. In certain embodiments, the bacterium is mcr-1 positive. In further embodiments, the bacterium is mcr-2 positive. In some embodiments, the bacterium is selected from the family of Enterobacteriaceae. In some embodiments, the bacterium is selected from the genera of gram-negative bacteria. In certain embodiments, the bacterium is selected from the genus *Escherichia*, *Acinetobacter*, *Klebsiella*, *Pseudomonas* and *Burkholderia*. In certain embodiments, the bacterium is *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* or *Burkholderia cepacia* (complex).

Some methods include administering the oligomer separately or concurrently with an antimicrobial agent, for example, where administration of the oligomer increases susceptibility of the bacterium to the antimicrobial agent. Some methods include administering the oligomer by aerosolization.

In some embodiments, the oligomer reduces the minimum inhibitory concentration (MIC) of one or more antimicrobial agent against an mcr-positive bacterium by at least about 10% relative to a control. In certain embodiments, the oligomer increases the susceptibility of an mcr-positive bacterium to one or more antimicrobial agent by at least about 10% relative to a control. In some

embodiments, the oligomer increases the susceptibility of an mcr-positive bacterium to one or more antimicrobial agent by at least about 2-fold relative to a control. In certain embodiments, the bacterium is mcr-1 positive.

In certain embodiments, the virulence factor is an antibiotic resistance protein selected from one or more of MCR polymyxin resistance protein. In some embodiments, the one or more polymyxin resistance protein is a phosphoethanolamine transferase enzyme. In further embodiments, the one or more polymyxin resistance protein is encoded by *mcr-1*. In yet further embodiments, the one or more polymyxin resistance protein is encoded by *mcr-2*.

In some embodiments, the virulence factor is a protein associated with one or more essential biochemical pathways and/or cellular processes. In some embodiments, the protein associated with one or more essential biochemical pathways and/or cellular processes is a protein associated with fatty acid biosynthesis. In some embodiments, the protein associated with fatty acid biosynthesis is an acyl carrier protein (ACP). In further embodiments, the acyl carrier protein is encoded by *acpP*. In some embodiments, the protein associated with one or more essential biochemical pathways and/or cellular processes is a protein associated with murein biosynthesis. In some embodiments, the protein associated with murein biosynthesis is a peptidoglycan biosynthesis protein. In certain embodiments, the peptidoglycan biosynthesis protein is a UDP-N-acetylglucosamine 1-carboxyvinyltransferase. In particular embodiments, the UDP-N-acetylglucosamine 1-carboxyvinyltransferase is encoded by *murA*.

In certain embodiments, administration of the antisense oligomer sensitizes mcr-positive bacteria to one or more polymyxin antibiotic. In some embodiments, the one or more polymyxin antibiotic is selected from polysporin, Neosporin, Bacitracin, colistimethate, colistin (polymyxin E), and polymyxin B. In certain embodiments, the bacteria are mcr-1 positive.

In certain embodiments, the bacterium is a gram-negative bacterium, the virulence factor is an antibiotic resistance protein encoded by one or more of *mcr*, and the antimicrobial agent is selected from one or more of polymyxin, aminoglycoside antibiotics, tetracycline antibiotics, and β-lactam antibiotics.

In some embodiments, the bacterium is a gram-negative bacterium resistant to polymyxin and the antisense oligomer targets one or more genes encoding one or more proteins associated with polymyxin resistance. In certain embodiments, the one or more genes encode one or more MCR polymyxin resistance proteins.

In some embodiments, the bacterium is a gram-negative bacterium resistant to polymyxin and the antisense oligomer targets one or more genes encoding one or more proteins associated with one or more essential biochemical pathways and/or cellular processes. In certain embodiments,

the one or more genes encode one or more proteins associated with fatty acid biosynthesis and/or murein biosynthesis. In further embodiments, the one or more genes is *acpP* and/or *murA*.

Also included are pharmaceutical compositions, comprising an antisense oligomer described herein and a pharmaceutically-acceptable carrier. Certain pharmaceutical compositions can further comprise one or more antimicrobial agents.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A shows an exemplary morpholino oligomer structure with a phosphorodiamidate linkage. **FIG. 1B-1E** show the repeating subunit segment of exemplary morpholino oligomers, designated B through E. **FIG. 1F-1H** show exemplary peptide PMO conjugates structures used in the exemplary PPMOs.

FIG. 2 shows peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) designed to target the *mcr-1* and *mcr-2* genes. PPMOs were designed and synthesized to the *mcr-1* gene which confers transmissible colistin resistance. The start site is indicated in italics and sequence variations in *mcr-2* are in bold. PPMOs have also been designed to target the more recently described *mcr-2*.

FIG. 3 shows minimum inhibitory concentration (MIC) of polymyxins against *mcr-1* strains. Colistimethate, colistin sulfate (polymyxin E), and polymyxin B sulfate MICs are expressed as (μg/mL) in four clinical strains (AF##) and a standard laboratory strain (25922). *mcr-1* was also expressed on a pBAD vector in the standard TOP10 cloning *E. coli*. MICs from the originating publication (Poirel L. et al. (2016) Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*, The Lancet Infectious Diseases, 2016, vol. 16, no. 3, p. 281) are indicated in the last column.

FIG. 4 shows *mcr-1* PPMOs resensitize *E. coli* to colistin. *E. coli* were treated with the PPMOs mcr1-0545 and mcr1-0638 at 16 μM during standard MIC testing with colistimethate. MICs are expressed as the fold enhancement of MIC compared to vehicle (H_2O) or control PPMO (ctrl). Green demonstrates two-fold or greater sensitization and red demonstrates no sensitization in MIC.

FIG. 5A-5D show minimum bactericidal concentration (MBC) is enhanced by *mcr-1* PPMOs. *E. coli* were treated with the PPMOs mcr1-0545 and mcr1-0638 at 16 μM during standard MBC testing with colistimethate. AF23 (**Figure 5A**), AF24 (**Figure 5B**), and AF31 (**Figure 5C**) demonstrated a decreased MBC for colistimethate when treated with mcr1-0545 compared to vehicle or control PPMO. AF24 also demonstrated a decreased MBC with mcr1-0638. Dashed line represents the limit of detection (LOD).

FIG. 6 shows PPMOs targeting essential genes are efficacious in *mcr-1*-positive *E. coli*. Standard MIC testing of *E. coli* with PPMOs targeted to the essential genes *acpP* or *murA*

demonstrate that PPMOs are efficacious compared to control PPMOs (represented as μM). Peptide conjugation was assessed both by site of attachment on PPMO (5' vs. 3') as well as peptide sequence. Colors represent equivalent peptides in targeted versus control PPMOs. Peptides are as follows: RXR – $(RXR)_4XB$, R6G – R_6G, and RFR – $(RFR)_4XB$ where F is phenylalanine, G is glycine, R is arginine, and X is aminohexanoic acid.

DETAILED DESCRIPTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the disclosure belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, preferred methods and materials are described. For the purposes of the present disclosure, the following terms are defined below.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

By "about" is meant a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight, or length that varies by as much as 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight, or length.

By "coding sequence" is meant any nucleic acid sequence that contributes to the code for the polypeptide product of a gene. By contrast, the term "non-coding sequence" refers to any nucleic acid sequence that does not directly contribute to the code for the polypeptide product of a gene.

Throughout this specification, unless the context requires otherwise, the words "comprise," "comprises," and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of:" Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but

that other elements are optional and may or may not be present depending upon whether or not they materially affect the activity or action of the listed elements.

As used herein, the terms "contacting a cell", "introducing" or "delivering" include delivery of the oligomers of this disclosure into a cell by methods routine in the art, e.g., transfection (e.g., liposome, calcium-phosphate, polyethyleneimine), electroporation (e.g., nucleofection), microinjection), transformation, and administration.

The terms "cell penetrating peptide" (CPP) or "a peptide moiety which enhances cellular uptake" are used interchangeably and refer to cationic cell penetrating peptides, also called "transport peptides", "carrier peptides", or "peptide transduction domains". In some aspects, the peptides have the capability of inducing cell penetration within about or at least about 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% of cells of a given population and/or allow macromolecular translocation to or within multiple tissues in vivo upon systemic administration. Particular examples of CPPs include "arginine-rich peptides." CPPs are well-known in the art and are disclosed, for example, in U.S. Application No. 2010/0016215, which is incorporated by reference in its entirety.

"An electron pair" refers to a valence pair of electrons that are not bonded or shared with other atoms.

"Homology" refers to the percentage number of amino acids that are identical or constitute conservative substitutions. Homology may be determined using sequence comparison programs such as GAP (Deveraux et al., 1984, Nucleic Acids Research 12, 387-395) or BLAST. In this way sequences of a similar or substantially different length to those cited herein could be compared by insertion of gaps into the alignment, such gaps being determined, for example, by the comparison algorithm used by GAP.

By "isolated" is meant material that is substantially or essentially free from components that normally accompany it in its native state. For example, an "isolated polynucleotide" or "isolated oligomer," as used herein, may refer to a polynucleotide that has been purified or removed from the sequences that flank it in a naturally-occurring state, e.g., a DNA fragment that is removed from the sequences that are adjacent to the fragment in the genome. The term "isolating" as it relates to cells refers to the purification of cells (e.g., fibroblasts, lymphoblasts) from a source subject (e.g., a subject with a polynucleotide repeat disease). In the context of mRNA or protein, "isolating" refers to the recovery of mRNA or protein from a source, e.g., cells.

The term "modulate" includes to "increase" or "decrease" one or more quantifiable parameters, optionally by a defined and/or statistically significant amount. By "increase" or "increasing," "enhance" or "enhancing," or "stimulate" or "stimulating," refers generally to the ability of one or antisense compounds or compositions to produce or cause a greater physiological

response (i.e., downstream effects) in a cell or a subject relative to the response caused by either no antisense compound or a control compound. Relevant physiological or cellular responses (*in vivo* or *in vitro*) will be apparent to persons skilled in the art. An "increased" or "enhanced" amount is typically a "statistically significant" amount, and may include an increase that is 1.1, 1.2, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50 or more times (e.g., 500, 1000 times) (including all integers and ranges between and above 1), e.g., 1.5, 1.6, 1.7. 1.8) the amount produced by no antisense compound (the absence of an agent) or a control compound. The term "reduce" or "inhibit" may relate generally to the ability of one or more antisense compounds or compositions to "decrease" a relevant physiological or cellular response, such as a symptom of a disease or condition described herein, as measured according to routine techniques in the diagnostic art. Relevant physiological or cellular responses (*in vivo* or *in vitro*) will be apparent to persons skilled in the art, and may include reductions in bacterial cell growth, reductions in the minimum inhibitory concentration (MIC) of an antimicrobial agent, and others. A "decrease" in a response may be "statistically significant" as compared to the response produced by no antisense compound or a control composition, and may include a 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% decrease, including all integers and ranges in between.

As used herein, an "antisense oligomer," "oligomer" or "oligomer" refers to a linear sequence of nucleotides, or nucleotide analogs, which allows the nucleobase (for example a purine or pyrimidine base-pairing moiety) to hybridize to a target sequence in an RNA by Watson-Crick base pairing, to form an oligomer:RNA heteroduplex within the target sequence. The terms "antisense oligomer", "antisense oligomer", "oligomer" and "compound" may be used interchangeably to refer to an oligomer. The cyclic subunits may be based on ribose or another pentose sugar or, in certain embodiments, a morpholino group (see description of morpholino oligomers below).

The term "oligomer," "oligomer," or "antisense oligomer" also encompasses an oligomer having one or more additional moieties conjugated to the oligomer, e.g., at its 3'- or 5'-end, such as a polyethylene glycol moiety or other hydrophilic polymer, e.g., one having 10-100 monomeric subunits, which may be useful in enhancing solubility, or a moiety such as a lipid or peptide moiety that is effective to enhance the uptake of the compound into target bacterial cells and/or enhance the activity of the compound within the cell, e.g., enhance its binding to a target polynucleotide.

A "nuclease-resistant" oligomers refers to one whose backbone is substantially resistant to nuclease cleavage, in non-hybridized or hybridized form; by common extracellular and intracellular nucleases in the body or in a bacterial cell (for example, by exonucleases such as 3'-exonucleases, endonucleases, RNase H); that is, the oligomer shows little or no nuclease cleavage under normal

nuclease conditions to which the oligomer is exposed. A "nuclease-resistant heteroduplex" refers to a heteroduplex formed by the binding of an antisense oligomer to its complementary target, such that the heteroduplex is substantially resistant to in vivo degradation by intracellular and extracellular nucleases, which are capable of cutting double-stranded RNA/RNA or RNA/DNA complexes. A "heteroduplex" refers to a duplex between an antisense oligomer and the complementary portion of a target RNA.

As used herein, "nucleobase" (Nu), "base pairing moiety" or "base" are used interchangeably to refer to a purine or pyrimidine base found in native DNA or RNA (uracil, thymine, adenine, cytosine, and guanine), as well as analogs of the naturally occurring purines and pyrimidines, that confer improved properties, such as binding affinity to the oligomer. Exemplary analogs include hypoxanthine (the base component of the nucleoside inosine); 2, 6-diaminopurine; 5-methyl cytosine; C5-propynyl-modifed pyrimidines; 9-(aminoethoxy)phenoxazine (G-clamp) and the like.

A nucleobase covalently linked to a ribose, sugar analog or morpholino comprises a nucleoside. "Nucleotides" are composed of a nucleoside together with one phosphate group. The phosphate groups covalently link adjacent nucleotides to one another to form an oligomer.

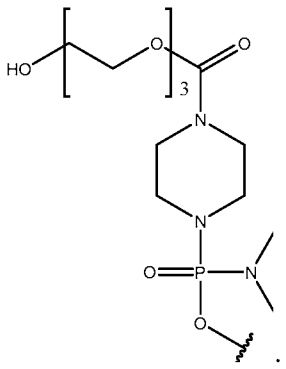
An oligomer "specifically hybridizes" to a target sequence if the oligomer hybridizes to the target under physiological conditions, with a Tm substantially greater than 40°C or 45°C, preferably at least 50°C, and typically 60°C-80°C or higher. Such hybridization preferably corresponds to stringent hybridization conditions. At a given ionic strength and pH, the Tm is the temperature at which 50% of a target sequence hybridizes to a complementary polynucleotide. Such hybridization may occur with "near" or "substantial" complementarity of the antisense oligomer to the target sequence, as well as with exact complementarity.

As used herein, "sufficient length" includes an antisense oligomer that is complementary to at least about 8, more typically about 8-10, 8-11, 8-12, 8-13, 8-14, 8-15, 8-16, 8-17, 8-18, 8-19, 8-20, 8-30, 8-40, or 10-11, 10-12, 10-13, 10-14, 10-15, 10-16, 10-17, 10-18, 10-19, 10-20, 10-30, 10-40 (including all integers and ranges in between) contiguous or non-contiguous nucleobases in a region of a bacterial mRNA target sequence. An antisense oligomer of sufficient length has at least a minimal number of nucleotides to be capable of specifically hybridizing to a region of the bacterial mRNA target. In some embodiments, an oligomer of sufficient length is from 10 to 40 or 10 to 30 nucleotides in length, for example, about 10-11, 10-12, 10-13, 10-14, 10-15, 10-16, 10-17, 10-18, 10-19, 10-20, 10-25, 10-28,10-30, 10-40, 11-12, 11-13, 11-14, 11-15, 11-16, 11-17, 11-18, 11-19, 11-20, 11-25, 11-28, 11-30, or 11-40 nucleotides in length, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides in length.

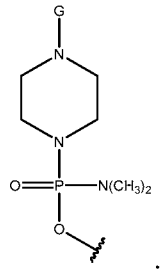
The terms "sequence identity" or, for example, comprising a "sequence 50% identical to," as used herein, refer to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" may be calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, I) or the identical amino acid residue (e.g., Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, Wis., USA) or by inspection and the best alignment (i.e., resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul et al., Nucl. Acids Res. 25:3389, 1997.

A "subject" or a "subject in need thereof" includes a mammalian subject such as a human subject.

The terms "TEG," "EG3," or "triethylene glycol tail" refer to triethylene glycol moieties conjugated to the oligomer, e.g., at its 3'- or 5'-end. For example, in some embodiments, "TEG" includes, for example, wherein T of the compound of formula (I), (II), or (III) is of the formula:



The term "pip-PDA" refers to a 5' terminal piperazine-phosphorodiamidate moiety that connects a G group, where the G group comprises a cell-penetrating peptide (CPP) and linker moiety further discussed below, to the 5'end of the oligomer by way of an amide bond between the G group linker and the piperazinyl nitrogen. For example, in some embodiments, "pip-PDA" includes wherein T of the compound of formula (I) or (II) is of the formula:



The term "target sequence" refers to a portion of the target RNA, for example, a bacterial mRNA, against which the antisense oligomer is directed, that is, the sequence to which the oligomer will hybridize by Watson-Crick base pairing of a complementary sequence. In certain embodiments, the target sequence may be a contiguous region of the translation initiation region of a bacterial gene.

The "translational start codon region" refers to a region that is 30 bases upstream or downstream of a translation initiation codon of a gene.

The term "targeting sequence" or "antisense targeting sequence" refers to the sequence in an oligomer that is complementary or substantially complementary to the target sequence in the RNA, e.g., the bacterial mRNA. The entire sequence, or only a portion, of the antisense compound may be complementary to the target sequence. For example, in an oligomer of about 10-30 bases, about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29 of the bases may be targeting sequences that are complementary to the target region. Typically, the targeting sequence is formed of contiguous bases, but may alternatively be formed of non-contiguous sequences that when placed together, e.g., from opposite ends of the oligomer, constitute sequence that spans the target sequence.

A "targeting sequence" may have "near" or "substantial" complementarity to the target sequence and still function for the purpose of the present disclosure, that is, still be "complementary." Preferably, the oligomer analog compounds employed in the present disclosure have at most one mismatch with the target sequence out of 10 nucleotides, and preferably at most one mismatch out of 20. Alternatively, the antisense oligomers employed have at least 90% sequence homology, and preferably at least 95% sequence homology, with the exemplary targeting sequences as designated herein.

As used herein, the term "quantifying", "quantification" or other related words refer to determining the quantity, mass, or concentration in a unit volume, of a nucleic acid, polynucleotide, oligomer, peptide, polypeptide, or protein.

As used herein, "treatment" of a subject (e.g. a mammal, such as a human) or a cell is any type of intervention used in an attempt to alter the natural course of the individual or cell. Treatment includes, but is not limited to, administration of a pharmaceutical composition, and may

be performed either prophylactically or subsequent to the initiation of a pathologic event or contact with an etiologic agent. Also included are "prophylactic" treatments, which can be directed to reducing the rate of progression of the disease or condition being treated, delaying the onset of that disease or condition, or reducing the severity of its onset. "Treatment" or "prophylaxis" does not necessarily indicate complete eradication, cure, or prevention of the disease or condition, or associated symptoms thereof.

Sequences for Targeting Bacterial Virulence Factors

Certain embodiments relate to antisense oligomers, and related compositions and methods, which are of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor. General examples of virulence factors include antibiotic resistance genes, biofilm formation genes, essential genes and their encoded proteins. In addition, virulence factors include genes that encode regulatory proteins that control the expression (transcription and/or translation) of other genes which provide a benefit to the bacterium during the process of infection.

In certain embodiments, the target sequence contains all or a portion (e.g., 1 or 2 nucleotides) of a translational start codon of the bacterial mRNA. In some embodiments, the target sequence contains a sequence that is about or within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 bases upstream or downstream of a translational start codon (e.g., ATG; AUG) of the bacterial mRNA target sequence. For example, in particular embodiments, the 5'-end of the target sequence is the adenine, uracil, or guanine nucleotide in a translational start codon of the bacterial mRNA. In some embodiments, the 5'-end or 3'-end of the target sequence begins at residue 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 downstream of the last nucleotide (e.g., guanine) of a translational start codon of the bacterial mRNA. In some embodiments, the 5'-end or 3'-end of the target sequence begins at residue 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 upstream of the first nucleotide (e.g., adenine) of a translational start codon of the bacterial mRNA

In some embodiments, the virulence factor is an antibiotic resistance gene or its encoded protein, i.e., a gene or protein that is associated with resistance of the bacteria to at least one antimicrobial agent. General examples of antibiotic resistance genes include beta-lactamases, which can enzymatically deactivate certain antimicrobial agents, proteins that increase the permeability or active efflux (pumping-out) of an antimicrobial agent, and proteins that modify the affinity of lipid A for one or more antimicrobial agent. Particular examples of antibiotic resistance genes include beta-

lactamase TEM-1, beta-lactamase CTX-M-1-ATG, and polymyxin resistance genes (*mcr*) encoding one or more phosphoethanolamine transferase enzyme. Exemplary translational start codon region sequences of polymyxin resistance genes are provided in **Table 1A** below.

In some embodiments, the virulence factor is a biofilm formation gene or its encoded protein, i.e., a gene or protein that is associated with or contributes to the formation of biofilm. A biofilm can include any group of bacterial cells that adhere to each other on a surface, for example, a tissue surface or a surface of an implanted medical device. Such adherent cells are often embedded within a self-produced matrix of extracellular polymeric substance (EPS), a polymeric mixture composed, for example, of extracellular DNA, proteins, and polysaccharides. Bacteria form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of cells to sub-inhibitory concentrations of antibiotics. The microbial cells growing in a biofilm are physiologically distinct from individual cells of the same organism. For example, when a bacterial cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behavior in which certain genes (e.g., biofilm formation-associated) are differentially regulated.

In some embodiments, the bacterial target is a gene or protein that is associated with biosynthesis of fatty acids. General examples of proteins associated with fatty acid biosynthesis include: acyl carrier protein (ACP), such as AcpP, that plays an essential role in stabilizing and shuttling the intermediate fatty acid chain to each of the enzymes in the fatty acid synthase complex; acyl carrier protein synthase (AcpS), an enzyme that transfers the 4'-phosphopantetheine prosthetic group to apo-ACP to form the functional holo-ACP; acetyl-CoA carboxylase, an enzyme composed of four proteins that catalyzes the conversion of acetyl-CoA to malonyl-CoA in the first committed step of fatty acid biosynthesis: AccA (carboxyltransferase alpha subunit catalyzing the transfer of the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA), AccB (biotin carboxyl carrier protein, BCCP, carrying the biotin prosthetic group covalently attached to a lysine residue proximal to the carboxyl terminus), AccC (biotin carboxylase catalyzing the carboxylation of protein bound biotin with bicarbonate), AccD (carboxyltransferase beta subunit catalyzing the transfer of the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA); fatty acid biosynthesis (Fab) enzymes, such as FabA, FabB, FabI, FabF, FabD, FabH, FabG and FabZ, that each catalyze either elongation or tailoring steps on the growing fatty acid chain. Particular examples of genes associated with fatty acid biosynthesis include *acpP*, the carboxyltransferase alpha subunit *accA*, and the acyl carrier protein synthase *fabB*.

A specific embodiment therefore relate to antisense oligomers, and related compositions and methods, which are of sufficient length and complementarity to specifically hybridize to an

mRNA target sequence of a bacterial *acpP* gene, which encodes an acyl carrier protein (ACP). In some embodiments, the *acpP* gene is from *Klebsiella, e.g., Klebsiella pneumoniae*. In some embodiments, the *acpP* gene is from *Pseudomonas, e.g., Pseudomonas aeruginosa*. In some embodiments, the *acpP* gene is from *Acinetobacter*, e.g., *Acinetobacter baumannii*. In some embodiments, the *acpP* gene is from *Escherichia*, e.g., *E. coli*.

The bacterial cell wall peptidoglycan is an essential cellular component involved in the maintenance of shape and protection from osmotic shock lysis. Typically, peptidoglycan is assembled from a basic building block composed of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid with an attached pentapeptide. In some embodiments, the bacterial target is a gene or protein that is associated with peptidoglycan biosynthesis. A particular example of a gene associated with peptidoglycan biosynthesis include *murA* (formerly known as *murZ*), which encodes a UDP-N-acetylglucosamine 1-carboxyvinyltransferase, which catalyzes the first committed step of peptidoglycan biosynthesis. The enzyme catalyzes the transfer of enolpyruvate from phosphoenolpyruvate to the 3-OH of UDP-N-acetylglucosamine. In some embodiments, the *murA* gene is from *Klebsiella, e.g., Klebsiella pneumoniae*. In some embodiments, the *murA* gene is from *Pseudomonas, e.g., Pseudomonas aeruginosa*. In some embodiments, the *murA* gene is from *Acinetobacter, e.g., Acinetobacter baumanii*. In some embodiments, the murA gene is from *Escherichia*, e.g., *E. coli*.

Table 1: Exemplary Target Sequences

Table 1A: Exemplary Antibiotic Resistance Target Sequences

Description	Sequence*	SEQ ID NO:
E. coli mcr-1	TATTTTTTGAGTAGTTTCTCATGATGCAGCATACTTCTGT	1
E. coli mcr-2	GGCATTTGTGGGTAATTTCTATGACATCACATCACTCTTG	2

Table 1B: Exemplary Fatty Acid Biosynthesis-Associated Target Sequence

Description	Sequence*	SEQ ID NO:
acpP acyl carrier protein	GCGCACTTGTAAATCTGAACTTTCCCTCGGAGGGGTAATGGACAAC ATCGAACAACGTGTCAAGAAGATGTCGCTGAACAA	3

*The thymines (T) can be uracils (U)

Thus, in certain embodiments, antisense targeting sequences are designed to hybridize to a region of one or more of the target sequences listed in **Table 1** or a target gene described herein. Selected antisense targeting sequences can be made shorter, e.g., about 8, 9, 10, 11, 12, 13, 14, or 15 bases, or longer, e.g., about 20, 30, or 40 bases, and include a small number of mismatches, as long as the sequence is sufficiently complementary to reduce transcription or translation upon

hybridization to the target sequence, and optionally forms with the RNA a heteroduplex having a Tm of 45°C or greater.

In certain embodiments, the degree of complementarity between the target sequence and antisense targeting sequence is sufficient to form a stable duplex. The region of complementarity of the antisense oligomers with the target RNA sequence may be as short as 8-9 bases, 8-10 bases, 8-11 bases, 8-12 bases, 10-11 bases, 10-12 bases, but can be 12-15 bases or more, e.g., 10-40 bases, 12-30 bases, 12-25 bases, 15-25 bases, 12-20 bases, or 15-20 bases, including all integers in between these ranges. An antisense oligomer of about 10-15 bases is generally long enough to have a unique complementary sequence. In certain embodiments, a minimum length of complementary bases may be required to achieve the requisite binding Tm, as discussed herein.

In certain embodiments, oligomers as long as 40 bases may be suitable, where at least a minimum number of bases, e.g., 10-12 bases, are complementary to the target sequence. In general, however, facilitated or active uptake in cells is optimized at oligomer lengths of less than about 30 or less than about 20 bases. Included are antisense oligomers that consist of about 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 bases, in which at least about 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 contiguous or non-contiguous bases are complementary to a target gene described herein, for example, a target sequence of **Table 1** (e.g., SEQ ID NOs: 1-3).

In certain embodiments, antisense oligomers may be 100% complementary to the target sequence, or may include mismatches, e.g., to accommodate variants, as long as a heteroduplex formed between the oligomer and target sequence is sufficiently stable to withstand the action of cellular nucleases and other modes of degradation which may occur in vivo, and reduce expression of the targeted mRNA. Hence, certain oligomers may have about or at least about 70% sequence complementarity, e.g., 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence complementarity, between the oligomer and the target sequence. Oligomer backbones that are less susceptible to cleavage by nucleases are discussed herein. Mismatches, if present, are typically less destabilizing toward the end regions of the hybrid duplex than in the middle. The number of mismatches allowed will depend on the length of the oligomer, the percentage of G:C base pairs in the duplex, and the position of the mismatch(es) in the duplex, according to well understood principles of duplex stability. Although such an antisense oligomer is not necessarily 100% complementary to the target sequence, it is effective to stably and specifically bind to the target sequence, for example, such that translation of the target RNA is reduced.

The stability of the duplex formed between an oligomer and a target sequence is a function of the binding Tm and the susceptibility of the duplex to cellular enzymatic cleavage. The Tm of an oligomer with respect to complementary-sequence RNA may be measured by conventional methods, such as those described by Hames et al., Nucleic Acid Hybridization, IRL Press, 1985, pp. 107-108 or as described in Miyada C. G. and Wallace R. B., 1987, Oligomer Hybridization Techniques, Methods Enzymol. Vol. 154 pp. 94-107. In certain embodiments, antisense oligomers may have a binding Tm, with respect to a complementary-sequence RNA, of greater than body temperature and preferably greater than about 45°C or 50°C. Tm's in the range 60-80°C or greater are also included. According to well-known principles, the Tm of an oligomer, with respect to a complementary-based RNA hybrid, can be increased by increasing the ratio of C:G paired bases in the duplex, and/or by increasing the length (in base pairs) of the heteroduplex. At the same time, for purposes of optimizing cellular uptake, it may be advantageous to limit the size of the oligomer.

Tables 2A-2B below shows exemplary targeting sequences (in a 5'-to-3' orientation) of antisense oligomers described herein. **Table 2C** shows control targeting sequences.

Table 2A: Exemplary Antibiotic Resistance Targeting Sequences

Target Gene	Targeting Sequence (TS)*	TS SEQ ID NO:
mcr	GATGTCATAGA	4
mcr	CATAGAAATTA	5
mcr	CATGAGAAACT	6
mcr	TGCTGCATCAT	7
TEM-1	TCATACTCTTC	8
CTX-M-1-ATG	TTTTTAACCAT	9

Table 2B: Exemplary Essential Targeting Sequences

Target Gene	Targeting Sequence (TS)*	TS SEQ ID NO:
acpP	TGCTCATACTC	10
acpP	CTTCGATAGTG	11
murA	ATCCATTTAGT	12

Table 2C: Control Targeting Sequences

	Control Targeting Sequence (TS)*	TS SEQ ID NO:
	TCTCAGATGGT	13
	ATCGTTGCATC	14

*The thymines (T) can be uracils (U).

Certain antisense oligomers thus comprise, consist, or consist essentially of a targeting sequence in **Tables 2A-2B** (e.g., SEQ ID NOS: 4-12) or a variant or contiguous or non-contiguous portion(s) thereof. For instance, certain antisense oligomers comprise about or at least about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27 contiguous or non-contiguous nucleotides of any of the targeting sequences in **Tables 2A-2B** (e.g., SEQ ID NOS: 4-12). For non-contiguous portions, intervening nucleotides can be deleted or substituted with a different nucleotide, or intervening nucleotides can be added. Additional examples of variants include oligomers having about or at least about 70% sequence identity or homology, e.g., 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity or homology, over the entire length of any of the targeting sequences in **Tables 2A-2B** (e.g., SEQ ID NOS: 4-12).

The activity of antisense oligomers and variants thereof can be assayed according to routine techniques in the art (see, e.g., the Examples).

I. Antisense Oligomer Compounds

The antisense oligomers typically comprises a base sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor, and thereby reduce expression (e.g., translation) of the virulence factor protein. This requirement is optionally met when the oligomer compound has the ability to be actively taken up by bacterial cells, and once taken up, form a stable duplex (or heteroduplex) with the target mRNA, optionally with a Tm greater than about 40°C or 45°C.

A. Antisense Oligomer Chemical Features

In certain embodiments, the backbone of the antisense oligomer is substantially uncharged, and is optionally recognized as a substrate for active or facilitated transport across a cell wall and/or cell membrane. The ability of the oligomer to form a stable duplex with the target RNA may also relate to other features of the backbone, including the length and degree of complementarity of the antisense oligomer with respect to the target, the ratio of G:C to A:T base matches, and the positions of any mismatched bases. The ability of the antisense oligomer to resist cellular nucleases may promote survival and ultimate delivery of the agent to the cell. Exemplary antisense oligomer targeting sequences are listed in Tables 2A-2B (supra).

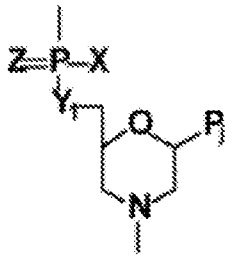
In certain embodiments, the antisense oligomer is a morpholino-based oligomer, for example, a phosphorodiamidate morpholino oligomer (PMO). Morpholino-based oligomers refer to

an oligomer comprising morpholino subunits supporting a nucleobase and, instead of a ribose, contains a morpholine ring. Exemplary internucleoside linkages include, for example, phosphoramidate or phosphorodiamidate internucleoside linkages joining the morpholine ring nitrogen of one morpholino subunit to the 4' exocyclic carbon of an adjacent morpholino subunit. Each morpholino subunit comprises a purine or pyrimidine nucleobase effective to bind, by base-specific hydrogen bonding, to a base in an oligonucleotide.

Morpholino-based oligomers (including antisense oligomers) are detailed, for example, in U.S. Patent Nos. 5,698,685; 5,217,866; 5,142,047; 5,034,506; 5,166,315; 5,185,444; 5,521,063; 5,506,337 and pending US Patent Application Nos. 12/271,036; 12/271,040; and PCT Publication No. WO/2009/064471 and WO/2012/043730 and Summerton et al. 1997, Antisense and Nucleic Acid Drug Development, 7, 187-195, which are hereby incorporated by reference in their entirety.

Within the oligomer structure, the phosphate groups are commonly referred to as forming the "internucleoside linkages" of the oligomer. The naturally occurring internucleoside linkage of RNA and DNA is a 3' to 5' phosphodiester linkage. A "phosphoramidate" group comprises phosphorus having three attached oxygen atoms and one attached nitrogen atom, while a "phosphorodiamidate" group comprises phosphorus having two attached oxygen atoms and two attached nitrogen atoms. In the uncharged or the cationic internucleoside linkages of the morpholino-based oligomers described herein, one nitrogen is always pendant to the linkage chain. The second nitrogen, in a phosphorodiamidate linkage, is typically the ring nitrogen in a morpholine ring structure.

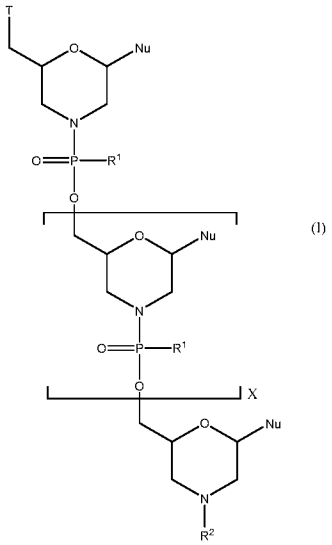
Accordingly, various embodiments of the disclosure include a substantially uncharged antisense morpholino oligomer, composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit, and having (a) about 10-40 nucleotide bases, and (b) a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor; where the oligomer is conjugated to a cell-penetrating peptide (CPP). In particular embodiments, the morpholino subunits are joined by phosphorous-containing intersubunit linkages in accordance with the structure:



where Y_1= oxygen (O) or sulfur, nitrogen, or carbon; Z=oxygen or sulfur, preferably oxygen; Pj is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide, and X is –NRR' where R and R' are the same or different and are either H or alkyl. In particular embodiments, X is –NRR', where R and R' are the same or different and are either H or methyl.

Also included are antisense oligomer that comprise a sequence of nucleotides of the formula in **FIG. 1A-1E**. In **FIG. 1A**, B is a purine or pyrimidine base-pairing moiety effective to bind, by base-specific hydrogen bonding, to a base in a polynucleotide. Y_1 or Y_2 may be oxygen, sulfur, nitrogen, or carbon, preferably oxygen. The X moiety pendant from the phosphorus may be fluorine, an alkyl or substituted alkyl, an alkoxy or substituted alkoxy, a thioalkoxy or substituted thioalkoxy, or unsubstituted, monosubstituted, or disubstituted nitrogen, including cyclic structures, such as morpholines or piperidines. Alkyl, alkoxy and thioalkoxy include 1-6 carbon atoms. The Z moieties may be sulfur or oxygen, and are preferably oxygen.

In various aspects, an antisense oligomer of the disclosure includes a compound of formula (I):



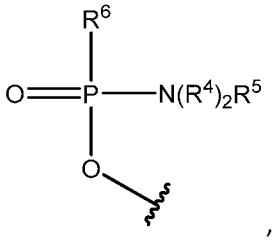
(I)

or a pharmaceutically acceptable salt thereof,

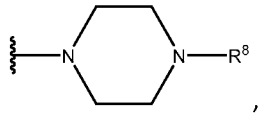
where each Nu is a nucleobase which taken together forms a targeting sequence;

X is an integer from 9 to 38;

T is selected from OH and a moiety of the formula:



where each R^4 is independently C_1-C_6 alkyl, and R^5 is selected from an electron pair and H, and R^6 is selected from OH, $–N(R^7)CH_2C(O)NH_2$, and a moiety of the formula:



where:

R^7 is selected from H and C_1-C_6 alkyl, and

R^8 is selected from G, -C(O)-R^9OH, acyl, trityl, and 4-methoxytrityl, where:

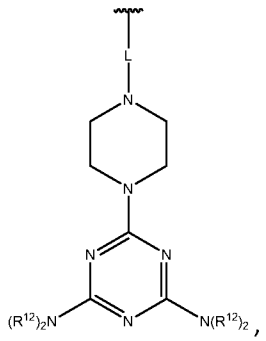
R^9 is of the formula -(O-alkyl)$_y$- wherein y is an integer from 3 to 10 and each of

the y alkyl groups is independently selected from C_2-C_6 alkyl;

each instance of R^1 is $–N(R^{10})_2R^{11}$ wherein each R^{10} is independently C_1-C_6 alkyl, and R^{11} is

selected from an electron pair and H;

R^2 is selected from H, G, acyl, trityl, 4-methoxytrityl, benzoyl, stearoyl, and a moiety of the formula:



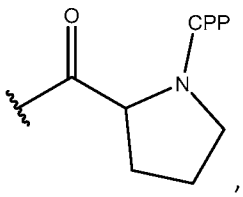
where L is selected from $–C(O)(CH_2)_6C(O)–$ and $-C(O)(CH_2)_2S_2(CH_2)_2C(O)–$, and each R^{12} is of the formula $–(CH_2)_2OC(O)N(R^{14})_2$ wherein each R^{14} is of the formula $-(CH_2)_6NHC(=NH)NH_2$; and

R^3 is selected from an electron pair, H, and C_1-C_6 alkyl,

wherein G is a cell penetrating peptide (“CPP”) and linker moiety selected from

$-C(O)(CH_2)_5$NH-CPP, $-C(O)(CH_2)_2$NH-CPP, $-C(O)(CH_2)_2NHC(O)(CH_2)_5$NH-CPP,

and $-C(O)CH_2$NH-CPP, or G is of the formula:



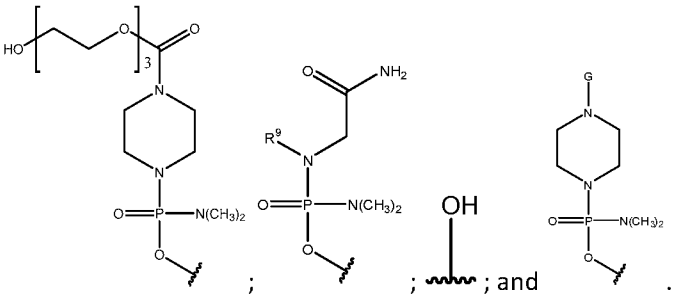
,

wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus, with the proviso that only one instance of G is present,

wherein the targeting sequence specifically hybridizes to a bacterial mRNA target sequence that encodes a virulence factor.

In some embodiments, X is from 9 to 18. In certain embodiments, X is 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30. In various embodiments, X is 9.

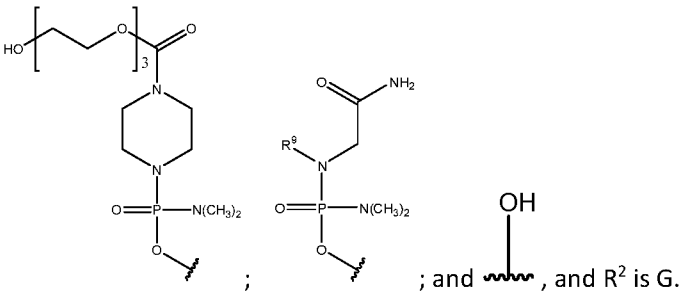
In certain embodiments, T is selected from:



; ; ; and .

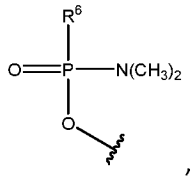
In some embodiments, R^2 is selected from H, G, acyl, trityl, 4-methoxytrityl, benzoyl, and stearoyl.

In various embodiments, T is selected from:

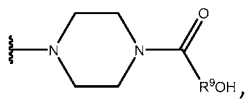


; ; and , and R^2 is G.

In some embodiments, T is of the formula:

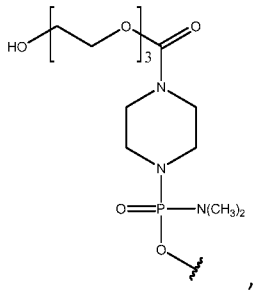


R^6 is of the formula:



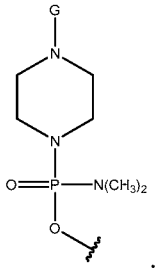
and R^2 is G.

In certain embodiments, T is of the formula:

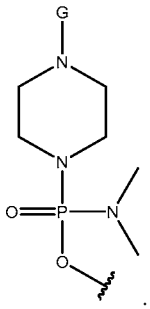


and R^2 is G.

In certain embodiments, T is of the formula:



In some embodiments, R^2 is G or T is of the formula:

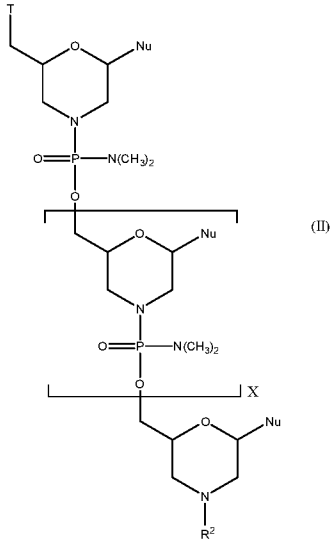


In some embodiments, R^2 is selected from H, acyl, trityl, 4-methoxytrityl, benzoyl, and stearoyl.

In various embodiments, R^2 is selected from H or G, and R^3 is selected from an electron pair or H. In a particular embodiment, R^2 is G. In some embodiments, R^2 is H or acyl. In some

embodiments, each R^1 is $-N(CH_3)_2$. In some embodiments, at least one instance of R^1 is $-N(CH_3)_2$. In certain embodiments, each instance of R^1 is $-N(CH_3)_2$.

In various embodiments of the disclosure, an antisense oligomer of the disclosure includes a compound of formula (II):



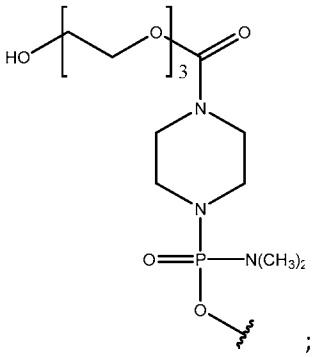
(II)

or a pharmaceutically acceptable salt thereof,

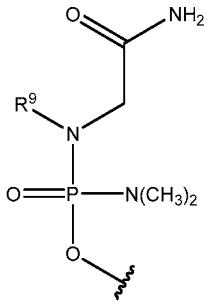
where each Nu is a nucleobase which taken together forms a targeting sequence;

X is an integer from 9 to 28;

T is selected from:

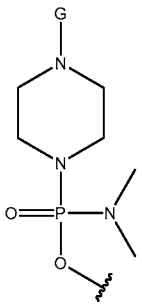


;



;

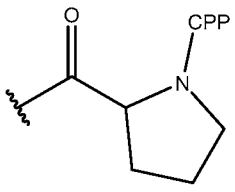
; and



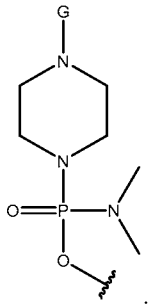
; and

R^2 is selected from H, G, acyl, trityl, 4-methoxytrityl, benzoyl, and stearoyl; and

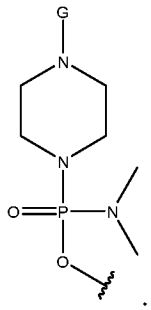
wherein G is a cell penetrating peptide ("CPP") and linker moiety selected from $-C(O)(CH_2)_5NH$-CPP, $-C(O)(CH_2)_2NH$-CPP, $-C(O)(CH_2)_2NHC(O)(CH_2)_5NH$-CPP, and $-C(O)CH_2NH$-CPP, or G is of the formula:



, wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus, with the proviso that only one instance of G is present. In various embodiments, R^2 is G or T is of the formula:

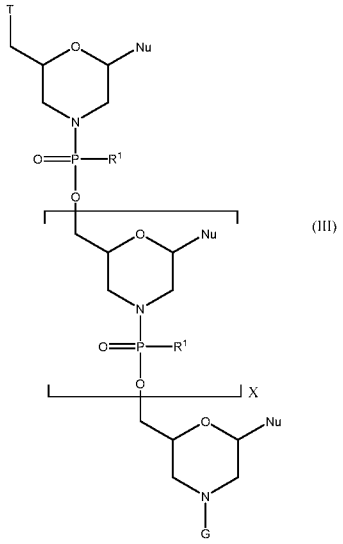


In some embodiments, T is TEG as defined above, R^2 is G, and R^3 is an electron pair or H. In certain embodiments, R^2 is selected from H, acyl, trityl, 4-methoxytrityl, benzoyl, and stearoyl and T is of the formula:



In various embodiments, X is 9.

In various aspects, an antisense oligomer of the disclosure includes a compound of formula (III):



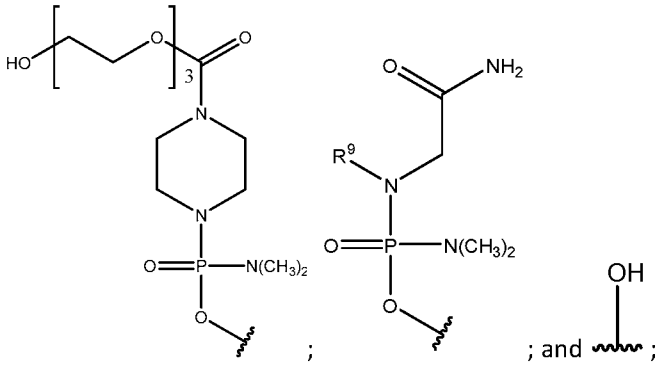
(III)

or a pharmaceutically acceptable salt thereof,

where each Nu is a nucleobase which taken together forms a targeting sequence;

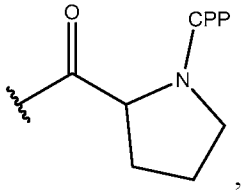
X is an integer from 9 to 28;

T is selected from:



each instance of R^1 is $-N(R^{10})_2R^{11}$ wherein each R^{10} is independently C_1-C_6 alkyl, and R^{11} is selected from an electron pair and H; and

G is a cell penetrating peptide ("CPP") and linker moiety selected from -C(O)$(CH_2)_5$NH-CPP, -C(O)$(CH_2)_2$NH-CPP, -C(O)$(CH_2)_2$NHC(O)$(CH_2)_5$NH-CPP, and -C(O)CH_2NH-CPP, or G is of the formula:



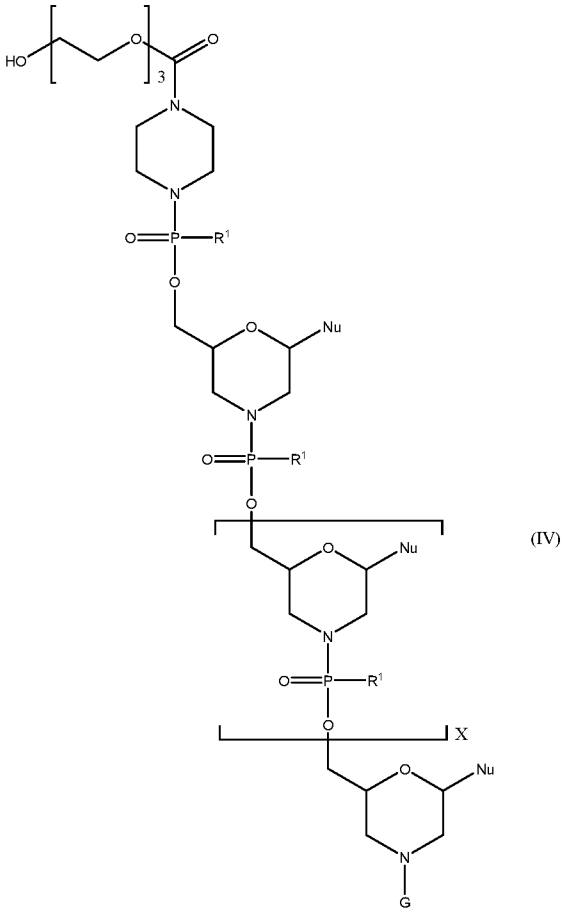
,

wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus.

In some embodiments, at least one instance of R^1 is $-N(CH_3)_2$. In certain embodiments, each instance of R^1 is $-N(CH_3)_2$.

In various embodiments, X is 9.

In various aspects, an antisense oligomer of the disclosure includes a compound of formula (IV):



(IV)

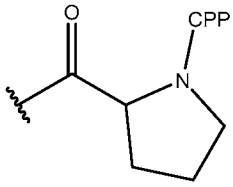
or a pharmaceutically acceptable salt thereof, wherein:

X is an integer from 9 to 28;

each Nu is a nucleobase which taken together forms a targeting sequence;

each instance of R^1 is $-N(R^{10})_2R^{11}$ wherein each R^{10} is independently C_1-C_6 alkyl, and R^{11} is selected from an electron pair and H; and

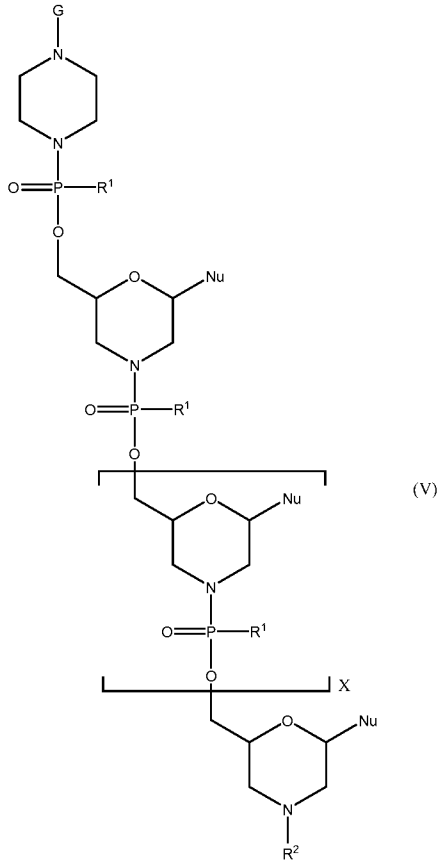
G is a cell penetrating peptide ("CPP") and linker moiety selected from -C(O)$(CH_2)_5$NH-CPP, -C(O)$(CH_2)_2$NH-CPP, -C(O)$(CH_2)_2$NHC(O)$(CH_2)_5$NH-CPP, and -C(O)CH_2NH-CPP, or G is of the formula:



wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus. In some embodiments, at least one instance of R^1 is -N$(CH_3)_2$. In certain embodiments, each instance of R^1 is -N$(CH_3)_2$.

In various embodiments, X is 9.

In various aspects, an antisense oligomer of the disclosure can be a compound of formula (V):



(V)

wherein:

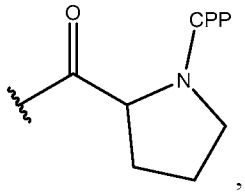
X is an integer from 9 to 18;

each Nu is a nucleobase which taken together forms a targeting sequence;

each instance of R^1 is $-N(R^{10})_2R^{11}$ wherein each R^{10} is independently C_1-C_6 alkyl, and R^{11} is selected from an electron pair and H; and

R^2 is selected from H, trityl, 4-methoxytrityl, acyl, benzoyl, and stearoyl,

wherein G is a cell penetrating peptide ("CPP") and linker moiety selected from -C(O)$(CH_2)_5$NH-CPP, -C(O)$(CH_2)_2$NH-CPP, -C(O)$(CH_2)_2$NHC(O)$(CH_2)_5$NH-CPP, and -C(O)CH_2NH-CPP, or G is of the formula:

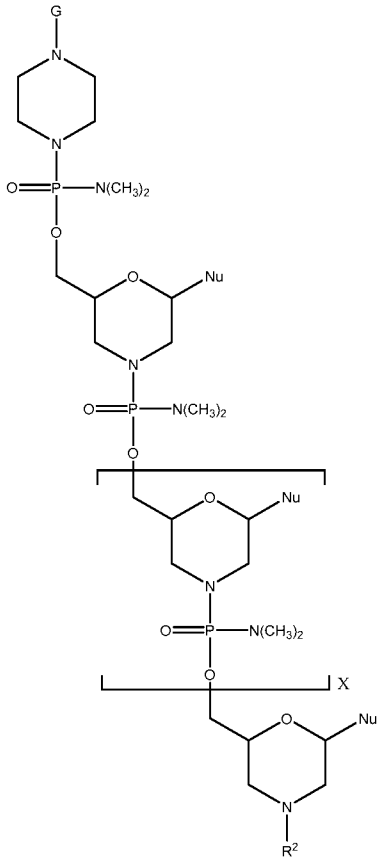


,

wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus. In some embodiments, at least one instance of R^1 is -N$(CH_3)_2$. In certain embodiments, each instance of R^1 is -N$(CH_3)_2$.

In various embodiments, X is 9.

In various aspects, an antisense oligomer of the disclosure includes a compound of formula (VI):



(VI)

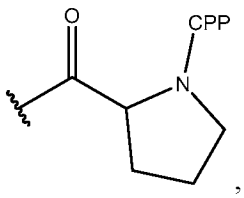
or a pharmaceutically acceptable salt thereof, wherein:

X is an integer from 9 to 28;

each Nu is a nucleobase which taken together forms a targeting sequence;

R^2 is selected from H or acyl; and

G is a cell penetrating peptide ("CPP") and linker moiety selected from $-C(O)(CH_2)_5NH$-CPP, $-C(O)(CH_2)_2NH$-CPP, $-C(O)(CH_2)_2NHC(O)(CH_2)_5NH$-CPP, and $-C(O)CH_2NH$-CPP, or G is of the formula:



,

wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus.

In various embodiments, X is 9.

The antisense oligomers can be prepared by stepwise solid-phase synthesis, employing methods known in the art and described in the references cited herein.

B. Cell-Penetrating Peptides

In certain embodiments, the antisense oligomer is conjugated to a cell-penetrating peptide (CPP). In some embodiments, the CPP is an arginine-rich peptide. By "arginine-rich carrier peptide" is meant that the CPP has at least 2, and preferably 2, 3, 4, 5, 6, 7, or 8 arginine residues, each optionally separated by one or more uncharged, hydrophobic residues, and optionally containing about 6-14 amino acid residues. **FIGs. 1F-1H** show exemplary chemical structures of CPP-PMO conjugates used in the Examples, including 5' and 3' PMO conjugates.

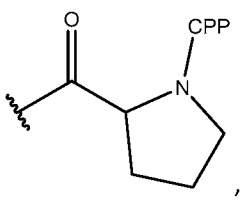
Exemplary CPPs are provided in **Table C1** (SEQ ID NOS: 15-22).

Table C1: Exemplary Cell-Penetrating Peptides

Name	Sequence	SEQ ID NO:
$(RXR)_4$	RXRRXRRXRRXR	15
$(RFF)_3R$	RFFRFFRFFR	16
$(RXR)_4XB$	RXRRXRRXRRXRXB	17
$(RFF)_3RXB$	RFFRFFRFFRXB	18
$(RFF)_3RG$	RFFRFFRFFR	19
R_6G	RRRRRRG	20
R_6	RRRRRR	21
$(RFR)_4XB$	RFRRFRRFRRFRXB	22
R is arginine; X is 6-aminohexanoic acid; B is β-alanine; F is phenylalanine; G is glycine		

CPPs, their synthesis, and methods of conjugating a CPP to an oligomer are detailed, for example, in International Patent Application Publication Nos. WO 2004/097017, WO 2009/005793, and WO 2012/150960, which are all incorporated by reference in their entirety.

In some embodiments, the CPP is linked at its C-terminus to the 3'-end or the 5'-end of the oligomer via a 1, 2, 3, 4, or 5 amino acid linker. In particular embodiments, including antisense oligomer compounds of formula (I)-(VI), the linkers can include: $-C(O)(CH_2)_5NH$-CPP (X linker), $-C(O)(CH_2)_2NH$-CPP (B linker), $-C(O)(CH_2)_2NHC(O)(CH_2)_5NH$-CPP (XB peptide linker), and $-C(O)CH_2NH$-CPP (Gly linker), or G is of the formula:

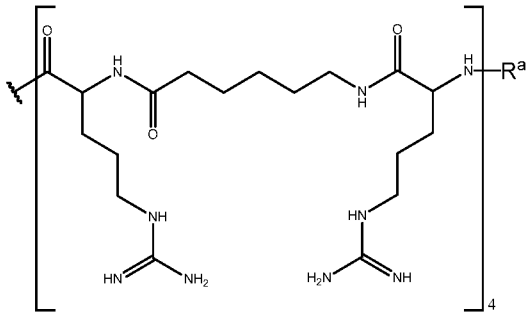


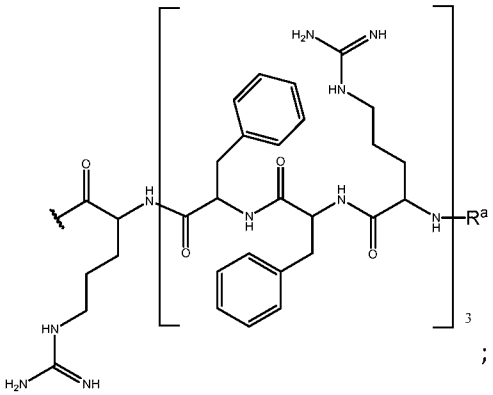
,

wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus. In some embodiments of the disclosure, including antisense oligomer compounds of formula (I)-(VI), G

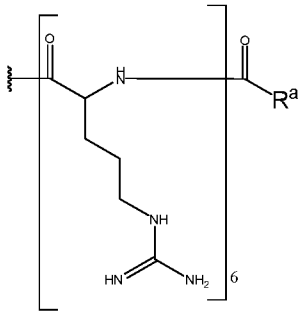
is selected from SEQ ID NOs: 17, 20, and 22. In various embodiments, including antisense oligomer compounds of formula (I)-(VI), the CPP is selected from SEQ ID NO: 17, 20, and 22, and the linker is selected from the group described above.

In some embodiments, including antisense oligomer compounds of formula (I)-(VI), the CPP is selected from:


;

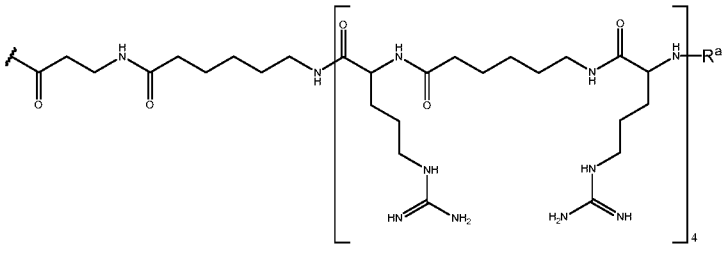

;

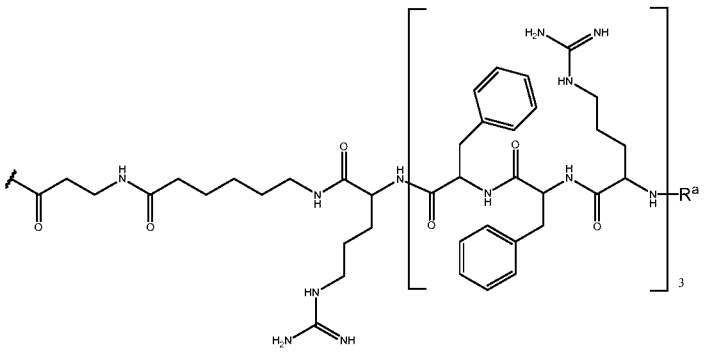
and


,

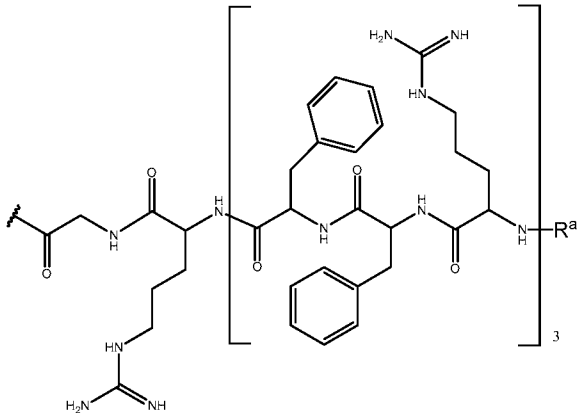
wherein R^a is selected from H, acetyl, benzoyl, and stearoyl.

In some embodiments, including antisense oligomer compounds of formula (I)-(VI), G is selected from:

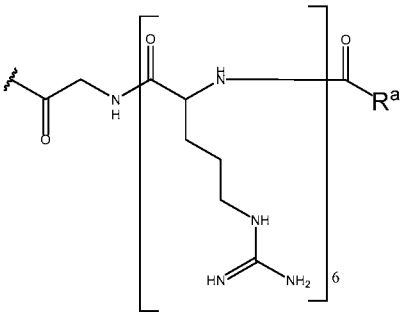

;



;



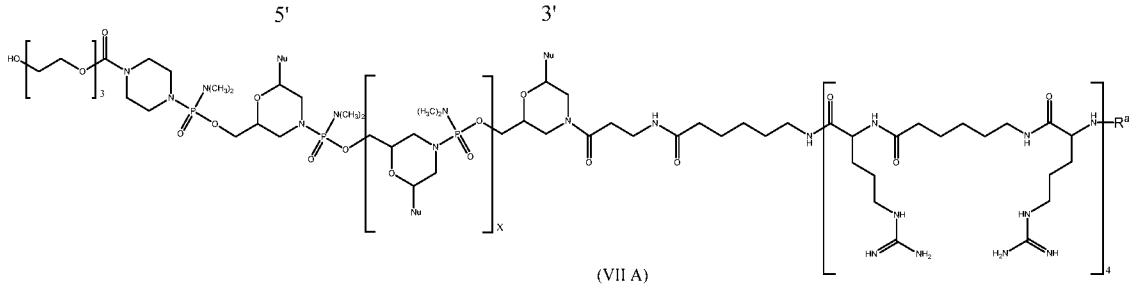
; and



,

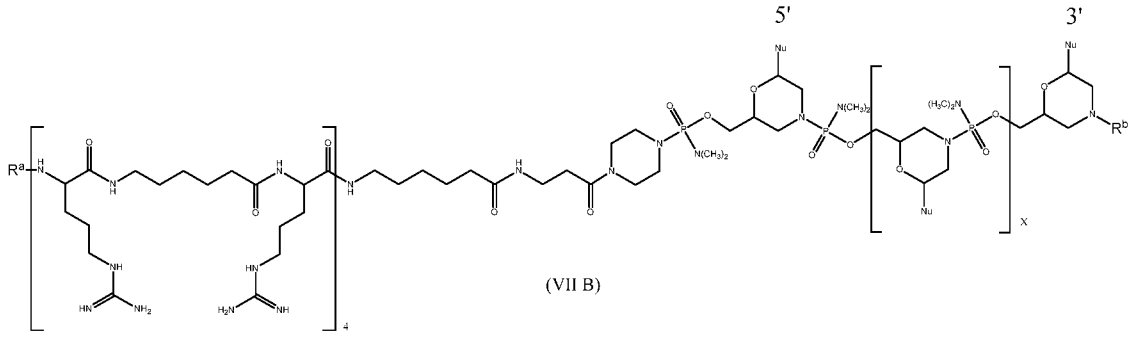
wherein R^a is selected from H, acetyl, benzoyl, and stearoyl.

In various aspects, an antisense oligomer of the disclosure, or a pharmaceutically acceptable salt thereof, includes an antisense oligomer of the formula (VII) selected from:



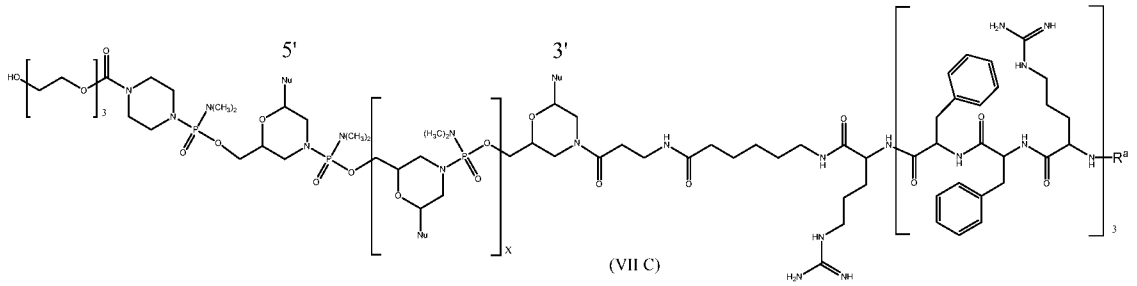
(VII A)

;



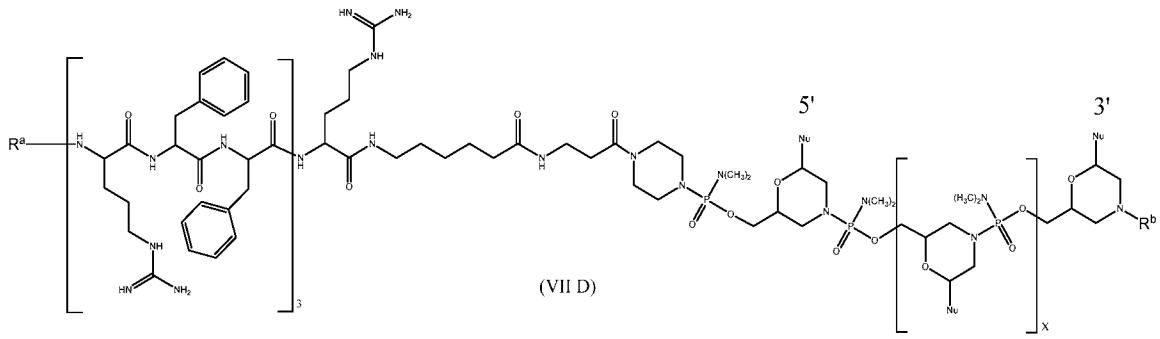
(VII B)

;



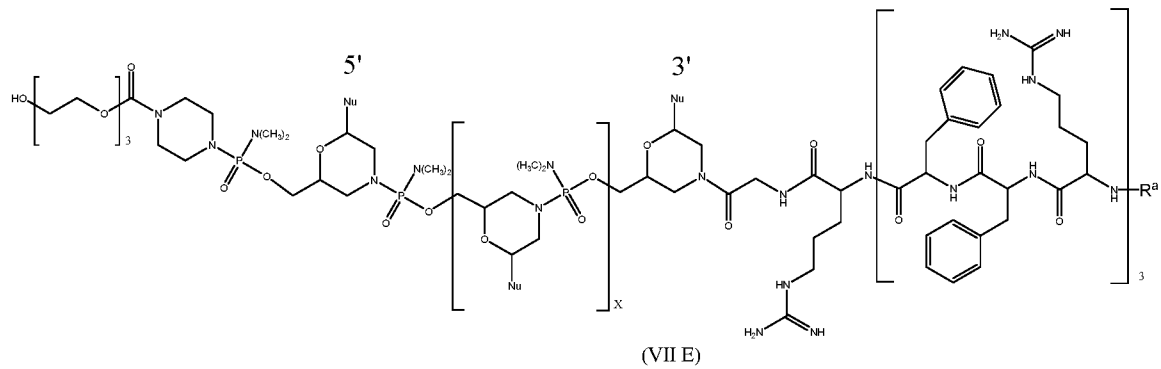
(VII C)

;



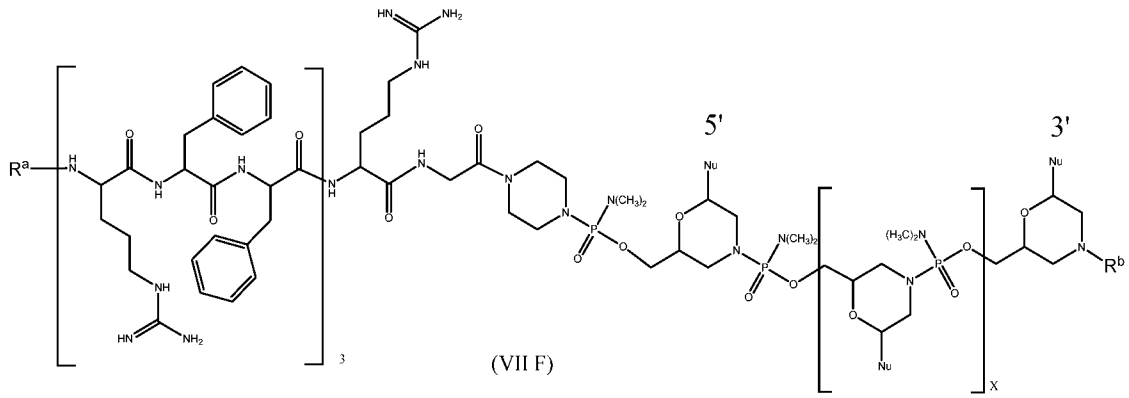
(VII D)

;



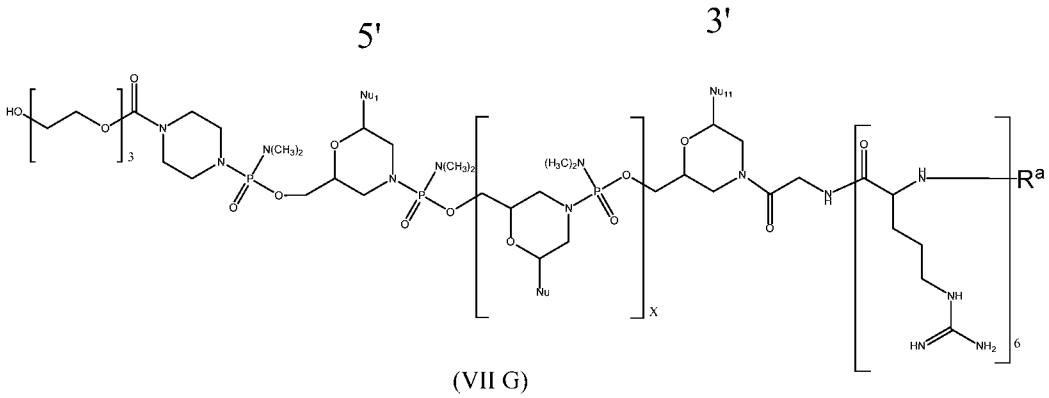
(VII E)

;



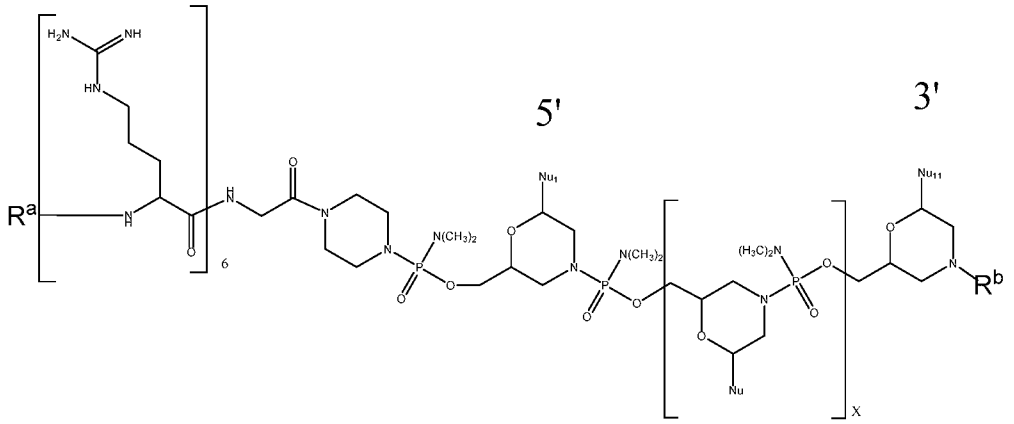
(VII F)

;



(VII G)

; and



(VII H)

,

wherein X is an integer from 9 to 38, R^a is selected from H, acetyl, benzoyl, and stearoyl, R^b is selected from H, acetyl, benzoyl, stearoyl, trityl, and 4-methoxytrityl, and each Nu is a purine or pyrimidine base-pairing moiety which taken together form a targeting sequence described above.

C. Antisense Oligomer Targeting Sequence

In various embodiments of the antisense oligomers of the disclosure, including the antisense oligomer compounds of formulas (I)-(VII), the targeting sequence can specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor. In some embodiments, the target sequence comprises a translational start codon of the bacterial mRNA and/or a sequence within about 30 bases upstream or downstream of the translational start codon of the bacterial mRNA. In certain embodiments, the virulence factor can be an antibiotic resistance protein, a biofilm formation protein or an essential protein. In some embodiments, the antibiotic resistance protein may be selected from at least one of beta-lactamase TEM-1, beta-lactamase CTX-M-1-ATG, and polymyxin resistance gene (*mcr*). In some embodiments, the target sequence can be selected from SEQ ID NOs: 1 and 2, wherein thymine bases (T) are optionally uracil bases (U). In certain embodiments, the targeting sequence may be one of the targeting sequences set forth in SEQ ID NOs: 4-9, may comprise a fragment of at least 10 contiguous nucleotides of SEQ ID NOs: 4-9, or may comprise a variant having at least 80% sequence identity to SEQ ID NOs: 4-9, wherein thymine bases (T) are optionally uracil bases (U). In some embodiments, an essential protein may be encoded by at least one of acpP or MurA. In some embodiments, the target sequence can be SEQ ID NO: 3, wherein thymine bases (T) are optionally uracil bases (U). In some embodiments, the targeting sequence may be a targeting sequence set forth in SEQ ID NOs: 10-12, may comprise a fragment of at least 10 contiguous nucleotides of SEQ ID NOs: 10-12, or may comprise a variant having at least 80% sequence identity to SEQ ID NOs: 10-12, wherein thymine bases (T) are optionally uracil bases (U).In some embodiments of the disclosure, including the antisense oligomer compounds of formulas (I)-(VII), the targeting sequence is selected from:

a) SEQ ID NO: 4 (GAT GTC ATA GA);

b) SEQ ID NO: 5 (CAT AGA AAT TA);

c) SEQ ID NO: 6 (CAT GAG AAA CT);

d) SEQ ID NO: 7 (TGC TGC ATC AT);

e) SEQ ID NO: 8 (TCA TAC TCT TC); and

f) SEQ ID NO: 9 (TTT TTA ACC AT),

wherein X is 9, and thymine bases (T) may be uracil bases(U).

In various embodiments of the disclosure, including the antisense oligomer compounds of formulas (I)-(VII), the targeting sequence is selected from:

a) SEQ ID NO: 10 (TGC TCA TAC TC);

b) SEQ ID NO: 11 (CTT CGA TAG TG); and

c) SEQ ID NO: 12 (ATC CAT TTA GT),

wherein X is 9, and thymine bases (T) may be uracil bases(U).

D. Exemplary Antisense Oligomers

Exemplary antisense oligomers (AONs) of the disclosure include those described in **Tables 3A-3B** below. Control AONs include those described in Table 3C.

Table 3A: Exemplary Antibiotic Resistance Targeting AONs

PMO Name	Target Gene	Targeting Sequence (TS)*	TS SEQ ID NO:	5' Attachment ***	3' Attachment **	CPP SEQ ID NO.
PPMO#1	mcr	GAT GTC ATA GA	4		$(RXR)_4XB$-	17
PPMO#2	mcr	CAT AGA AAT TA	5		$(RXR)_4XB$-	17
PPMO#3	mcr	CAT GAG AAA CT	6	HO-(CH2CH2O)3-CO-(pip-PDA)-	$(RXR)_4XB$-	17
PPMO#4	mcr	TGC TGC ATC AT	7	HO-(CH2CH2O)3-CO-(pip-PDA)-	$(RXR)_4XB$-	17
PPMO#5	mcr	TCA TAC TCT TC	8	HO-(CH2CH2O)3-CO-(pip-PDA)-	$(RXR)_4XB$-	17
PPMO#6	mcr	TTT TTA ACC AT	9	HO-(CH2CH2O)3-CO-(pip-PDA)-	$(RXR)_4XB$-	17

* The thymines (T) can be uracils (U);
** R is arginine, X is 6-aminohexanoic acid and B is beta-alanine.
*** a 5' CPP is linked through a pip-PDA moiety described above.

Table 3B: Exemplary Essential Genes Targeting AONs

PMO Name	Target Gene	Targeting Sequence (TS)*	TS SEQ ID NO:	5' Attachment ***	3' Attachment **	CPP SEQ ID NO.
PPMO#7	acpP	TGC TCA TAC TC	10	$(RXR)_4XB$-	H	17
PPMO#8	acpP	TGC TCA TAC TC	10	H	$(RXR)_4XB$-	17
PPMO#9	acpP	TGC TCA TAC TC	10	TEG	$(RXR)_4XB$-	17
PPMO#10	acpP	TGC TCA TAC TC	10	TEG	R_6G	20
PPMO#11	acpP	CTT CGA TAG TG	11	$(RFR)_4XB$-	H	22
PPMO#12	murA	ATC CAT TTA GT	12	TEG	$(RXR)_4XB$-	17

* The thymines (T) can be uracils (U);
** R is arginine, X is 6-aminohexanoic acid, B is beta-alanine, and G is glycine.
*** R is arginine, X is 6-aminohexanoic acid, B is beta-alanine, F is phenylalanine and TEG is described above.

Table 3C: Control Targeting AONs

PMO Name	Targeting Sequence (TS)*	TS SEQ ID NO:	5' Attachment ***	3' Attachment **	CPP SEQ ID NO.
PPMO#13	TCT CAG ATG GT	13	TEG	$(RXR)_4XB$-	17
PPMO#14	ATC GTT GCA TC	14	$(RXR)_4XB$-	H	17
PPMO#15	TCT CAG ATG GT	13	$(RFR)_4XB$-	H	22
PPMO#16	TCT CAG ATG GT	13	TEG	R_6G	20
PPMO#17	TCT CAG ATG GT	13	$(RXR)_4XB$-	H	17

* The thymines (T) can be uracils (U);
** R is arginine, X is 6-aminohexanoic acid, B is beta-alanine, and G is glycine.
*** R is arginine, X is 6-aminohexanoic acid, B is beta-alanine, F is phenylalanine and TEG is described above.

II. Methods of Use and Formulations

Embodiments of the present disclosure include methods of using the antisense oligomers described herein to reduce the expression and activity of one or more bacterial virulence factors. Certain embodiments include methods of using the antisense oligomers to reduce replication, proliferation, virulence factors, or growth of bacteria, for example, to treat bacterial infections in a subject, either alone or in combination with one or more additional antimicrobial agents. In some instances, the antisense oligomers increase the susceptibility of the bacterium to antibiotics. Certain embodiments include methods of using the antisense oligomers described herein to reduce the formation or existence of bacterial biofilms, for instance, to treat bacterial infections in a subject, either alone or in combination with one or more additional antimicrobial agents.

Also included are pharmaceutical compositions comprising the antisense oligomers, typically in combination with a pharmaceutically-acceptable carrier. The methods provided herein can be practiced *in vitro* or *in vivo*.

For example, certain embodiments include methods of treating a bacterial infection in a subject, comprising administering to a subject in need thereof (e.g., subject having or at risk for having a bacterial infection) an antisense oligomer or pharmaceutical composition described herein. Also included are methods of reducing virulence and/or biofilm formation of a bacteria or bacterium which comprises a gene encoding a virulence factor, comprising contacting the bacteria or bacterium with an antisense oligomer described herein.

In some embodiments, the bacterium is mcr-positive. In certain embodiments, the bacterium is mcr-1 positive. In further embodiments, the bacterium is mcr-2 positive. In some embodiments, the bacterium is selected from the family of Enterobacteriaceae. In some embodiments, the bacterium is selected from the genera of gram-negative bacteria. In certain embodiments, the bacterium is selected from the genus *Escherichia*, *Acinetobacter*, *Klebsiella*, *Pseudomonas* and *Burkholderia*. In certain embodiments, the bacterium is *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* or *Burkholderia cepacia* (complex).

Escherichia is a genus of Gram-negative, non-spore forming, facultatively anaerobic, rod-shaped bacteria from the family Enterobacteriaceae, and includes the species *Escherichia coli*, which is responsible for the vast majority of *Escherichia*-related pathogenesis.

Acinetobacter is a genus of Gram-negative bacteria belonging to the class of Gammaproteobacteria. Examples of clinically-relevant *Acinetobacter* complexes include the *Acinetobacter calcoaceticus-baumanii* complex (glucose-oxidizing nonhemolytic), *Acinetobacter*

lwoffii (glucose-negative nonhemolytic), and *Acinetobacter haemolyticus* (hemolytic). Specific examples include *Acinetobacter baumannii*. *Acinetobacter baumannii* is a ubiquitous organism that has emerged over recent years to be a significant cause of hospital-acquired infections. It is all the more concerning given that *A. baumannii* has become one of the most antibiotic resistant Gram-negative pathogens that the medical community currently faces worldwide. The rapid increase in multidrug-resistance in *A. baumannii* has left few therapeutic choices for the treating physician. Older drugs such as colistin are now frequently used, although colistin-resistant strains have now emerged. *A. baumannii* can cause a variety of clinical infections, with pneumonia being one of the most frequent.

Klebsiella is a genus of non-motile, Gram-negative, oxidase-negative, rod-shaped bacteria with a prominent polysaccharide-based capsule. *Klebsiella* organisms can lead to a wide range of disease states, such as pneumonia, urinary tract infections, septicemia, meningitis, diarrhea, and soft tissue infections. The majority of human infections are caused by *Klebsiella pneumoniae* and *Klebsiella oxytoca*. *Klebsiella* has become increasingly drug resistant. A recent outbreak of *Klebsiella* infections at the National Institutes of Health Clinical Center illustrates the difficulty in treating patients with these infections and the complexities that institutions can face in trying to eradicate these strains from the hospital environment. The spread of carbapenem-resistant *Enterobacteriaceae* (CRE) (including *K. pneumoniae*) has happened rapidly worldwide, including in the U.S. where carbapenemase-producing CRE has now been reported in most states.

Burkholderia (previously part of *Pseudomonas*) refers to a group of near ubiquitous gram-negative, motile, obligately aerobic rod-shaped bacteria. These protobacteria include pathogenic bacteria such as *Burkholderia mallei*, responsible for glanders; *Burkholderia pseudomallei*, causative agent of melioidosis; and *Burkholderia cepacia*, a significant pathogen of pulmonary infections, for example, in subjects with cystic fibrosis (CF). *Burkholderia cepacia* (or *Burkholderia cepacia* complex) is a Gram-negative bacterium composed of many different sub-species, including, for example, *Burkholderia cenocepacia*, *Burkholderia multivorans*, *Burkholderia vietnamiensis*, *Burkholderia stabilis*, *Burkholderia anthina*, *Burkholderia pyrrocinia*, *Burkholderia dolosa*, and/or *Burkholderia ambifaria*.

Pseudomonas is a genus of Gram-negative aerobic gammaproteobacteria, belonging to the family Pseudomonadaceae. *Pseudomonas aeruginosa* is increasingly recognized as an emerging opportunistic pathogen of clinical relevance. *Pseudomonas aeruginosa* can cause a variety of infections in the hospital setting including VAP, bacteremia and wound infections in burn patients. In addition, it is the major pathogen associated with lung infections in cystic fibrosis. Eighty percent of CF patients are infected with *P. aeruginosa* by adulthood, and chronic lung infections with this

pathogen are the primary cause of morbidity and mortality. In the CF patient, complete eradication of *P. aeruginosa* is rarely achieved. *P. aeruginosa* is naturally resistant to many antibiotics and is becoming resistant to those it was once sensitive to. Importantly, multi-drug resistant isolates of *P. aeruginosa* are now common in both CF and non-CF patients leaving virtually no therapeutic options. The formation of biofilm is a major virulence trait in *Pseudomonas*.

Thus, in some embodiments, the bacterium is any of the foregoing members of the genera *Escherichia*, *Acinetobacter*, *Klebsiella*, *Burkholderia*, and *Pseudomonas*. In some embodiments, the bacterium is any of the foregoing members of the genera *Escherichia*, *Acinetobacter*, *Klebsiella*, *Burkholderia*, and *Pseudomonas* and is mcr-positive.

In specific embodiments, the bacterium is one or more of *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Burkholderia cepacia* (complex), or *Pseudomonas aeruginosa*. In yet further embodiments, the bacterium is one or more of *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Burkholderia cepacia* (complex), or *Pseudomonas aeruginosa* and is mcr-positive. In certain embodiments, the bacterium is mcr-1 positive.

In certain embodiments, the bacterium is multi-drug resistance (MDR) bacteria or bacterium. Multiple drug resistance (MDR), multi-drug resistance or multiresistance is a condition enabling disease-causing microorganisms (bacteria, viruses, fungi or parasites) to resist distinct antimicrobials such as antibiotics, antifungal drugs, antiviral medications, antiparasitic drugs, and others. In particular embodiments, the bacterium is extensively-drug resistant (XDR) or pan-drug resistant (PDR). In some embodiments, the bacterium is a polymyxin resistant (*mcr*) Gram-negative bacterium. In some embodiments, the polymyxin resistance is plasmid-mediated. In further embodiments, the polymyxin resistance is transmissible from one bacterium to another.

As noted above, the bacteria or bacterium described herein typically comprise (e.g., encode) one or more virulence factors such as antibiotic resistance genes and/or essential genes. General examples of antibiotic resistance genes (and their related proteins) include beta-lactamases, which can enzymatically deactivate certain antimicrobial agents, genes/proteins which increase the permeability or active efflux (pumping out) of an antimicrobial agent, and proteins that modify the affinity of lipid A for one or more antimicrobial agent. Particular examples of antibiotic resistance genes include beta-lactamase TEM-1, beta-lactamase CTX-M-1-ATG, and polymyxin resistance genes (*mcr*) encoding one or more phosphoethanolamine transferase enzyme. In some embodiments, the *mcr* gene is from *Klebsiella, e.g., Klebsiella pneumoniae*. In some embodiments, the *mcr* gene is from *Pseudomonas, e.g., Pseudomonas aeruginosa*. In some embodiments, the *mcr* gene is from *Acinetobacter*, e.g., *Acinetobacter baumannii*. In some embodiments, the *mcr* gene is from

Escherichia, e.g., *E. coli*. In some embodiments, the *mcr* gene is from *Burkholderia*, e.g., *Burkholderia cepacia* (complex).

In some embodiments, the bacteria or bacterium described herein are mcr-1 positive and comprise (e.g., encode) a protein that is associated with biosynthesis of fatty acids. In some embodiments, the bacteria or bacterium described herein are resistant to one or more polymyxin antibiotic and comprise (e.g., encode) a protein that is associated with biosynthesis of fatty acids. General examples of proteins associated with fatty acid biosynthesis include: acyl carrier protein (ACP), such as AcpP, that plays an essential role in stabilizing and shuttling the intermediate fatty acid chain to each of the enzymes in the fatty acid synthase complex; acyl carrier protein synthase (AcpS), an enzyme that transfers the 4'-phosphopantetheine prosthetic group to apo-ACP to form the functional holo-ACP; acetyl-CoA carboxylase, an enzyme composed of four proteins that catalyzes the conversion of acetyl-CoA to malonyl-CoA in the first committed step of fatty acid biosynthesis: AccA (carboxyltransferase alpha subunit catalyzing the transfer of the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA), AccB (biotin carboxyl carrier protein, BCCP, carrying the biotin prosthetic group covalently attached to a lysine residue proximal to the carboxyl terminus), AccC (biotin carboxylase catalyzing the carboxylation of protein bound biotin with bicarbonate), AccD (carboxyltransferase beta subunit catalyzing the transfer of the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA); fatty acid biosynthesis (Fab) enzymes, such as FabA, FabB, FabI, FabF, FabD, FabH, FabG and FabZ, that each catalyze either elongation or tailoring steps on the growing fatty acid chain. Particular examples of genes associated with fatty acid biosynthesis include *acpP*, the carboxyltransferase alpha subunit *accA*, and the acyl carrier protein synthase *fabB*.

A specific embodiment therefore relate to antisense oligomers, and related compositions and methods, which are of sufficient length and complementarity to specifically hybridize to an mRNA target sequence of a bacterial *acpP* gene, which encodes an acyl carrier protein (ACP). In some embodiments, the *acpP* gene is from *Klebsiella, e.g., Klebsiella pneumoniae*. In some embodiments, the *acpP* gene is from *Pseudomonas, e.g., Pseudomonas aeruginosa*. In some embodiments, the *acpP* gene is from *Acinetobacter*, e.g., *Acinetobacter baumannii*. In some embodiments, the *acpP* gene is from *Escherichia*, e.g., *E. coli*. In some embodiments, the *acpP* gene is from *Burkholderia*, e.g., *Burkholderia cepacia* (complex).

In some embodiments, the bacteria or bacterium described herein are mcr-1 positive and comprise (e.g., encode) a protein that is associated with peptidoglycan biosynthesis. In some embodiments, the bacteria or bacterium described herein are resistant to one or more polymyxin antibiotic and comprise (e.g., encode) a protein that is associated with peptidoglycan biosynthesis. A particular example of a gene associated with peptidoglycan biosynthesis include *murA* (formerly

known as *murZ*), which encodes a UDP-N-acetylglucosamine 1-carboxyvinyltransferase, which catalyzes the first committed step of peptidoglycan biosynthesis. The enzyme catalyzes the transfer of enolpyruvate from phosphoenolpyruvate to the 3-OH of UDP-N-acetylglucosamine. In some embodiments, the *murA* gene is from *Klebsiella, e.g., Klebsiella pneumoniae*. In some embodiments, the *murA* gene is from *Pseudomonas, e.g., Pseudomonas aeruginosa*. In some embodiments, the *murA* gene is from *Acinetobacter, e.g., Acinetobacter baumanii*. In some embodiments, the *murA* gene is from *Escherichia*, e.g., *E. coli*. In some embodiments, the *murA* gene is from *Burkholderia*, e.g., *Burkholderia cepacia* (complex).

In some embodiments, the bacteria or bacterium described herein is mcr-positive and/or resistant to one or more polymyxin antibiotic and the antisense oligomer targets one or more genes encoding one or more proteins associated with polymyxin resistance. In certain embodiments, the one or more genes encode one or more MCR polymyxin resistance proteins. In certain embodiments, the one or more genes encoding one or more MCR polymyxin resistance proteins is selected from *mcr-1* and *mcr-2*. In some embodiments, the one or more polymyxin antibiotic is selected from polysporin, Neosporin, Bacitracin, colistimethate, colistin (polymyxin E), and polymyxin B.

In some embodiments, the bacteria or bacterium described herein is mcr-positive and/or resistant to one or more polymyxin antibiotic and the antisense oligomer targets one or more genes encoding one or more proteins associated with one or more essential biochemical pathways and/or cellular processes. In certain embodiments, the one or more genes encode one or more proteins associated with fatty acid biosynthesis and/or murein biosynthesis. In further embodiments, the one or more genes is *acpP* and/or *murA*. In certain embodiments, the bacterium is mcr-1 positive.

In some embodiments, the antisense oligomer reduces or inhibits the growth of the bacterium. For instance, in some embodiments, the antisense oligomer reduces growth of the bacterium by about or at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000% or more (including all integers and ranges in between), relative to a control (e.g., absence of the antisense oligomer, scrambled oligomer, prior to contacting with the oligomer), or by about or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100-fold or more (including all integers and ranges in between), relative to a control. Bacterial growth can be measured *in vitro* (see, e.g., the Examples) or *in vivo*. In some embodiments, as described herein, the antisense oligomer is employed in combination with one or more antimicrobial agents.

In some embodiments, the antisense oligomer reduces beta-lactamase (e.g., carbapenemase) activity in the periplasm of the bacterium by about or at least about 5, 10, 15, 20,

25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000% or more (including all integers and ranges in between), relative to a control, or by at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100-fold or more (including all integers and ranges in between), relative to a control. In some embodiments, the antisense oligomer reduces meropenemase enzymatic activity in the periplasm of the bacterium. In particular embodiments, the antisense oligomer that reduces beta-lactamase (e.g., carbapenemase) activity is targeted against TEM-1, and the bacterium is an *Acinetobacter*, *Escherichia*, *Pseudomonas*, *Burkholderia* or *Klebsiella* species, for example, *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* (complex) or *Klebsiella pneumoniae* which comprises or expresses TEM-1. These are exemplary bacterial species and it is expected that any bacterium expressing the TEM-1 gene is susceptible to the compounds and methods described herein. In particular embodiments, the antisense oligomer that reduces beta-lactamase (e.g., carbapenemase) activity is targeted against CTX-M-1-ATG, and the bacterium is an *Acinetobacter*, *Escherichia*, *Pseudomonas*, *Burkholderia* or *Klebsiella* species, for example, *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* (complex) or *Klebsiella pneumoniae* which comprises or expresses CTX-M-1-ATG. These are exemplary bacterial species and it is expected that any bacterium expressing the CTX-M-1-ATG gene is susceptible to the compounds and methods described herein. Beta-lactamase (e.g., carbapenemase) activity can be measured according to routine techniques in the art.

In some embodiments, the antisense oligomer reduces the minimum inhibitory concentration (MIC) of the antimicrobial agent against the bacterium by about or at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000% or more (including all integers and ranges in between), relative to a control, or by at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100-fold or more (including all integers and ranges in between), relative to a control.

In some embodiments, the antisense oligomer increases the susceptibility of a bacterium to one or more antimicrobial agent by about or at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000% or more (including all integers and ranges in between), relative to a control, or by at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100-fold or more (including all integers and ranges in between), relative to a control.

In some embodiments, the antisense oligomer reduces phosphoethanolamine transferase enzymatic activity in the bacterium. In particular embodiments, the antisense oligomer that reduces

phosphoethanolamine transferase activity is targeted against *mcr*, and the bacterium is an *Acinetobacter*, *Escherichia*, *Pseudomonas*, *Burkholderia* or *Klebsiella* species, for example, *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* (complex) or *Klebsiella pneumoniae* which comprises or expresses *mcr*. These are exemplary bacterial species and it is expected that any bacterium expressing an *mcr* gene is susceptible to the compounds and methods described herein. Phosphoethanolamine transferase activity can be measured according to routine techniques in the art.

In some embodiments, the antisense oligomer reduces acyl carrier protein activity in the bacterium. In particular embodiments, the antisense oligomer that reduces acyl carrier protein activity is targeted against *acpP*, and the bacterium is an *Acinetobacter*, *Escherichia*, *Pseudomonas*, *Burkholderia* or *Klebsiella* species, for example, *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* (complex) or *Klebsiella pneumoniae* which comprises or expresses *acpP* and mcr-1. These are exemplary bacterial species and it is expected that any bacterium expressing an *acpP* gene and an mcr gene is susceptible to the compounds and methods described herein. Acyl carrier protein activity can be measured according to routine techniques in the art.

In some embodiments, the antisense oligomer reduces UDP-N-acetylglucosamine 1-carboxyvinyltransferase enzymatic activity in the bacterium. In particular embodiments, the antisense oligomer that reduces UDP-N-acetylglucosamine 1-carboxyvinyltransferase activity is targeted against *murA*, and the bacterium is an *Acinetobacter*, *Escherichia*, *Pseudomonas*, *Burkholderia* or *Klebsiella* species, for example, *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* (complex) or *Klebsiella pneumoniae* which comprises or expresses *murA* and mcr-1. These are exemplary bacterial species and it is expected that any bacterium expressing a *murA* gene and an mcr gene is susceptible to the compounds and methods described herein. UDP-N-acetylglucosamine 1-carboxyvinyltransferase activity can be measured according to routine techniques in the art.

In some embodiments, the methods are practiced *in vivo*, and comprise administering the antisense oligomer to a subject in need thereof, for example, a subject in need thereof that is infected or at risk for being infected by one or more of the bacteria or bacterium described herein. The antisense oligomers of the disclosure can thus be administered to subjects to treat (prophylactically or therapeutically) an infection by any of the bacteria or bacterium described herein. In conjunction with such treatment, pharmacogenomics (e.g., the study of the relationship between an individual's genotype/phenotype and that individual's response to a foreign compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or

therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug.

Thus, a physician or clinician may consider applying knowledge obtained in relevant pharmacogenomics studies in determining whether to administer a therapeutic agent as well as tailoring the dosage and/or therapeutic regimen of treatment with a therapeutic agent.

Effective delivery of the antisense oligomer to the target nucleic acid is one aspect of treatment. Routes of antisense oligomer delivery include, but are not limited to, various systemic routes, including oral and parenteral routes, e.g., intravenous, subcutaneous, intraperitoneal, and intramuscular, as well as inhalation, transdermal, and topical delivery. The antisense oligomer may be aerosolized for delivery. The appropriate route may be determined by one of skill in the art, as appropriate to the condition of the subject under treatment. Vascular or extravascular circulation, the blood or lymph system, and the cerebrospinal fluid are some non-limiting sites where the antisense oligomers may be introduced. Direct CNS delivery may be employed, for instance, intracerebral, intraventricular, or intrathecal administration may be used as routes of administration.

In certain embodiments, the antisense oligomers of the disclosure can be delivered by transdermal methods (e.g., via incorporation of the antisense oligomers into, e.g., emulsions, with such antisense oligomers optionally packaged into liposomes). Such transdermal and emulsion/liposome-mediated methods of delivery are described for delivery of antisense oligomers in the art, e.g., in U.S. Pat. No. 6,965,025, the contents of which are incorporated in their entirety by reference herein.

In certain embodiments, the antisense oligomers of this disclosure can be delivered by aerosolization. Advantages to administering medications to the lung as an aerosol include: a more rapid onset of action compared to oral therapy; high local concentration by delivery directly to the airways; needle-free systemic delivery of drugs with poor oral bioavailability; and pain- and needle-free delivery for drugs that require subcutaneous or intravenous injection. Traditional aerosol therapies with the lung as the target consist of short-acting β2-adrenergic agonists and long-acting β2–adrenergic agonists (LABA), anticholinergics, inhaled corticosteroids (ICSs), nonsteroidal antiinflammatories, antibiotics and mucolytics. Devices that deliver these drugs include pressurized metered-dose inhalers (pMDIs), used either alone, or attached to spacers, or valved holding chambers (VHCs), breathactuated (BA)-pMDIs, dry powder inhalers (DPIs), jet nebulizers, vibrating mesh nebulizers and soft mist inhalers. Well-established treatment guidelines for the management of asthma and chronic obstructive pulmonary disease (COPD) each recommend inhaled therapy as

the primary route to administer these medications. Treatment guidelines for cystic fibrosis (CF) also include recommendations for inhalation of aerosolized medications.

The antisense oligomers described herein may also be delivered via an implantable device. Design of such a device is an art-recognized process, with, e.g., synthetic implant design described in, e.g., U.S. Pat. No. 6,969,400, the contents of which are incorporated by reference.

Antisense oligomers can be introduced into cells using art-recognized techniques (e.g., transfection, electroporation, fusion, liposomes, colloidal polymeric particles and viral and non-viral vectors as well as other means known in the art). The method of delivery selected will depend at least on the oligomer chemistry, the cells to be treated and the location of the cells and will be apparent to the skilled artisan. For instance, localization can be achieved by liposomes with specific markers on the surface to direct the liposome, direct injection into tissue containing target cells, specific receptor-mediated uptake, or the like.

As known in the art, antisense oligomers may be delivered using, e.g., methods involving liposome-mediated uptake, lipid conjugates, polylysine-mediated uptake, nanoparticle-mediated uptake, and receptor-mediated endocytosis, as well as additional non-endocytic modes of delivery, such as microinjection, permeabilization (e.g., streptolysin-O permeabilization, anionic peptide permeabilization), electroporation, and various non-invasive non-endocytic methods of delivery that are known in the art (see, e. g., Dokka and Rojanasakul, Advanced Drug Delivery Reviews 44:35-49, incorporated by reference in its entirety).

The antisense oligomers may be administered in any convenient vehicle or carrier which is physiologically and/or pharmaceutically acceptable. Such a composition may include any of a variety of standard pharmaceutically acceptable carriers employed by those of ordinary skill in the art. Examples include, but are not limited to, saline, phosphate buffered saline (PBS), water, aqueous ethanol, emulsions, such as oil/water emulsions or triglyceride emulsions, tablets and capsules. The choice of suitable physiologically acceptable carrier will vary dependent upon the chosen mode of administration. “Pharmaceutically acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions

The compounds (e.g., antisense oligomers, antimicrobial agents) described herein may generally be utilized as the free acid or free base. Alternatively, the compounds of this disclosure

may be used in the form of acid or base addition salts. Acid addition salts of the free amino compounds of the present disclosure may be prepared by methods well known in the art, and may be formed from organic and inorganic acids. Suitable organic acids include maleic, fumaric, benzoic, ascorbic, succinic, methanesulfonic, acetic, trifluoroacetic, oxalic, propionic, tartaric, salicylic, citric, gluconic, lactic, mandelic, cinnamic, aspartic, stearic, palmitic, glycolic, glutamic, and benzenesulfonic acids.

Suitable inorganic acids include hydrochloric, hydrobromic, sulfuric, phosphoric, and nitric acids. Base addition salts included those salts that form with the carboxylate anion and include salts formed with organic and inorganic cations such as those chosen from the alkali and alkaline earth metals (for example, lithium, sodium, potassium, magnesium, barium and calcium), as well as the ammonium ion and substituted derivatives thereof (for example, dibenzylammonium, benzylammonium, 2-hydroxyethylammonium, and the like). Thus, the term "pharmaceutically acceptable salt" is intended to encompass any and all acceptable salt forms.

In addition, prodrugs are also included within the context of this disclosure. Prodrugs are any covalently bonded carriers that release a compound in vivo when such prodrug is administered to a patient. Prodrugs are generally prepared by modifying functional groups in a way such that the modification is cleaved, either by routine manipulation or in vivo, yielding the parent compound. Prodrugs include, for example, compounds of this disclosure wherein hydroxy, amine or sulfhydryl groups are bonded to any group that, when administered to a patient, cleaves to form the hydroxy, amine or sulfhydryl groups. Thus, representative examples of prodrugs include (but are not limited to) acetate, formate and benzoate derivatives of alcohol and amine functional groups of the antisense oligomers of the disclosure. Further, in the case of a carboxylic acid (-COOH), esters may be employed, such as methyl esters, ethyl esters, and the like.

In some instances, liposomes may be employed to facilitate uptake of the antisense oligomer into cells (see, e.g., Williams, S.A., Leukemia 10(12):1980-1989, 1996; Lappalainen et al., Antiviral Res. 23:119, 1994; Uhlmann et al., antisense oligomers: a new therapeutic principle, Chemical Reviews, Volume 90, No. 4, 25 pages 544-584, 1990; Gregoriadis, G., Chapter 14, Liposomes, Drug Carriers in Biology and Medicine, pp. 287-341, Academic Press, 1979). Hydrogels may also be used as vehicles for antisense oligomer administration, for example, as described in WO 93/01286. Alternatively, the oligomers may be administered in microspheres or microparticles. (See, e.g., Wu, G.Y. and Wu, C.H., J. Biol. Chem. 262:4429-4432, 30 1987). Alternatively, the use of gas-filled microbubbles complexed with the antisense oligomers can enhance delivery to target tissues, as described in US Patent No. 6,245,747. Sustained release compositions may also be used. These

may include semipermeable polymeric matrices in the form of shaped articles such as films or microcapsules.

In certain embodiments, the antisense oligomer is administered to a mammalian subject, e.g., human or domestic animal, exhibiting the symptoms of a bacterial infection (e.g., antibiotic resistance or MDR bacterial infection), in a suitable pharmaceutical carrier. In some aspects, the subject is a human subject, e.g., a patient diagnosed as having a bacterial infection. In particular embodiments, the antisense oligomer is contained in a pharmaceutically acceptable carrier, and is delivered orally. In some embodiments, the antisense oligomer is contained in a pharmaceutically acceptable carrier, and is delivered intravenously (i.v.).

In some embodiments, the antisense oligomer is administered in an amount and manner effective to result in a peak blood concentration of at least 200-400 nM antisense oligomer. Typically, one or more doses of antisense oligomer are administered, generally at regular intervals, for a period of about one to two weeks. Certain doses for oral administration are from about 1-1000 mg oligomer per 70 kg. In some cases, doses of greater than 1000 mg oligomer/patient may be necessary. For i.v. administration, some doses are from about 0.5 mg to 1000 mg oligomer per 70 kg. The antisense oligomer may be administered at regular intervals for a short time period, e.g., daily for two weeks or less. However, in some cases the antisense oligomer is administered intermittently over a longer period of time. Administration may be followed by, or concurrent with, administration of an antimicrobial (e.g., antibiotic) or other therapeutic treatment, as described herein. The treatment regimen may be adjusted (dose, frequency, route, etc.) as indicated, based on the results of immunoassays, other biochemical tests and physiological examination of the subject under treatment.

An effective *in vivo* treatment regimen using the antisense oligomers of the disclosure may vary according to the duration, dose, frequency and route of administration, as well as the condition of the subject under treatment (i.e., prophylactic administration versus administration in response to localized or systemic infection). Accordingly, such *in vivo* therapy will often include monitoring by tests appropriate to the particular type of disorder or bacterial infection under treatment, and corresponding adjustments in the dose or treatment regimen, in order to achieve an optimal therapeutic outcome.

Treatment may be monitored, e.g., by general indicators of disease known in the art. The efficacy of an *in vivo* administered antisense oligomer of the disclosure may be determined from biological samples (tissue, blood, urine etc.) taken from a subject prior to, during and subsequent to administration of the antisense oligomer. Assays of such samples include (1) monitoring the presence or absence of heteroduplex formation with target and non-target sequences, using

procedures known to those skilled in the art, e.g., an electrophoretic gel mobility assay; (2) monitoring the amount of a mutant mRNA in relation to a reference normal mRNA or protein as determined by standard techniques such as RT-PCR, Northern blotting, ELISA or Western blotting.

III. Combination Therapies

Certain embodiments include combination therapies, for example, the administration of antisense oligomers in combination with antimicrobial agents such as antibiotics. Combination therapies can be employed, for example, to increase the sensitivity or susceptibility of a given bacteria to one or more antimicrobial agents, and thereby improve the therapeutic outcome (e.g., resolution of the infection). Likewise, certain combination therapies can be employed, for example, to reduce or reverse the antibiotic resistance of a given bacteria to one or more antimicrobial agents. In particular embodiments, the antisense oligomer reduces the minimum inhibitory concentration (MIC) of an antibiotic against a bacterium. Also included are pharmaceutical compositions, as described herein, which comprise an antisense oligomer and an antimicrobial agent such as antibiotic.

In some embodiments, the antisense oligomer and the antimicrobial agent are administered separately. In certain embodiments, the antisense oligomer and the antimicrobial agent are administered sequentially. In some embodiments, the antisense oligomer and the antimicrobial agent are administered concurrently, for example, as part of the same or different pharmaceutical composition.

Examples of antimicrobial agents (e.g., antibiotics) that can be administered in combination with an antisense oligomer include beta-lactam antibiotics such as carbapenems, penicillin and penicillin derivatives (or penams), cephalosporins (e.g., Cefacetrile (cephacetrile), Cefadroxil (cefadroxyl; Duricef), Cephalexin (cefalexin; Keflex), Cefaloglycin (cephaloglycin), Cefalonium (cephalonium), Cefaloridine (cephaloradine), Cefalotin (cephalothin; Keflin), Cefapirin (cephapirin; Cefadryl), Cefatrizine, Cefazaflur, Cefazedone, Cefazolin (cephazolin; Ancef, Kefzol), Cefradine (cephradine; Velosef), Cefroxadine, Ceftezole, Cefaclor (Ceclor, Distaclor, Keflor, Raniclor), Cefonicid (Monocid), Cefprozil (cefproxil; Cefzil), Cefuroxime (Zefu, Zinnat, Zinacef, Ceftin, Biofuroksym, Xorimax), Cefuzonam, Cefmetazole, Cefotetan, Cefoxitin, loracarbef (Lorabid); Cephamycins: cefbuperazone, cefmetazole (Zefazone), cefminox, cefotetan (Cefotan), cefoxitin (Mefoxin), Cefotiam (Pansporin), Cefcapene, Cefdaloxime, Cefdinir (Sefdin, Zinir, Omnicef, Kefnir), Cefditoren, Cefetamet, Cefixime (Fixx, Zifi, Suprax), Cefmenoxime, Cefodizime, Cefotaxime (Claforan), Cefovecin (Convenia), Cefpimizole, Cefpodoxime (Vantin, PECEF), Cefteram, Ceftibuten (Cedax), Ceftiofur, Ceftiolene, Ceftizoxime (Cefizox), Ceftriaxone (Rocephin), Cefoperazone (Cefobid), Ceftazidime

(Meezat, Fortum, Fortaz), latamoxef (moxalactam), Cefclidine, cefepime (Maxipime), cefluprenam, cefoselis, Cefozopran, Cefpirome (Cefrom), Cefquinome, flomoxef, Ceftobiprole, Ceftaroline, Cefaloram, Cefaparole, Cefcanel, Cefedrolor, Cefempidone, Cefetrizole, Cefivitril, Cefmatilen, Cefmepidium, Cefoxazole, Cefrotil, Cefsumide, Ceftioxide, Cefuracetime), and monobactams (e.g., aztreonam, tigemonam, nocardin A, tabtoxin); aminoglycosides such as tobramycin, gentamicin, kanamycin a, amikacin, dibekacin, sisomicin, netilmicin, neomycin B, neomycin C, neomycin E (paromomycin), and streptomycin; tetracyclines such as tetracycline, chlortetracycline, oxytetracycline, demeclocycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline, and doxycyline; sulfonamides such as sulfacetamide, sulfadiazine, sulfadimidine, sulfafurazole, sulfisomidine, sulfadoxine, sulfamethoxazole, sulfamoxole, sulfadimethoxine, sulfamethoxypyridazine, sulfametoxydiazine, sulfadoxine, and sulfametopyrazine; quinolones such as cinoxacin, nalidixic acid, oxolinic acid (Uroxin), piromidic acid (Panacid), pipemidic acid (Dolcol) rosoxacin (Eradacil), ciprofloxacin (Alcipro,Ciprobay, Cipro, Ciproxin, ultracipro), enoxacin (Enroxil, Penetrex), fleroxacin (Megalone, Roquinol), lomefloxacin (Maxaquin), nadifloxacin (Acuatim, Nadoxin, Nadixa), norfloxacin (Lexinor, Noroxin, Quinabic, Janacin), ofloxacin (Floxin, Oxaldin, Tarivid), pefloxacin (Peflacine), rufloxacin (Uroflox), balofloxacin (Baloxin), grepafloxacin (Raxar), levofloxacin (Cravit, Levaquin, Tavanic), pazufloxacin (Pasil, Pazucross), sparfloxacin (Zagam), temafloxacin (Omniflox), tosufloxacin (Ozex, Tosacin), clinafloxacin, gatifloxacin (Zigat, Tequin) (Zymar -opth.), gemifloxacin (Factive), moxifloxacin (Acflox Woodward, Avelox,Vigamox, sitafloxacin (Gracevit), trovafloxacin (Trovan), prulifloxacin (Quisnon); oxazolidinones such as eperezolid, linezolid, posizolid, radezolid, ranbezolid, sutezolid, and tedizolid; polymyxins such as polysporin, neosporin, polymyxin B, polymyxin E (colistin); rifamycins such as rifampicin or rifampin, rifabutin, rifapentine, and rifaximin; lipiarmycins such as fidaxomicin; macrolides such as azithromycin, clarithromycin, dirithromycin, erythromycin, roxithromycin, telithromycin, carbomycin A, josamycin, kitasamycin, midecamycin/midecamycin acetate, oleandomycin, solithromycin, spiramycin, and troleandomycin; lincosamides such as lincomycin, clindamycin, and pirlimycin; cyclic lipopeptides such as daptomycin; glycopeptides such as vancomycin and teichoplanin; glycylcyclines such as tigecycline. Thus, any one or more of the foregoing antibiotics can be combined with any of the antisense oligomers described herein, for the treatment of any of the bacteria described herein.

In some embodiments, the antimicrobial agent is a beta-lactam antibiotic, as described herein. In certain of these and related embodiments, the bacterium comprises or expresses a beta-lactamase such as TEM-1 or CTX-M-1-ATG, and the antisense oligomer is targeted against the beta-lactamase. In particular embodiments, the antimicrobial agent is a carbapenem. Examples of carbapenems include meropenem, imipenem, ertapenem, doripenem, panipenem, biapenem,

razupenem, tebipenem, lenapenem, and tomopenem. In certain of these and related embodiments, the bacterium comprises or expresses a carbapenemase such as TEM-1 and CTX-M-1-ATG, and the antisense oligomer is targeted against the carbapenemase. In specific embodiments, the bacterium is *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* (complex) or *Klebsiella pneumoniae*.

In some embodiments, the antimicrobial agent is a polymyxin antibiotic, as described herein. In certain of these and related embodiments, the bacterium comprises or expresses a phosphoethanolamine transferase such as MCR-1 or MCR-2, and the antisense oligomer is targeted against the phosphoethanolamine transferase. In some embodiments, the one or more polymyxin antibiotic is selected from polysporin, Neosporin, Bacitracin, colistimethate, colistin (polymyxin E), and polymyxin B. In specific embodiments, the bacterium is *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* (complex) or *Klebsiella pneumoniae*.

In certain embodiments, the antimicrobial agent includes one or more of polymyxin, aminoglycoside antibiotics, tetracycline antibiotics, and/or β-lactam antibiotics. In some of these and related embodiments, the bacterium is selected from an *Escherichia*, *Acinetobacter*, *Klebsiella*, *Pseudomonas* and *Burkholderia* species that comprises or expresses one or more essential genes such as *acpP*, and the antisense oligomer is targeted against the essential gene. In some embodiments, the bacterium is polymyxin resistant. In certain embodiments, the bacterium is mcr-1 positive.

In certain embodiments, the antimicrobial agent includes one or more of polymyxin, aminoglycoside antibiotics, tetracycline antibiotics, and/or β-lactam antibiotics. In some of these and related embodiments, the bacterium is selected from an *Escherichia*, *Acinetobacter*, *Klebsiella*, *Pseudomonas* and *Burkholderia* species that comprises or expresses one or more essential genes such as *murA*, and the antisense oligomer is targeted against the essential gene. In some embodiments, the bacterium is polymyxin resistant. In certain embodiments, the bacterium is mcr-1 positive.

In some embodiments, the antisense oligomer increases the sensitivity of a given bacteria to the antimicrobial agent, relative to the antimicrobial agent alone. For example, in certain embodiments, the antisense oligomer increases the sensitivity of the bacterium to the antimicrobial agent by increasing the bactericidal (cell-killing) and/or bacteriostatic (growth-slowing) activity of the antimicrobial agent against the bacterium being targeted, relative to the antimicrobial agent alone. In particular embodiments, the antisense increases the sensitivity by about or at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000% or more (including all integers and ranges in between),

relative to the antimicrobial agent alone, or by about or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100-fold or more (including all integers and ranges in between), relative to the antimicrobial agent alone.

In some embodiments, the antisense oligomer reduces the minimum inhibitory concentration (MIC) of an antimicrobial agent against the bacterium being targeted, relative to the antimicrobial agent alone. The "minimum inhibitory concentration" or "MIC" refers to the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight (*in vitro*) incubation. Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. The MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against a bacterial organism. Thus, in certain embodiments, the oligomer reduces the minimum inhibitory concentration (MIC) of an antimicrobial agent against the bacterium by at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000% or more (including all integers and ranges in between), relative to the antimicrobial agent alone. In certain embodiments, the oligomer reduces the minimum inhibitory concentration (MIC) of an antimicrobial agent against the bacterium by about or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100-fold or more (including all integers and ranges in between), relative to the antimicrobial agent alone.

In some embodiments, the antisense oligomer that increases the sensitivity or reduces the MIC is targeted against *mcr-1*, the bacterium is an *Escherichia*, *Acinetobacter*, *Klebsiella*, *Pseudomonas* or *Burkholderia* species that comprises or expresses mcr-1, and the antimicrobial agent is one or more polymyxin. In some embodiments, the antisense oligomer that increases the sensitivity or reduces the MIC is targeted against mcr-2, the bacterium is an *Escherichia*, *Acinetobacter*, *Klebsiella*, *Pseudomonas* or *Burkholderia* species that comprises or expresses mcr-2, and the antimicrobial agent is one or more polymyxin.

In some embodiments, the antisense oligomer that increases the sensitivity or reduces the MIC is targeted against *acpP*, the bacterium is an *Escherichia*, *Acinetobacter*, *Klebsiella*, *Pseudomonas* or *Burkholderia* species that comprises or expresses *acpP* and mcr-1, and the antimicrobial agent is one or more of polymyxin, aminoglycoside antibiotics, tetracycline antibiotics, and/or β-lactam antibiotics.

In some embodiments, the antisense oligomer that increases the sensitivity or reduces the MIC is targeted against *murA*, the bacterium is an *Escherichia*, *Acinetobacter*, *Klebsiella*, *Pseudomonas* or *Burkholderia* species that comprises or expresses *murA* and mcr-1, and the

antimicrobial agent is one or more of polymyxin, aminoglycoside antibiotics, tetracycline antibiotics, and/or β-lactam antibiotics.

IV. Treatment Monitoring Methods

The efficacy of a given therapeutic regimen involving the methods described herein may be monitored, for example, by general indicators of bacterial infection, such as complete blood count (CBC), nucleic acid detection methods, immunodiagnostic tests, or bacterial culture.

In some aspects, identification and monitoring of bacterial infection involves one or more of (1) nucleic acid detection methods, (2) serological detection methods, i.e., conventional immunoassay, (3) culture methods, and (4) biochemical methods. Such methods may be qualitative or quantitative.

Nucleic acid probes may be designed based on publicly available bacterial nucleic acid sequences, and used to detect target genes or metabolites (i.e., toxins) indicative of bacterial infection, which may be specific to a particular bacterial type, e.g., a particular species or strain, or common to more than one species or type of bacteria (i.e., Gram positive or Gram negative bacteria). Nucleic amplification tests (e.g., PCR) may also be used in such detection methods.

Serological identification may be accomplished using a bacterial sample or culture isolated from a biological specimen, e.g., stool, urine, cerebrospinal fluid, blood, etc. Immunoassay for the detection of bacteria is generally carried out by methods routinely employed by those of skill in the art, e.g., ELISA or Western blot. In addition, monoclonal antibodies specific to particular bacterial strains or species are often commercially available.

Culture methods may be used to isolate and identify particular types of bacteria, by employing techniques including, but not limited to, aerobic versus anaerobic culture, growth and morphology under various culture conditions. Exemplary biochemical tests include Gram stain (Gram, 1884; Gram positive bacteria stain dark blue, and Gram negative stain red), enzymatic analyses, and phage typing.

It will be understood that the exact nature of such diagnostic, and quantitative tests as well as other physiological factors indicative of bacterial infection will vary dependent upon the bacterial target, the condition being treated and whether the treatment is prophylactic or therapeutic.

In cases where the subject has been diagnosed as having a particular type of bacterial infection, the status of the bacterial infection is also monitored using diagnostic techniques typically used by those of skill in the art to monitor the particular type of bacterial infection under treatment.

The PMO or PPMO treatment regimen may be adjusted (dose, frequency, route, etc.), as indicated, based on the results of immunoassays, other biochemical tests and physiological examination of the subject under treatment.

From the foregoing, it will be appreciated how various objects and features of the disclosure are met. The method provides an improvement in therapy against bacterial infection, for example, multi-drug resistant (MDR) bacteria and/or biofilm-forming bacteria, using anti-virulence antisense oligomers to achieve enhanced cell uptake and anti-bacterial action. As a result, drug therapy is more effective and less expensive, both in terms of cost and amount of compound required.

One exemplary of the disclosure is that compounds effective against virtually any pathogenic bacterial can be readily designed and tested, e.g., for rapid response against new drug-resistant strains.

The following examples are intended to illustrate but not to limit the disclosure. Each of the patent and non-patent references referred to herein is incorporated by reference in its entirety.

EXAMPLES

Example 1

Development of PPMOs against colistin resistance genes *mcr-1* and *mcr-2*

Liu *et al.* (Liu, Y-Y et al. (2016) Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study, Lancet Infect Dis 16: 161-168) reported the first example of a transferrable polymyxin resistance mechanism, mcr-1, in gram-negative pathogens. Since that report mcr-1 has been found in many major gram-negative pathogens, including those already harboring high-level resistance mechanisms. The mechanism of mcr-1 was also rapidly described; an ethanolamine is attached to lipid A phosphate groups, rendering the membrane more electropositive, repelling positively-charge polymyxins. Acquisition of mcr-1 is clinically frightening because polymyxins are last-line antibiotics used to treat extensively resistant organisms, so acquisition would lead to pan-resistance. Therefore, the ability to inhibit mcr-1 and restore polymyxin sensitivity would be clinically significant. Peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) are antisense molecules that target mRNA and prevent translation of the protein and can be designed to basically any gene target. As a rapid response proof of principle, PPMOs targeting polymyxin resistance mcr genes were designed (**FIG. 2**) and tested for effectiveness against mcr-positive bacterial strains.

Example 2

Minimum inhibitory concentration (MIC) of polymyxins against mcr-1 strains

The minimum inhibitory concentrations (MIC) of polymyxins against four clinical *E. coli mcr-1* strains (AF23, AF24, AF31, and AF45) and a standard laboratory *E. coli* strain (25922) were determined (**FIG. 3**). *mcr-1* was also expressed on a pBAD vector in the standard TOP10 cloning *E. coli*. The MICs from the clinical strains and TOP10 expressing mcr-1 were comparable to MICs from the originating publication (Poirel L. et al. (2016) Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*, The Lancet Infectious Diseases, 2016, vol. 16, no. 3, p. 281).

Example 3

***mcr-1* PPMOs resensitize *E. coli* to colistin**

mcr-positive *E. coli* were treated with PPMO #s 3 and 4 at 16 µM during standard MIC testing with colistimethate (**FIG. 4**). MICs were expressed as the fold enhancement of MIC compared to vehicle (H_2O) or control PPMO (ctrl). Lead PPMO #s 3 and 4 sensitize three of four mcr-1-positive *E. coli* to polymyxin E (colistin) 2- to 8-fold. This result is surprising and unexpected at least because mcr-1 polymyxin resistance mechanism alters the bacterial membrane charge to be more positive. Because the PPMOs are positively charged (positive charges associated with the CPP), one would not expect efficient or any uptake of the PPMOs across the positively charged bacterial membrane due to the +/+ charge interaction. As seen in FIG. 4, the results are contrary to this expectation. Namely, the PPMOs do in fact cross the positively charged membrane and restore colistin resistance.

Example 4

Minimum bactericidal concentration (MBC) is enhanced by *mcr-1* PPMOs

mcr-positive *E. coli* were treated with PPMO #s 3 and 4 at 16 µM during standard MBC testing with colistimethate. AF23 (**Figure 5A**), AF24 (**Figure 5B**), and AF31 (**Figure 5C**) demonstrated a decreased MBC for colistimethate when treated with PPMO #3 compared to vehicle or control PPMO. AF24 also demonstrated a decreased MBC with PPMO #4. This result is again surprising and unexpected at least due to the +/+ PPMO/membrane charge interaction.

Example 5

PPMOs targeting essential genes are efficacious in mcr-1-positive *E. coli*

Standard MIC testing of mcr-positive *E. coli* with PPMOs targeted to the essential genes *acpP* or *murA* demonstrate that PPMOs are efficacious compared to control PPMOs (**FIG. 6**, represented

as μM). Peptide conjugation was assessed both by site of attachment on PPMO (5' vs. 3') as well as peptide sequence. The lead antibacterial PPMO #s 7 (acpP-0276), 8 (acpP-0310), 10 (acpP-0399) and 11 (acpP-0621) targeting an essential gene, *acpP*, are as effective in mcr-1-positive *E. coli* as in a wild-type laboratory strain with minimum inhibitory concentrations from 1 to 8 μM (**FIG. 6**). Control PPMOs were: PPMO #s 14 (ctrl-0078), 13 (ctrl-0949), 16 (ctrl-0412) and 15 (ctrl-0431). This result is again surprising and unexpected at least due to the +/+ PPMO/membrane charge interaction. Taken together these data suggest PPMOs as a viable strategy for restoration of antibiotic sensitivity as well as directly having antibacterial activity in mcr-1-positive *E. coli*.

Example 6

***mcr* PPMOs are effective against many gram-negative genera**

mcr PPMOs are tested in many genera of gram-negative mcr-positive bacteria to demonstrate efficacy in multiple pathogens.

Example 7

Treatment of *mcr*-positive bacteria with *mcr* PPMOs reduces the transfer of ethanolamine to lipid A

mcr-positive bacteria are treated with *mcr* PPMOs, and the transfer of ethanolamine to lipid A is assessed by mass spectrometry and compared to controls.

Example 8

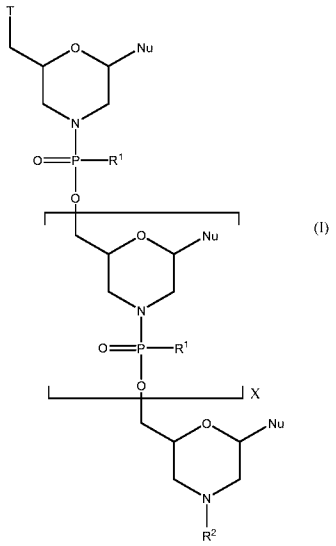
mcr* PPMOs are efficacious for treating bacterial infection *in vivo

Mice are infected with *mcr-1* strains and subsequently treated with polymyxin E with control and *mcr* PPMOs or without PPMOs. A significant reduction in bacterial CFU and an increase in survival of the mice are seen.

CLAIMS

1. An antisense morpholino oligomer, composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit, and having (a) about 10-40 nucleotide bases, and (b) a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor; where the oligomer is conjugated to a cell-penetrating peptide (CPP).

2. The antisense morpholino oligomer of claim 1, wherein the antisense morpholino oligomer is of formula (I):



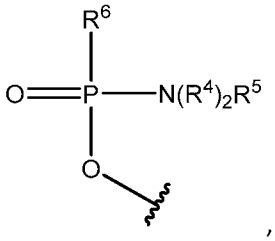
(I)

or a pharmaceutically acceptable salt thereof,

where each Nu is a nucleobase which taken together forms a targeting sequence;

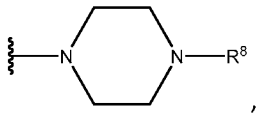
X is an integer from 9 to 38;

T is selected from OH and a moiety of the formula:



,

where each R^4 is independently C_1-C_6 alkyl, and R^5 is selected from an electron pair and H, and R^6 is selected from OH, $-N(R^7)CH_2C(O)NH_2$, and a moiety of the formula:



where:

R^7 is selected from H and C_1-C_6 alkyl, and

R^8 is selected from G, -C(O)-R^9OH, acyl, trityl, and 4-methoxytrityl, where:

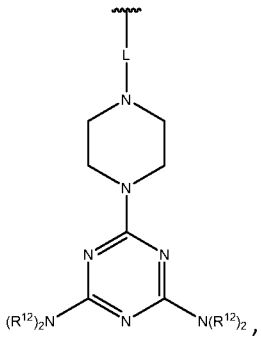
R^9 is of the formula -(O-alkyl)$_y$- wherein y is an integer from 3 to 10 and each of

the y alkyl groups is independently selected from C_2-C_6 alkyl;

each instance of R^1 is –N(R^{10})$_2$$R^{11}$ wherein each R^{10} is independently C_1-C_6 alkyl, and R^{11} is

selected from an electron pair and H; and

R^2 is selected from H, G, acyl, trityl, 4-methoxytrityl, benzoyl, stearoyl, and a moiety of the formula:

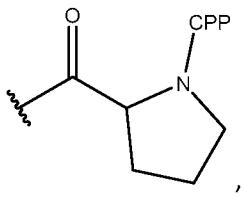


where L is selected from –C(O)(CH_2)$_6$C(O)– and -C(O)(CH_2)$_2$$S_2$($CH_2$)$_2$C(O)–, and each R^{12} is of the formula –(CH_2)$_2$OC(O)N(R^{14})$_2$ wherein each R^{14} is of the formula -(CH_2)$_6$NHC(=NH)NH_2,

wherein G is a cell penetrating peptide (“CPP”) and linker moiety selected from

-C(O)(CH_2)$_5$NH-CPP, -C(O)(CH_2)$_2$NH-CPP, -C(O)(CH_2)$_2$NHC(O)(CH_2)$_5$NH-CPP,

and -C(O)CH_2NH-CPP, or G is of the formula:



wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus, with the proviso that only one instance of G is present,

wherein the targeting sequence specifically hybridizes to a bacterial mRNA target sequence that encodes a virulence factor.

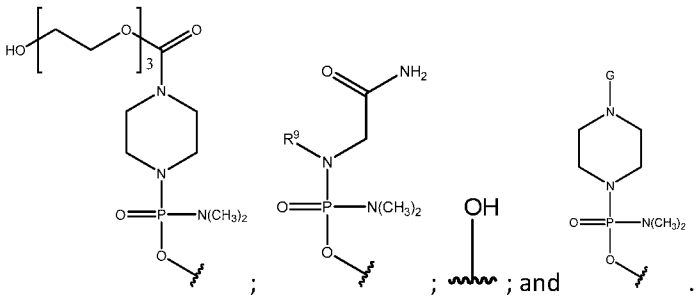
3. The antisense morpholino oligomer of claim 2, wherein the target sequence comprises a translational start codon of the bacterial mRNA and/or a sequence within about 30 bases upstream or downstream of the translational start codon of the bacterial mRNA.

4. The antisense morpholino oligomer of claim 2 or 3, wherein the virulence factor is an antibiotic resistance protein.

5. The antisense morpholino oligomer of claim 4, wherein the antibiotic resistance protein is selected from one or more of beta-lactamase TEM-1, beta-lactamase CTX-M-1-ATG, and polymyxin resistance protein (MCR).

6. The antisense morpholino oligomer of claim 5, wherein the targeting sequence is set forth in SEQ ID NOs: 4-9, comprises a fragment of at least 10 contiguous nucleotides of SEQ ID NOs: 4-9, or comprises a variant having at least 80% sequence identity to SEQ ID NOs: 4-9, wherein thymine bases (T) are optionally uracil bases (U).

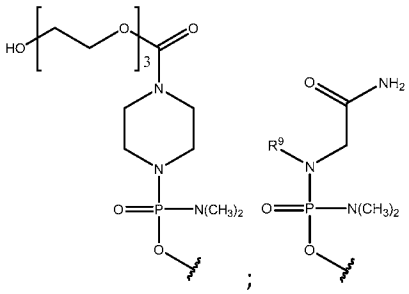
7. The antisense morpholino oligomer of claim 1, wherein T is selected from:

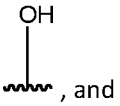


; ; ; and .

8. The antisense morpholino oligomer of claim 1 or 7, wherein R^2 is selected from H, G, acyl, trityl, 4-methoxytrityl, benzoyl, and stearoyl.

9. The antisense morpholino oligomer of claim 1, wherein T is selected from:

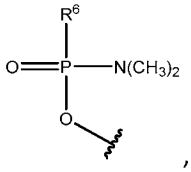




; ; and , and

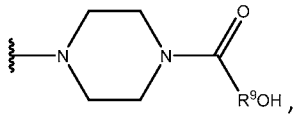
R^2 is G.

10. The antisense morpholino oligomer of claim 1, wherein T is of the formula:



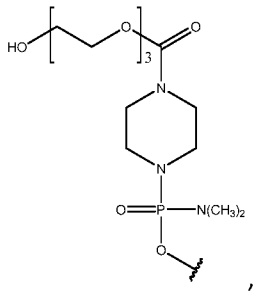
,

R^6 is of the formula:



and R^2 is G.

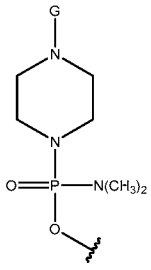
11. The antisense morpholino oligomer of claim 1, wherein T is of the formula:



,

and R^2 is G.

12. The antisense morpholino oligomer of claim 1, wherein T is of the formula:



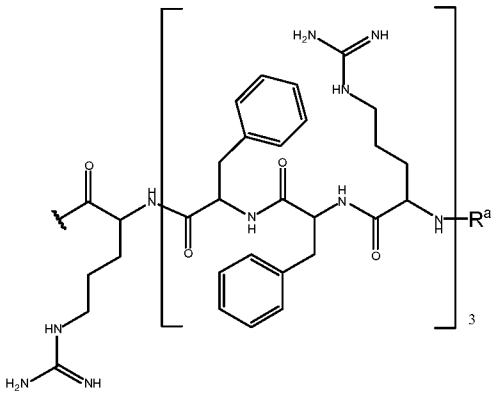
.

13. The antisense morpholino oligomer according to claim 1 or 12, wherein R^2 is selected from H, acyl, trityl, 4-methoxytrityl, benzoyl, and stearoyl.

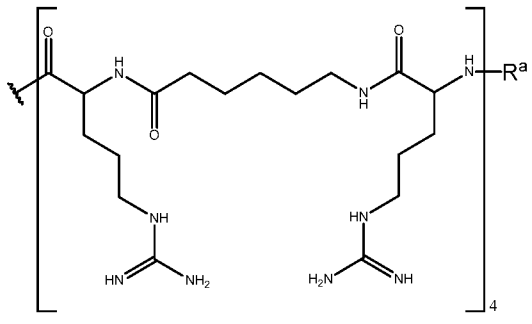
14. The antisense morpholino oligomer according to any one of claims 1 or 7-13, wherein at least one instance of R^1 is $-N(CH_3)_2$.

15. The antisense morpholino oligomer of claim 14, wherein each R^1 is $-N(CH_3)_2$.

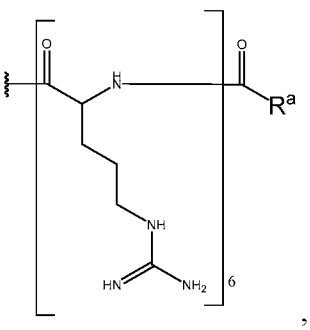
16. The antisense morpholino oligomer according to any one of claims 1 or 7-15, wherein the CPP is selected from:



;



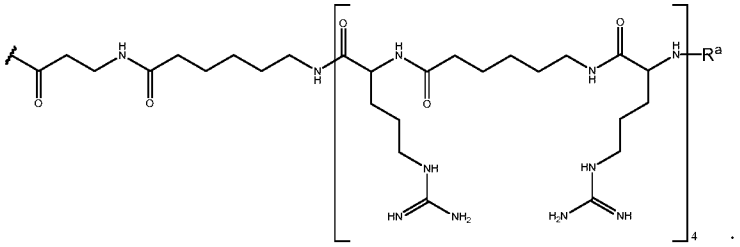
; and



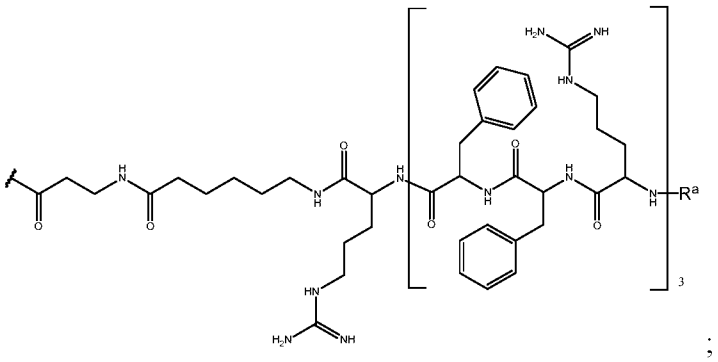
,

wherein R^a is selected from H, acetyl, benzoyl, and stearoyl.

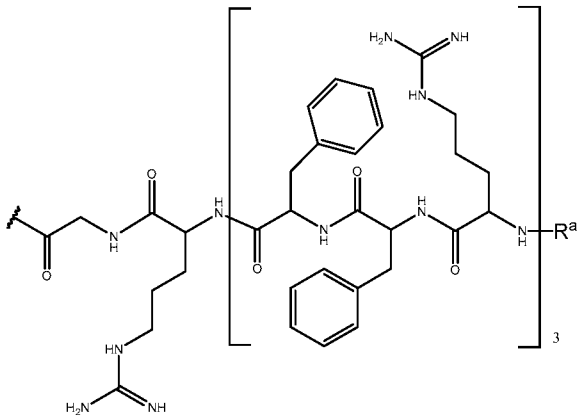
17. The antisense morpholino oligomer according to any one of claims 1 or 7-15, wherein G is selected from:



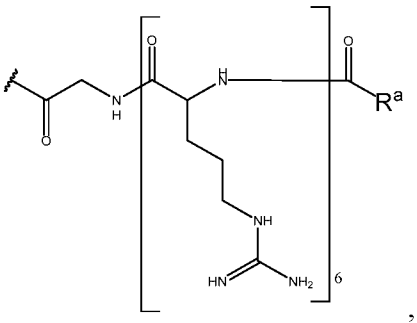
;



;



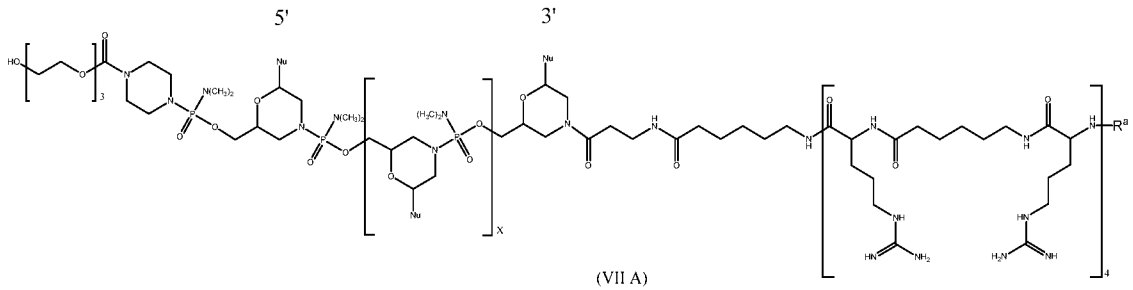
; and



,

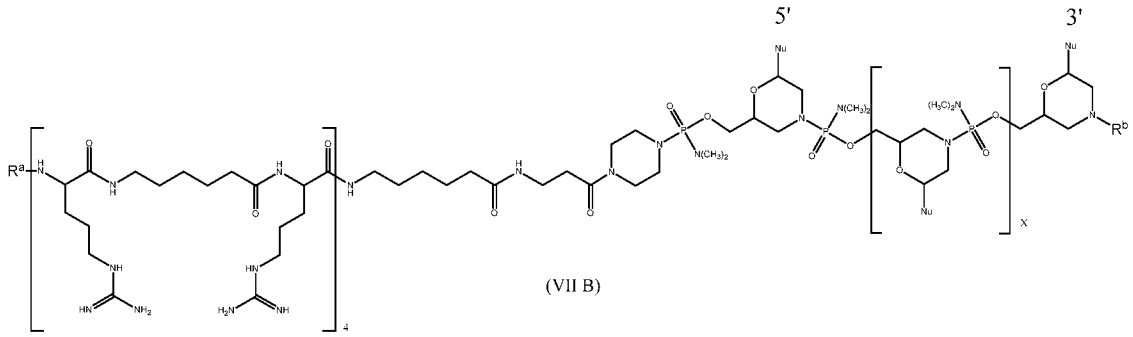
wherein R^a is selected from H, acetyl, benzoyl, and stearoyl.

18. The antisense morpholino oligomer of claim 1, wherein the antisense oligomer is of the formula (VII) selected from:



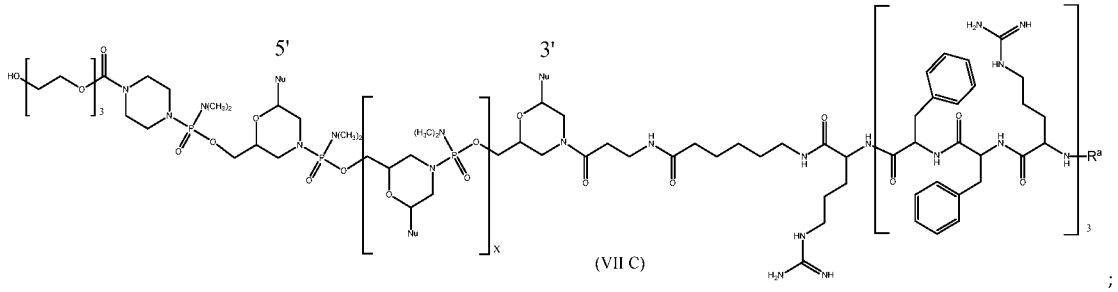
(VII A)

;



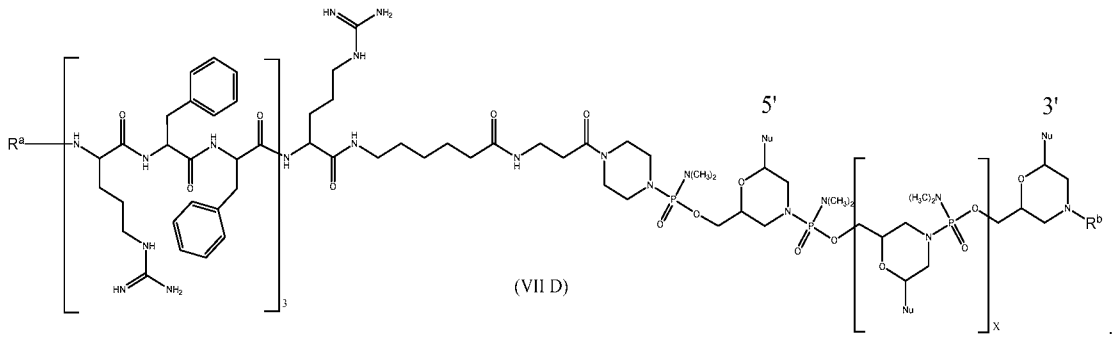
(VII B)

;



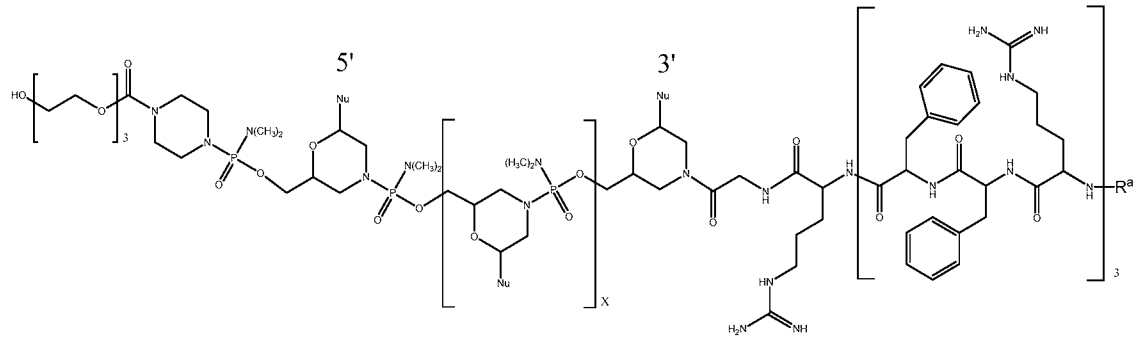
(VII C)

;



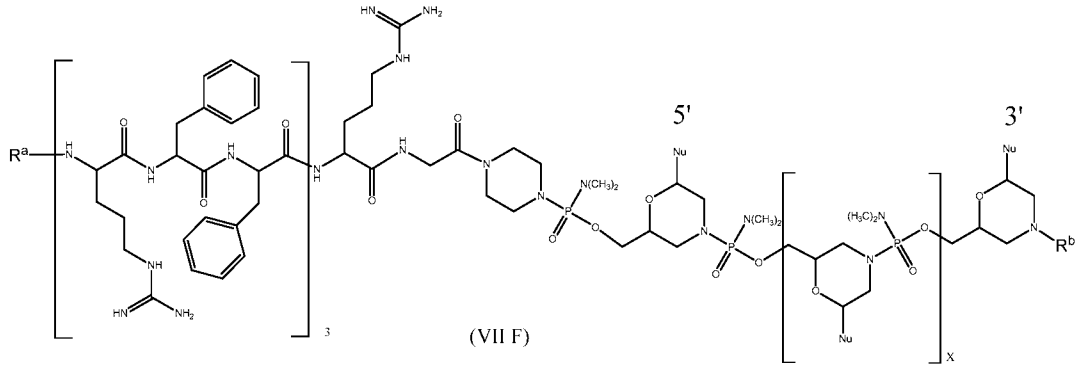
(VII D)

;



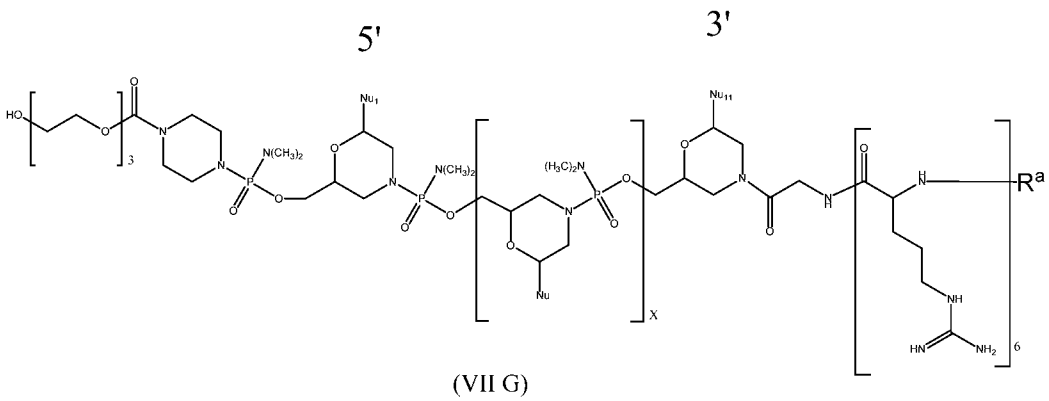
(VII E)

;



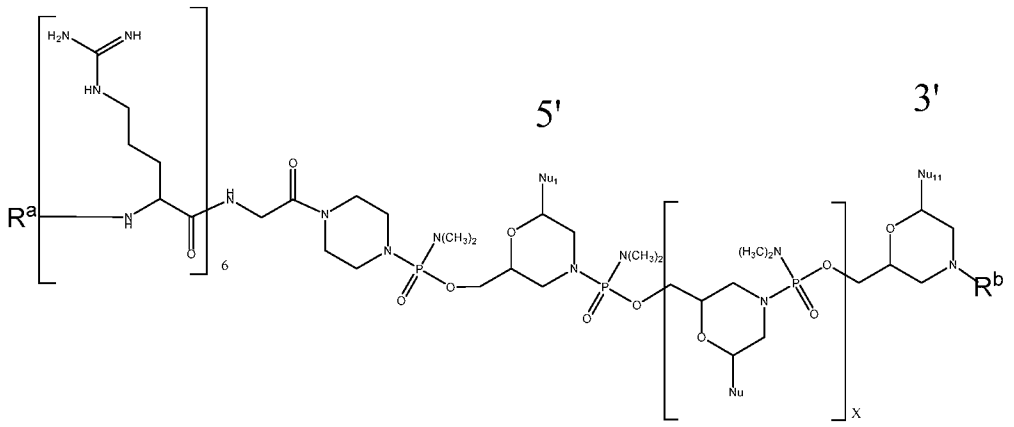
(VII F)

;



(VII G)

; and



(VII H)

,or a pharmaceutically acceptable salt of any of the foregoing,

wherein R^a is selected from H, acetyl, benzoyl, and stearoyl, R^b is selected from H, acetyl, benzoyl, stearoyl, trityl, and 4-methoxytrityl, and X and Nu are as defined in claim 1.

19. The antisense morpholino oligomer of claim 18, wherein R^a is acetyl and R^b is H.

20. The antisense morpholino oligomer according to any one of claims 7-19, wherein the targeting sequence is selected from:

a) SEQ ID NO: 4 (GAT GTC ATA GA);

b) SEQ ID NO: 5 (CAT AGA AAT TA);

c) SEQ ID NO: 6 (CAT GAG AAA CT);

d) SEQ ID NO: 7 (TGC TGC ATC AT);

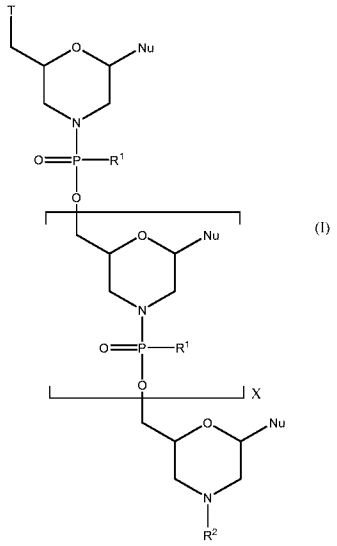
e) SEQ ID NO: 8 (TCA TAC TCT TC); and

f) SEQ ID NO: 9 (TTT TTA ACC AT),

wherein X is 9, and thymine bases (T) may be uracil bases(U).

21. A pharmaceutical composition, comprising a pharmaceutically acceptable carrier and an antisense morpholino oligomer, wherein the antisense morpholino oligomer is composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit, and having (a) about 10-40 nucleotide bases, and (b) a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor; where the oligomer is conjugated to a cell-penetrating peptide (CPP).

22. The pharmaceutical composition of claim 21, wherein the antisense morpholino oligomer is of formula (I):



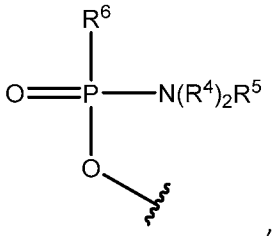
(I)

or a pharmaceutically acceptable salt thereof,

where each Nu is a nucleobase which taken together forms a targeting sequence;

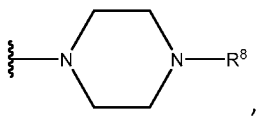
X is an integer from 9 to 38;

T is selected from OH and a moiety of the formula:



,

where each R^4 is independently C_1-C_6 alkyl, and R^5 is selected from an electron pair and H, and R^6 is selected from OH, $–N(R^7)CH_2C(O)NH_2$, and a moiety of the formula:



,

where:

R^7 is selected from H and C_1-C_6 alkyl, and

R^8 is selected from G, -C(O)-R^9OH, acyl, trityl, and 4-methoxytrityl, where:

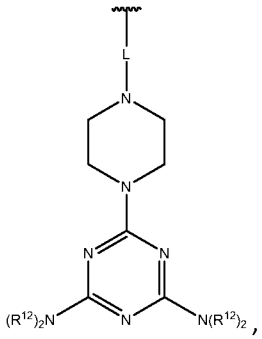
R^9 is of the formula -(O-alkyl)$_y$- wherein y is an integer from 3 to 10 and each of

the y alkyl groups is independently selected from C_2-C_6 alkyl;

each instance of R^1 is $-N(R^{10})_2R^{11}$ wherein each R^{10} is independently C_1-C_6 alkyl, and R^{11} is

selected from an electron pair and H; and

R^2 is selected from H, G, acyl, trityl, 4-methoxytrityl, benzoyl, stearoyl, and a moiety of the formula:

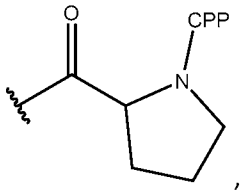


where L is selected from $-C(O)(CH_2)_6C(O)-$ and $-C(O)(CH_2)_2S_2(CH_2)_2C(O)-$, and each R^{12} is of the formula $-(CH_2)_2OC(O)N(R^{14})_2$ wherein each R^{14} is of the formula $-(CH_2)_6NHC(=NH)NH_2$,

wherein G is a cell penetrating peptide ("CPP") and linker moiety selected from

$-C(O)(CH_2)_5NH$-CPP, $-C(O)(CH_2)_2NH$-CPP, $-C(O)(CH_2)_2NHC(O)(CH_2)_5NH$-CPP,

and $-C(O)CH_2NH$-CPP, or G is of the formula:



wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus, with the proviso that only one instance of G is present,

wherein the targeting sequence specifically hybridizes to a bacterial mRNA target sequence that encodes a virulence factor.

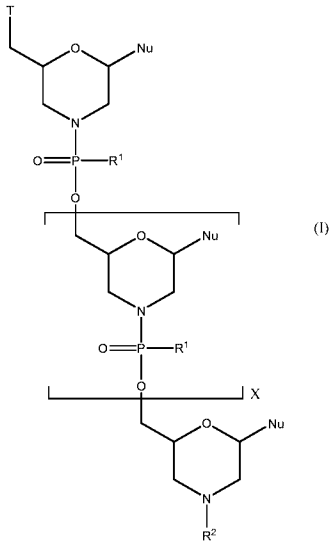
23. The pharmaceutical composition of claim 22, wherein the target sequence comprises a translational start codon of the bacterial mRNA and/or a sequence within about 30 bases upstream or downstream of the translational start codon of the bacterial mRNA.

24. The pharmaceutical composition of claim 22, wherein the targeting sequence is set forth in SEQ ID NOs: 4-9, comprises a fragment of at least 10 contiguous nucleotides of SEQ ID NOs:

4-9, or comprises a variant having at least 80% sequence identity to SEQ ID NOs: 4-9, wherein thymine bases (T) are optionally uracil bases (U).

25. A method of reducing expression and activity of a virulence factor in an mcr-1 positive bacterium or a method of treating an mcr-1 positive bacterial infection, comprising contacting the bacterium with an antisense morpholino oligomer, wherein the antisense morpholino oligomer is composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit, and having (a) about 10-40 nucleotide bases, and (b) a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor; where the oligomer is conjugated to a cell-penetrating peptide (CPP).

26. The method of claim 25, wherein the antisense morpholino oligomer is of formula (I):



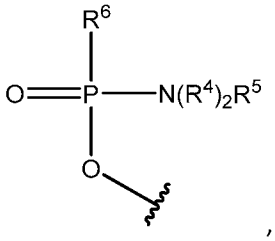
(I)

or a pharmaceutically acceptable salt thereof,

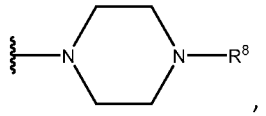
where each Nu is a nucleobase which taken together forms a targeting sequence;

X is an integer from 9 to 38;

T is selected from OH and a moiety of the formula:



where each R^4 is independently C_1-C_6 alkyl, and R^5 is selected from an electron pair and H, and R^6 is selected from OH, $–N(R^7)CH_2C(O)NH_2$, and a moiety of the formula:



where:

R^7 is selected from H and C_1-C_6 alkyl, and

R^8 is selected from G, -C(O)-R^9OH, acyl, trityl, and 4-methoxytrityl, where:

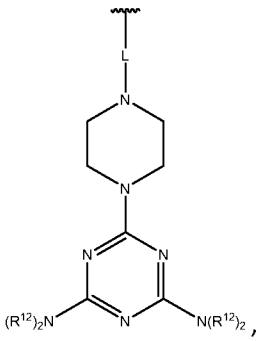
R^9 is of the formula -(O-alkyl)$_y$- wherein y is an integer from 3 to 10 and each of

the y alkyl groups is independently selected from C_2-C_6 alkyl;

each instance of R^1 is $–N(R^{10})_2R^{11}$ wherein each R^{10} is independently C_1-C_6 alkyl, and R^{11} is

selected from an electron pair and H; and

R^2 is selected from H, G, acyl, trityl, 4-methoxytrityl, benzoyl, stearoyl, and a moiety of the formula:

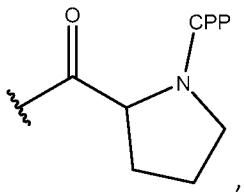


where L is selected from $–C(O)(CH_2)_6C(O)–$ and $-C(O)(CH_2)_2S_2(CH_2)_2C(O)–$, and each R^{12} is of the formula $–(CH_2)_2OC(O)N(R^{14})_2$ wherein each R^{14} is of the formula $-(CH_2)_6NHC(=NH)NH_2$,

wherein G is a cell penetrating peptide (“CPP”) and linker moiety selected from

$-C(O)(CH_2)_5$NH-CPP, $-C(O)(CH_2)_2$NH-CPP, $-C(O)(CH_2)_2NHC(O)(CH_2)_5$NH-CPP,

and $-C(O)CH_2$NH-CPP, or G is of the formula:



,

wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus, with the proviso that only one instance of G is present,

wherein the targeting sequence specifically hybridizes to a bacterial mRNA target sequence that encodes the virulence factor.

27. The method of claim 25, where the mcr-1 positive bacterium is in a subject, and the method comprises administering the antisense morpholino oligomer to the subject.

28. The method of any one of claims 25-27, wherein the mcr-1 positive bacterium is a gram-negative bacterium.

29. The method of any one of claims 25-27, wherein the mcr-1 positive bacterium is selected from the genus *Escherichia*, *Acinetobacter*, *Pseudomonas*, *Klebsiella*, and *Burkholderia*.

30. The method of claim 29, wherein the mcr-1 positive bacterium is *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa, Klebsiella pneumoniae*, or *Burkholderia cepacia* (complex).

31. The method of any one of claims 25-27, wherein the virulence factor is an antibiotic resistance protein selected from one or more of beta-lactamase TEM-1, beta-lactamase CTX-M-1-ATG, and polymyxin resistance protein MCR.

32. The method of any one of claims 25-27, wherein the mcr-1 positive bacterium is resistant to one or more polymyxin antibiotic.

33. The method of claim 32, wherein the virulence factor is an essential protein selected from one or more of acyl carrier protein and UDP-N-acetylglucosamine 1-carboxyvinyltransferase murA.

34. The method of any one of the preceding claims, comprising administering the oligomer separately or concurrently with one or more antimicrobial agent, optionally wherein administration of the oligomer increases susceptibility of the bacterium to the one or more antimicrobial agent.

35. The method of claim 34, wherein the one or more antimicrobial agent is selected from one or more of a β-lactam antibiotic and a polymyxin antibiotic.

36. The method of claim 34, where the combination of oligomer and the one or more antimicrobial agent increases the susceptibility of the bacterium to the one or more antimicrobial agent relative to the oligomer and/or the one or more antimicrobial agent alone.

37. The method of claim 25, wherein the targeting sequence is selected from:

a) SEQ ID NO: 4 (GAT GTC ATA GA);

b) SEQ ID NO: 5 (CAT AGA AAT TA);

c) SEQ ID NO: 6 (CAT GAG AAA CT);

d) SEQ ID NO: 7 (TGC TGC ATC AT);

e) SEQ ID NO: 8 (TCA TAC TCT TC); and

f) SEQ ID NO: 9 (TTT TTA ACC AT),

wherein X is 9, and thymine bases (T) may be uracil bases(U).

38. The method of claim 25, wherein the targeting sequence is selected from:

d) SEQ ID NO: 10 (TGC TCA TAC TC);

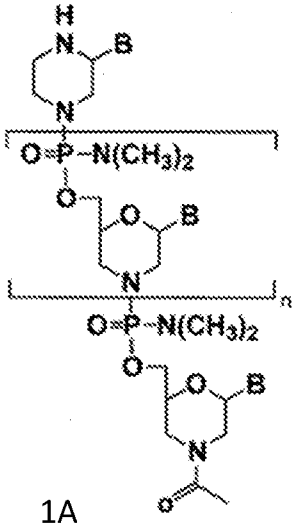
e) SEQ ID NO: 11 (CTT CGA TAG TG); and

f) SEQ ID NO: 12 (ATC CAT TTA GT),

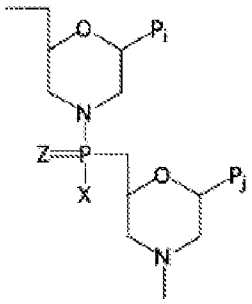
wherein X is 9, and thymine bases (T) may be uracil bases(U).

39. A method of sensitizing an mcr-1 positive bacterium to one or more polymyxin antibiotic, comprising contacting the bacterium with an antisense morpholino oligomer, wherein the antisense morpholino oligomer is composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit, and having (a) about 10-40 nucleotide bases, and (b) a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor; where the oligomer is conjugated to a cell-penetrating peptide

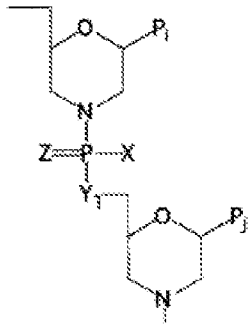
(CPP), and wherein the antisense morpholino oligomer targets one or more genes encoding a polymyxin resistance protein.



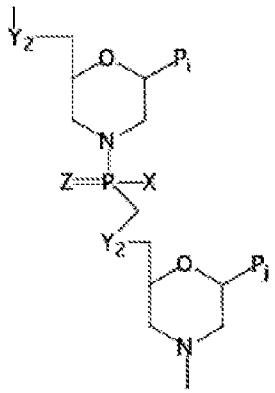
1A



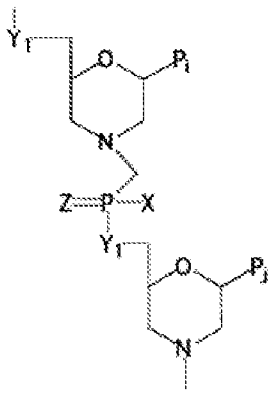
1B



1C



1D

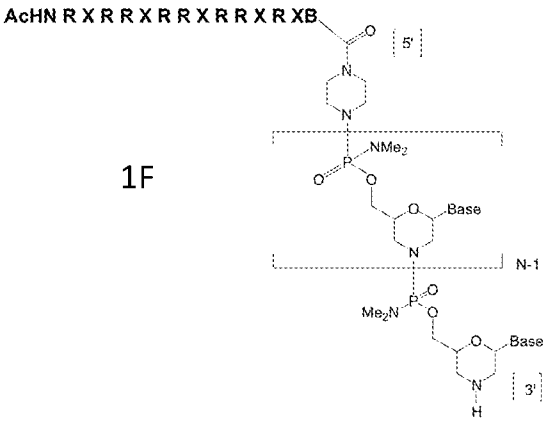


1E

FIGs. 1A-1H

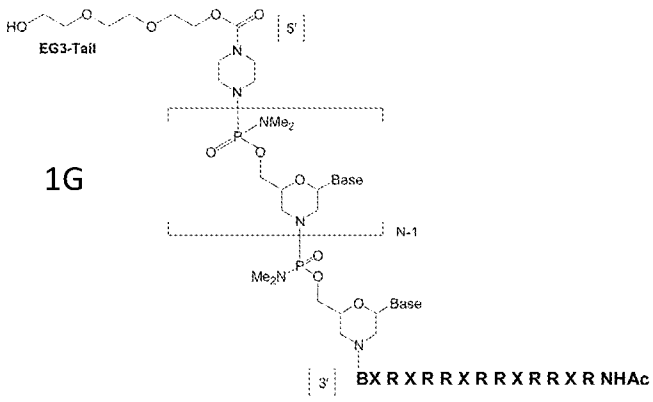
P007 5' PPMO

AcHN R X R R X R R X R R X R XB



1F

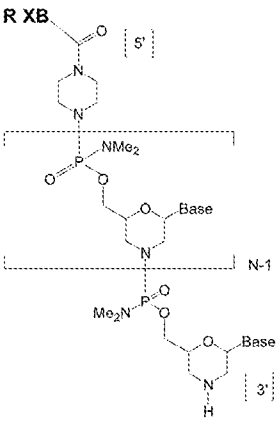
P007 3' PPMO



1G

CP05030 5' PPMO

AcHN R F F R F F R F F R XB



1H

FIGs. 1A-1H (CONT.)

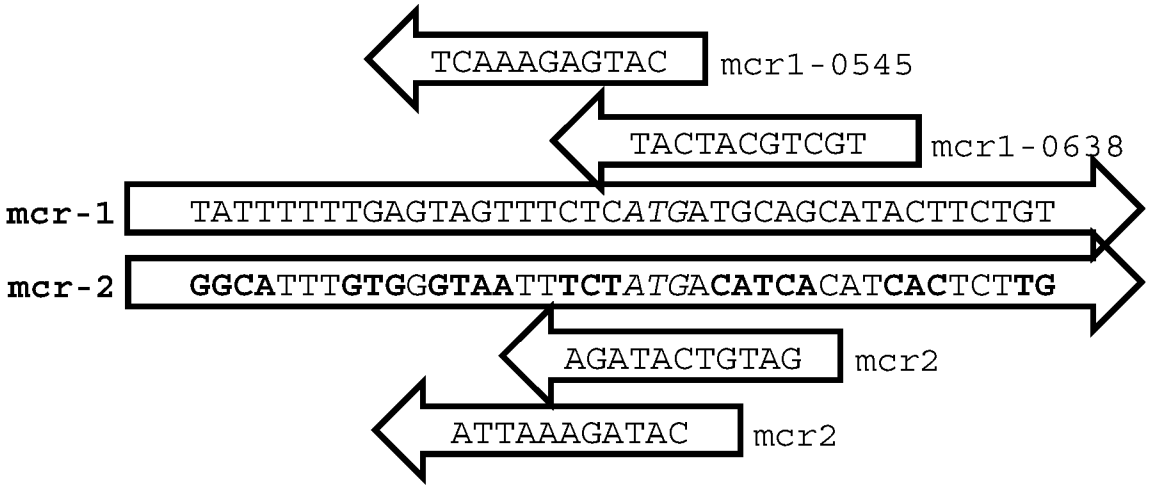

TCAAAGAGTAC
mcr1-0545
TACTACGTCGT
mcr1-0638
mcr-1
TATTTTTTGAGTAGTTTCTCATGATGCAGCATACTTCTGT
mcr-2
GGCATTTGTGGGTAATTTCTATGACATCACATCACTCTTG
AGATACTGTAG
mcr2
ATTAAAGATAC
mcr2

FIG. 2

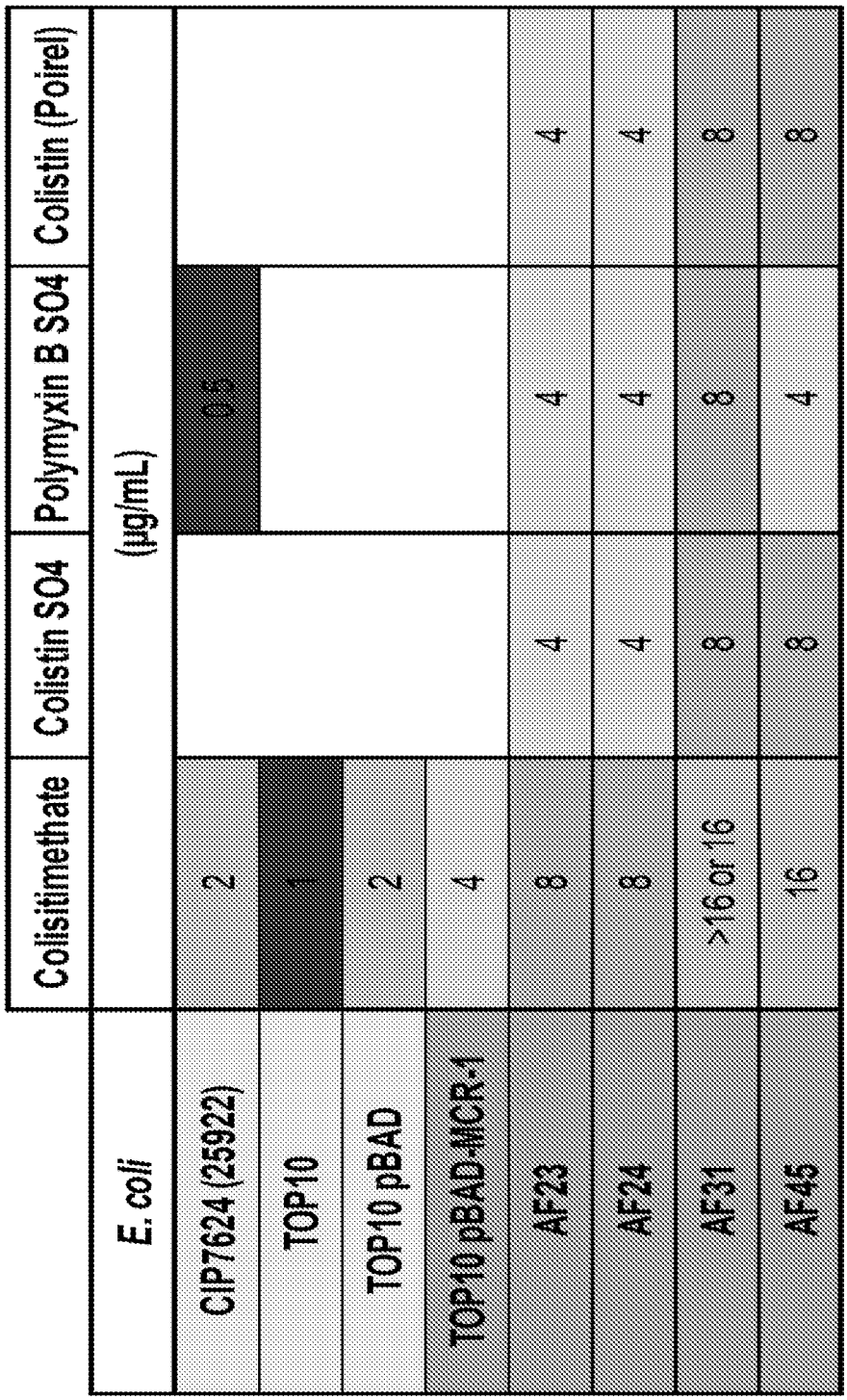
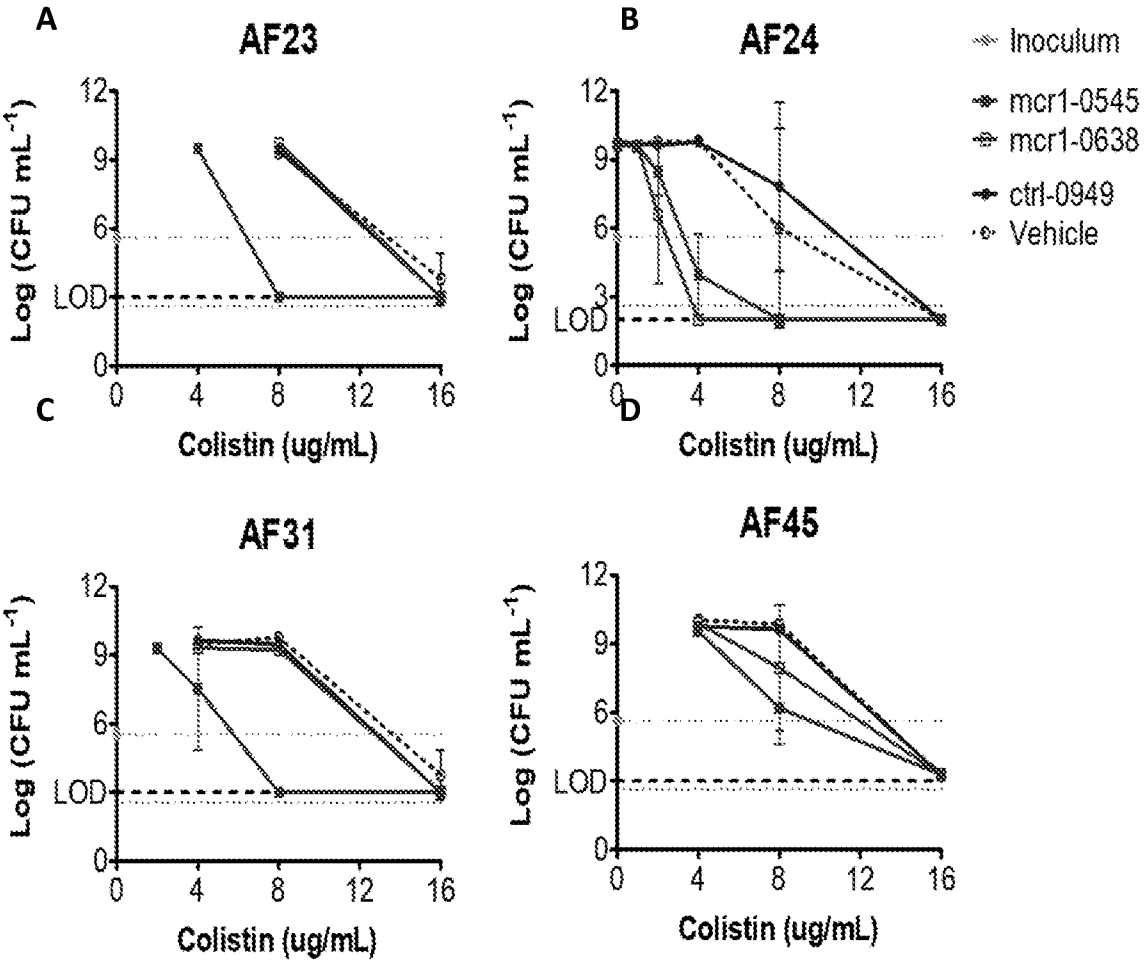

E. coli
Colisitimethate
Colistin SO4
Polymyxin B SO4
Colistin (Poirel)
(µg/mL)
CIP7624 (25922)
2
TOP10
1
0.5
TOP10 pBAD
2
TOP10 pBAD-MCR-1
4
AF23
8
4
4
4
AF24
8
4
4
4
AF31
>16 or 16
8
8
8
AF45
16
8
4
8

FIG. 3

	MIC$_{H2O}$ / MIC$_{mcr1}$		MIC$_{crtI}$ / MIC$_{mcr1}$	
E. coli	mcr1-545	mcr1-0638	mcr1-545	mcr1-0638
AF23	2.00	2.00	2.00	2.00
AF24	4.00	8.00	4.00	8.00
AF31	4.00	1.33	4.00	1.33
AF45	2.00	2.00	1.00	1.00

FIG. 4


A
AF23
B
AF24
C
AF31
D
AF45
Log (CFU mL-1)
Colistin (ug/mL)
LOD
Inoculum
mcr1-0545
mcr1-0638
ctrl-0949
Vehicle

FIGs. 5A-5D

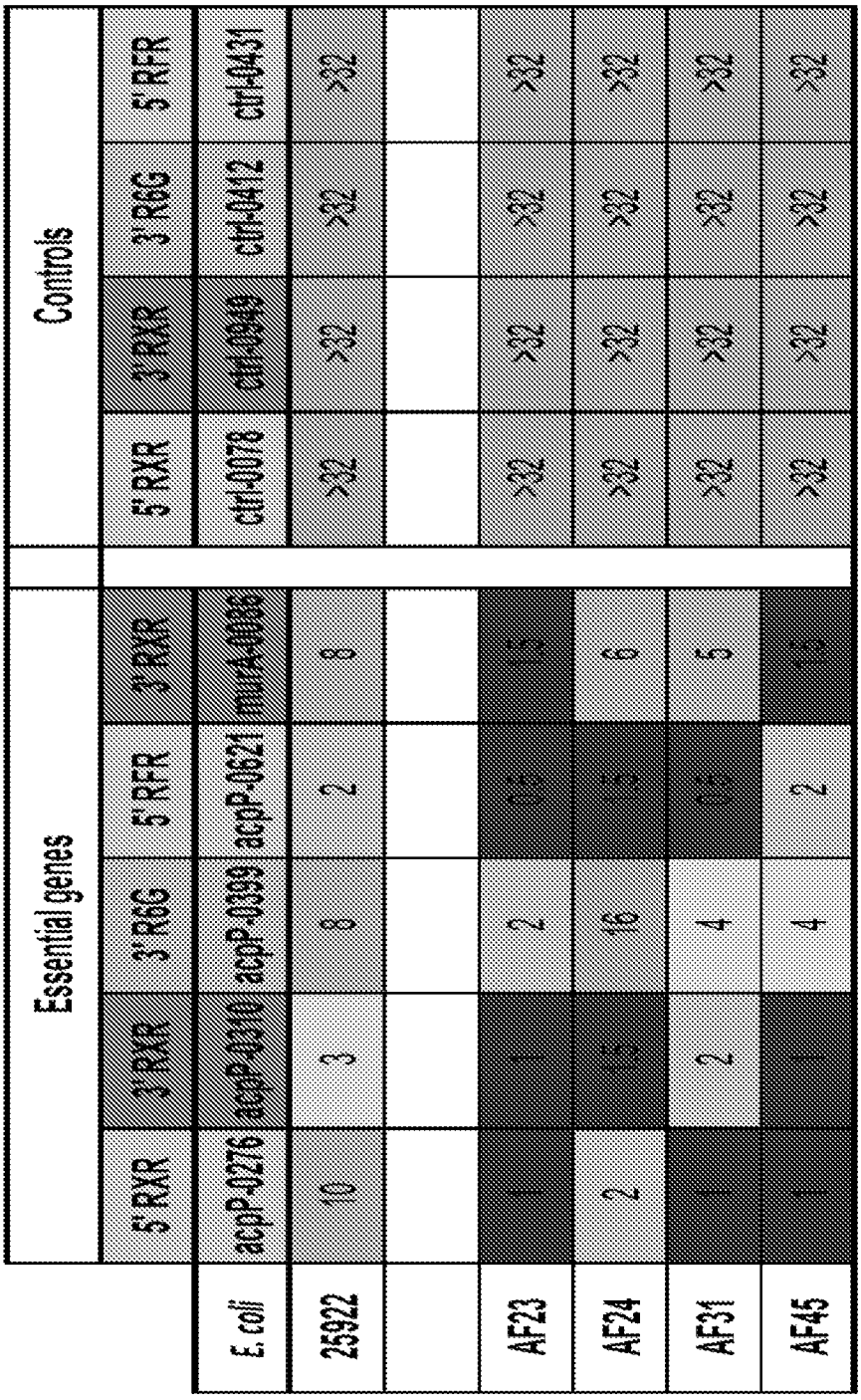

Essential genes
Controls
5' RXR
3' RXR
3' R6G
5' RFR
3' RXR
5' RXR
3' RXR
3' R6G
5' RFR
E. coli
acpP-0276
acpP-0310
acpP-0399
acpP-0621
murA-0036
ctrl-0078
ctrl-0949
ctrl-0412
ctrl-0431
25922
10
3
8
2
8
>32
>32
>32
AF23
1
1
2
1
1
>32
>32
>32
AF24
2
1
16
1
6
>32
>32
>32
AF31
1
2
4
1
5
>32
>32
>32
AF45
1
1
4
2
1
>32
>32
>32

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/20757

A. CLASSIFICATION OF SUBJECT MATTER
IPC - A61K 38/04, 47/64; C12N 15/11, 15/113 (2018.01)
CPC - A61K 38/04, 47/64, 47/6455; C12N 15/11, 15/113

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)
See Search History document

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

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

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 2015/0361425 A1 (GELLER, B et al.) 17 December 2015; paragraphs [0012], [0077], [0113], [0116], [0121]-[0137], [0205]	1-3, 4/2-3, 7-13, 18-19, 21-23 -------- 5/4/2-3, 25-27, 28/25-27, 29/25-27, 30/29/25-27, 31/25-27, 32/25-27, 33/32/25-27, 39
Y	(LIU, Y et al.) Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. The Lancet. Infection Diseases. February 2016, Epub 19 November 2015, No. 16, Vol. 2; pages 161-168; page 162, 1st column, 1st-2nd paragraph, page 164, 2nd column, 2nd paragraph; DOI: 10.1016/S1473-3099(15)00424-7	5/4/2-3, 25-27, 28/25-27, 29/25-27, 30/29/25-27, 31/25-27, 32/25-27, 33/32/25-27, 39
A	US 2011/0171633 A1 (COWENS, W et al.) 14 July 2011; Table A	6/5/4/2-3, 24, 37
A	(SULLY, EK et al.) Peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO) restores carbapenem susceptibility to NDM-1-positive pathogens in vitro and in vivo. The Journal of Antimicrobial Chemotherapy. 1 March 2017, No. 72, Vol. 3; pages 782-790; page 783, 1st column, 3rd paragraph; DOI: 10.1093/jac/dkw476	6/5/4/2-3, 24, 37

☐ Further documents are listed in the continuation of Box C. ☐ 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
13 June 2018 (13.06.2018)	03 JUL 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/20757

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 14-17, 20, and 34-36
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-***-Please See Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos..

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Groups I+, Claims 1-13, 18, 19, 21-33, 37-39; SEQ ID NO: 5 (antisense sequence)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US18/20757

-***-Continued from Box No. III Observations where unity of invention is lacking-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-13, 18, 19, 21-33, 37-39, SEQ ID NO: 4 are directed toward an antisense morpholino oligomer, a composition comprising the oligomer, and methods related thereto.

The antisense morpholino oligomer, composition and methods will be searched to the extent they encompass SEQ ID NO: 4 (antisense sequence). Applicant is invited to elect additional antisense sequence(s), with specified SEQ ID NO: for each, or with specified substitution(s) at specified site(s) of a SEQ ID NO:, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), to be searched. Additional antisense sequence(s) will be searched upon the payment of additional fees. It is believed that claims 1-5, 6 (in-part), 7-13, 18, 19, 21-23, 24 (in-part), 25-33, 37 (in-part) and 39 encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 4 (antisense sequence). Applicants must specify the claims that encompass any additionally elected antisense sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be an antisense sequence encompassing SEQ ID NO: 5 (antisense sequence).

No technical features are shared between the antisense sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a pharmaceutical composition, comprising a pharmaceutically acceptable carrier and an antisense morpholino oligomer, composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit, and having (a) about 10-40 nucleotide bases, and (b) a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor; where the oligomer is conjugated to a cell-penetrating peptide (CPP); and a method of reducing expression and activity of a virulence factor in an mcr-1 positive bacterium or a method of treating an mcr-1 positive bacterial infection by sensitizing an mcr-1 positive bacterium to one or more polymyxin antibiotic, comprising contacting the bacterium with an antisense morpholino oligomer, wherein the antisense morpholino oligomer is composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit, and having (a) about 10-40 nucleotide bases, and (b) a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor; where the oligomer is conjugated to a cell-penetrating peptide (CPP) and wherein the antisense morpholino oligomer targets one or more genes encoding a polymyxin resistance protein; these shared technical features are previously disclosed by US 2015/0361425 A1 to Geller et al. (hereinafter 'Geller') in view of the article 'Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study' by Liu et al. (herein after 'Liu').

Geller discloses a pharmaceutical composition (a pharmaceutical composition; paragraph [0049]), comprising a pharmaceutically acceptable carrier (comprising a pharmaceutically acceptable carrier; paragraph [0049]) and an antisense morpholino oligomer (and an antisense morpholino oligomer paragraphs [0012], [0018], [0049]), composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit (composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit; paragraphs [0012], [0018]), and having (a) about 10-40 nucleotide bases (having (a) about 10-40 nucleotide bases; paragraphs [0012], [0018], [0021]), and (b) a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor (a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor; paragraph [0012]); where the oligomer is conjugated to a cell-penetrating peptide (CPP) (where the oligomer is conjugated to a cell-penetrating peptide (CPP); paragraph [0012]); and a method of reducing expression and activity of a virulence factor in a bacterium (a method of reducing expression and activity of a virulence factor in a bacterium; paragraph [0038]) or a method of treating a bacterial infection (a method of treating a bacterial infection; paragraph [0214]) by sensitizing a bacterium (by increasing the sensitivity or susceptibility of a bacteria to an antimicrobial agent (by sensitizing a bacterium); paragraph [0249]) to one or more antibiotic (to one or more antimicrobials (antibiotics); paragraph [0249]), comprising contacting the bacterium with an antisense morpholino oligomer (comprising contacting the bacterium with an antisense morpholino oligomer; paragraph [0038], [0216]), wherein the antisense morpholino oligomer is composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit (wherein the antisense morpholino oligomer is composed of morpholino subunits and phosphorus-containing intersubunit linkages joining a morpholino nitrogen of one subunit to a 5'-exocyclic carbon of an adjacent subunit; paragraphs [0012], [0018]), and having (a) about 10-40 nucleotide bases (having (a) about 10-40 nucleotide bases; paragraphs [0012], [0018], [0021]), and (b) a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor (a targeting sequence of sufficient length and complementarity to specifically hybridize to a bacterial mRNA target sequence that encodes a virulence factor; paragraph [0012]); where the oligomer is conjugated to a cell-penetrating peptide (CPP) (where the oligomer is conjugated to a cell-penetrating peptide (CPP); paragraph [0012]); and wherein the virulence factor contributes to antibiotic resistance (wherein the virulence factor contributes to antibiotic resistance; abstract; paragraph [0014]).

-***-Continued Within the Next Supplemental Box-***-

-***-Continued from Previous Supplemental Box-***-

Geller does not disclose an mcr-1 positive bacterium or an mcr-1 positive bacterial infection; sensitizing an mcr-1 positive bacterium to one or more polymyxin antibiotic; and wherein the antisense morpholino oligomer targets one or more genes encoding a polymyxin resistance protein.

Liu discloses an mcr-1 positive bacterium (an mcr-1 positive bacterium; Summary); and wherein the phosphoethanolamine transferase activity of mcr-1 negated the efficacy of a polymyxin antibiotic (wherein the phosphoethanolamine transferase activity of mcr-1 negated the efficacy of colistin (a polymyxin antibiotic); Summary).

It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Geller to have included the use of sequences complementary to mcr-1, as disclosed by Liu, as a means to inhibit the production of the enzyme and reduce the corresponding phosphoethanolamine transferase activity that confers resistance to polymyxin antibiotics, thus sensitizing the bacteria to the antibiotics, and enabling treatment or prevention of an infection by mcr-1 expressing bacteria using a polymyxin antibiotic in combination with an appropriately-sequenced antisense morpholino-oligomer conjugate, as disclosed by Geller.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Geller and Liu references, unity of invention is lacking.