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(54) Title: ANTIMICROBIAL PHENAZINE COMPOUNDS

(57) Abstract

Phenazine compounds having antimicrobial activity are described. The phenazine compounds can be phenazine N-oxides, including phenazine-5,10-dioxides, which may optionally be substituted at one or more positions, preferably the 7- and/or 8-positions of the phenazine nucleus. Also described are methods for treating microbial infections, and methods for inhibiting the growth of a microbial cell. The microbes treated or inhibited can be multidrug resistant.
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ANTIMICROBIAL PHENAZINE COMPOUNDS

Background of the Invention

The appearance of drug-resistant microorganisms has caused concern among healthcare practitioners around the world. Drug-resistant microbes, especially bacteria, but also yeasts and fungi, have in some cases become multidrug resistant (MDR), that is, resistant to more than one drug. Clinical infections with such multidrug resistant strains can be difficult or impossible to treat with conventional antibiotics.

Summary of the Invention

The appearance of multidrug resistant pathogens has prompted a search for new classes of antimicrobial drugs which are structurally and/or functionally different from existing drugs. Drugs having new mechanisms of action may be effective against resistant organisms, where conventional drugs are no longer effective.

The invention provides methods and compositions for treating infections, including infections caused by drug-resistant microorganisms. In one embodiment, the invention provides a method for treating an infection due to a resistant microorganism in a subject. The method includes the step of administering to a subject in need thereof an effective amount of a phenazine compound, such that the infection is treated.

In one aspect, the invention provides a method for treating an infection due to a multidrug-resistant microorganism in a subject. The method includes the step of administering to a subject in need thereof an effective amount of a phenazine compound, or a pharmaceutically-acceptable salt thereof, such that the infection is treated. In preferred embodiments, the phenazine compound is a phenazine N-oxide, more preferably a phenazine 5,10-dioxide. In a particularly preferred embodiment, the phenazine N-oxide is a 7,8-disubstituted phenazine-5,10-dioxide. In certain embodiments, the multidrug resistant microorganism is a bacterium or other microbe, including a bacterium or other microbe selected from the group consisting of Hemophilus spp., E. coli, Enterobacter spp., Citrobacter spp., Proteus spp., Morganella spp., Shigella spp., Yersinia spp., Salmonella spp., and other bacteria in the family Enterobacteriaceae, Neisseria spp., Burkholderia spp., Campylobacter spp., Helicobacter spp., Vibrio spp., Staphylococcus spp., Enterococcus spp., Streptococcus spp., Chlamydia spp., Mycoplasma spp., Corynebacterium spp., and Clostridium spp., including azole- and amphotericin B-resistant strains.

In another aspect, the invention provides a method for treating an infection due to a microorganism in a subject, the method comprising the step of administering to a subject in need thereof an effective amount of a phenazine compound, or a pharmaceutically-acceptable salt thereof, such that the infection is treated, wherein the

In still another aspect, the invention provides a method for treating an infection due to a microorganism in a subject. The method includes the step of administering to a subject in need thereof an effective amount of a phenazine compound, or a pharmaceutically-acceptable salt thereof, such that the infection is treated, wherein the phenazine compound is represented by the formula:

![Chemical Structure](image)

in which \(R_1\) and \(R_2\) are selected independently for each occurrence from the group consisting of hydrogen, halogen (including fluoro, chloro, bromo, or iodo), lower alkyl, aryl (more preferably substituted or unsubstituted phenyl), hydroxy, lower alkoxy (such as methoxy), mercapto, lower alkylthio, nitro, cyano, amino, lower alkylamino, dialkylamino, and trifluoromethyl; \(R_3\) and \(R_4\) are selected independently for each occurrence from the group consisting of halogen, lower alkyl, hydroxy, lower alkoxy, mercapto, lower alkylthio, amino, lower alkylamino, dialkylamino, and trifluoromethyl; \(m\) is 0, 1 or 2; and \(n\) is an integer from 0 to 4; and \(X\) is, independently for each occurrence, oxygen or a pair of electrons. In certain embodiments, \(m\) and \(n\) are each 0. In certain embodiments, at least one occurrence of \(X\) is oxygen; in particularly preferred embodiments, both occurrences of \(X\) are oxygen (i.e., the compound is a phenazine-5,10-dioxide). In preferred embodiments, \(n\) is at least 1, and one occurrence of \(R_4\) is a 1-hydroxy group (i.e., the compound is a 1-hydroxyphenazine (optionally a 1-hydroxyphenazine-N-oxide)). \(R_1\) and \(R_2\) can be the same or different; in certain embodiments, \(R_1\) and \(R_2\) are the same, and are both methyl or both chlorine. In some embodiments, the organism is a multidrug resistant organism, including a bacterium.

In another aspect, the invention provides a method for inhibiting the growth of a drug-resistant microorganism. The method includes contacting the drug-resistant microorganism with an effective amount of a phenazine compound, such that growth of the drug-resistant microorganism is inhibited. In preferred embodiments, the microorganism is a bacterium, and the phenazine compound has bacteriostatic activity.
against the bacterium. In another embodiment, the microorganism is a bacterium, and the phenazine compound has bactericidal activity against the bacterium. In certain embodiments, the phenazine compound is a phenazine N-oxide, which can be a phenazine 5,10-dioxide. In certain preferred embodiments, the phenazine compound is a 7,8-disubstituted phenazine compound, including, without limitation, a 7,8-dimethylphenazine or a 7,8-dihalophenazine (e.g., a 7,8-dichlorophenazine, such as 7,8-dichlorophenazine-5,10-dioxide). In certain preferred embodiments, the phenazine is a 1-hydroxyphenazine, more preferably a 1-hydroxyphenazine-5,10-dioxide.

In another embodiment, the invention provides a method for targeting a drug-resistant microorganism in a subject for the purpose of treating an infection. The method includes administering an effective amount of a phenazine compound to a subject in need thereof, such that the drug is targeted to a drug-resistant microorganism known or suspected to be present in the subject and the infection is treated. In a preferred embodiment, the phenazine compound also targets a non-drug resistant microorganism. In a preferred embodiment, the phenazine compound is a phenazine-N-oxide, including a phenazine 5-oxide, a phenazine-10-oxide, or a phenazine-5,10-dioxide. In certain preferred embodiments, the phenazine compound is a 7,8-disubstituted phenazine compound, including, without limitation, a 7,8-dimethylphenazine or a 7,8-dihalophenazine (e.g., a 7,8-dichlorophenazine, such as 7,8-dichlorophenazine-5,10-dioxide). In certain preferred embodiments, the phenazine is a 1-hydroxyphenazine, more preferably a 1-hydroxyphenazine-5,10-dioxide.

In another embodiment, the invention provides a method for significantly decreasing the infective ability of a drug-resistant microorganism. The method includes contacting the drug-resistant microorganism with an effective amount of a phenazine compound, such that the infective ability of the drug-resistant microorganism is significantly decreased. In a preferred embodiment, the phenazine compound is a phenazine-N-oxide, including a phenazine 5-oxide, a phenazine-10-oxide, or a phenazine-5,10-dioxide. In certain preferred embodiments, the phenazine compound is a 7,8-disubstituted phenazine compound, including, without limitation, a 7,8-dimethylphenazine or a 7,8-dihalophenazine (e.g., a 7,8-dichlorophenazine, such as 7,8-dichlorophenazine-5,10-dioxide). In certain preferred embodiments, the phenazine is a 1-hydroxyphenazine, more preferably a 1-hydroxyphenazine-5,10-dioxide. In certain preferred embodiments, the phenazine compound is able to better stabilize a positive charge compared to unsubstituted phenazine.

In another aspect, the invention provides a packaged drug, comprising a composition including a phenazine compound in a container, packaged with instructions for administering the composition to a subject for treating an infection due to a microorganism in the subject, which microorganism may be drug-resistant. In a
preferred embodiment, the phenazine compound is a phenazine-N-oxide, including a phenazine 5-oxide, a phenazine-10-oxide, or a phenazine-5,10-dioxide. In certain preferred embodiments, the phenazine compound is a 7,8-disubstituted phenazine compound, including, without limitation, a 7,8-dimethylphenazine or a 7,8-dihalophenazine (e.g., a 7,8-dichlorophenazine, such as 7,8-dichlorophenazine-5,10-dioxide). In certain preferred embodiments, the phenazine is a 1-hydroxyphenazine, more preferably a 1-hydroxyphenazine-5,10-dioxide.

In still another embodiment, the invention provides a method for treating a subject having a tumor, the method comprising administering to the subject an effective anti-tumor amount of a phenazine compound, such that the tumor is treated.

**Detailed Description of the Invention**

Antimicrobial drugs are the mainstay of treatment and control of infectious diseases in humans and animals. Antimicrobial agents have been effective against all classes of pathogens; bacteria, fungi, viruses and parasites. Nevertheless, new classes of antimicrobial molecules with therapeutic potential are still required to provide treatment for pathogens that have or will become resistant to existing antimicrobial agents. The problem of resistant microorganisms has been known since the first uses of antibiotics in the 1920s, but in the last decade the number of organisms resistant to currently available antimicrobials has increased at an accelerated rate. So alarming has been this increase in microbial resistance that many government and nongovernment scientific organizations have published guidelines and position statements to draw attention to this problem (see, e.g., Lederberg J. et al. Emerging Infections: Microbial threats to health in the United States. Washington, DC: National Academy Press, 1992).

Preventing microbial infections can be accomplished by vaccination (e.g., pertussis, measles), physical isolation of infected individuals from the rest of the community (e.g., tuberculosis, measles, chickenpox), personal cleanliness (e.g., salmonellosis), reducing personal lifestyle risks (e.g., HIV, Hepatitis C), proper food handling (e.g., enterohemorrhagic E. coli 0157.H7), disposal of waste (e.g., salmonella, shigella, cholera), maintaining clean, fresh, potable water (e.g., cholera), or use of antimicrobial agents for prevention (e.g., malaria) or for treatment of active disease (e.g., meningitis, pneumonia).

Preventive measures, while critically important in controlling infectious diseases, are not 100% effective and therefore antimicrobial agents will always be very important in treating or preventing infectious diseases. This is especially true with an ever expanding population of citizens undergoing aggressive treatment for diseases which, as a consequence of the disease itself, or the treatment, are immune compromised (e.g., cancer chemotherapy, systemic lupus, AIDS, transplantation).
Despite a wide variety of antimicrobial agents effective against bacteria, fungi, viruses and parasites, resistance of these microorganisms to currently available antibiotics is increasing. Several recent examples (by no means exhaustive) are 1) penicillin-resistant *Streptococcus pneumoniae* (PRSP); 2) methicillin-resistant *Staphylococcus aureus* (MRSA); 3) vancomycin-resistant *Enterococci* (VRE); 4) cephalosporin-resistant *Enterobacteriaceae*; 5) malarial parasites resistant to chloroquine, mefloquine and other antimalarials; 6) fungi resistant to azoles, triazoles, and amphotericin B; 7) herpes simplex virus resistant to acyclovir and related drugs; and 8) HIV resistant to zidovudine and other antiviral drugs. The skilled artisan will appreciate that MRSA are actually multidrug resistant bacteria (not merely resistant to methicillin); however, for convenience, these organisms are referred to herein as MRSA. Similarly, multidrug resistant *S. epidermidis* is conventionally referred to as methicillin-resistant *S. epidermidis*; the latter terminology is used herein for convenience.

The causes of drug resistance can vary from one organism to another and from one drug to another. For example, it is generally accepted that MRSA and VRE are resistant to beta-lactams and glycopeptides, respectively, due to alterations in cell-wall proteins.

The present invention provides a class of antimicrobial drugs that is not being widely used for the treatment of infections and which shows significant antimicrobial properties against a broad range of microorganisms, bacteria, viruses, fungi and parasites, some of which are known to be highly resistant to currently available antimicrobial agents. The invention also provides phenazine compounds useful as antitumor agents. The phenazine compounds will provide new therapeutic options for the treatment of infections, both those susceptible and those resistant to currently available antimicrobial agents.

For convenience, certain terms used throughout the specification and claims which follow are defined.

The term "microorganism" as used herein, refer to microbes, including disease-causing microbes, such as bacteria, yeast, fungi, viruses, and parasites (such as the sporozoan malaria). (The terms "microbe" and "microorganism" are used interchangeably herein) As the skilled artisan will appreciate, the efficacy of the phenazine compounds of the invention against microorganisms will vary; that is, not every phenazine compound will have activity against all microorganisms. A phenazine compound having activity against a particular microorganism can be selected through use of assays such as are known in the art, including the assays described herein.

The term "inhibiting the growth of a microorganism," as used herein, refers to the process of preventing, arresting, slowing, or decreasing the growth of a selected
microorganism. Thus, "inhibiting the growth of a microorganism" includes both
destruction (i.e., killing) of a microorganism, e.g., a microbial cell, and inactivation of a
microbe, e.g., a virus, arresting, reducing or eliminating the ability of the microbe to
grow, reproduce, or cause infection of a subject. A treatment capable of "decreasing
infective ability," as used herein, refers to a treatment which kills, inactivates, or
otherwise inhibits or prevents a microorganism from infecting an uninfected subject or
continuing to infect an infected subject. Exemplary microbes include, but are not
limited to: Hemophilus spp. (including H. influenzae, H. parainfluenzae, and H.
aprophilus), E. coli, Enterobacter spp., Enterococcus spp. (including E. faecalis, E.
faecium, E. gallinarum, and E. cassillavus), Citrobacter spp., Proteus spp., Morganella
spp., Shigella spp., Salmonella spp. (including S. typhosa and S. typhimurium), Neisseria
spp., Burkholderia spp., Campylobacter spp., Yersinia spp. (including Y. pestis and Y.
entercolitica), Helicobacter spp. (including H. pylori), Vibrio spp. (including V. cholera
and V. vulnificus). Staphylococcus spp. (including coagulase-positive and coagulase-
negative Staphylococcus spp. (including S. epidermidis) and MRSA), Streptococcus spp.
(including Streptococci of the viridans group and S. pneumoniae (including penicillin
and multidrug-resistant)), Chlamydia spp. (including C. pneumoniae and C.
trachomatis), Mycoplasma spp. (including M. pneumoniae), Corynebacterium spp.
(including C. jeikeium and C. xerosis), and Clostridium spp. (including C. difficile),
Nocardia spp., Actinomyces spp., Cryptococcus spp., Histoplasma spp., Candida spp.,
Torulopsis spp., Blastomyces spp., Coccidioides spp. (including C. immitis), and
Aspergillus spp. Such exemplary microbes also include multidrug resistant strains of the
bacteria and fungi, including azole-, triazole- and amphotericin B-resistant strains of the
fungi.

The term "subject," as used herein, refers to an animal, preferably a warm-
blooded animal, more preferably a mammal, including, e.g., cattle, sheep, dogs, cats, and
humans.

The term "effective amount" is art-recognized, and, as used herein, refers to an
amount of a phenazine compound which is effective to achieve a desired result, e.g.,
killing of a microbe. An "effective amount" of a particular phenazine compound will
vary according to such factors as the phenazine compound, the microorganism to be
affected, the weight, age and condition of the subject (if any), and the like. The skilled
artisan will be able to determine an effective amount of a particular compound to
achieve a particular result in accordance with standard practices of the pharmaceutical
art and the teachings herein. In certain preferred embodiments, the phenazine
compounds of the invention have a minimum inhibitory concentration (MIC) of less
than about 25 μg/mL, more preferably less than about 10 μg/mL, more preferably less
than about 5 μg/mL, and most preferably less than about 1 μg/mL, against at least one
selected microorganism, preferably including a bacterium, which can be a drug-resistant bacterium.

The term "treating" is art-recognized, and, as used herein, refers to ameliorating or preventing a microbial infection and/or at least one symptom associated therewith. For example, treating a subject includes eradicating or arresting a microbial infection in a subject, preventing or slowing infection of the subject by a microorganism, decreasing the progression or extent of microbial infection in a subject (e.g., the duration, severity, or extent of tissue infected), and the like.

The term "contacting," as used herein, refers to both in vitro and in vivo contacting. The term "targeting," as used herein, refers to causing a micro-organism to be treated, e.g., by contacting the microorganism with a phenazine compound.

The term "phenazine compound" is known in the art and, as used herein, refers to substituted and unsubstituted compounds having the phenazine (dibenzo-1,4-diazone) nucleus, as well as analogs, derivatives, and congeners thereof; and salts thereof (preferably pharmaceutically acceptable salts). Thus, the term "phenazine compound" refers to the phenazine compounds set forth herein, including nitrogen oxides of phenazines (referred to herein as "phenazine N-oxides," and including phenazine-5-oxides, phenazine-10-oxides, and phenazine-5,10-dioxides). Phenazine compounds can be substituted, e.g., on the phenyl rings, with one or more substituents selected from the group consisting of halogen, alkyl, aryl, alkoxy, hydroxy, amino (including substituted amino), cyano, trifluoromethyl, carboxy, alkoxy carbonyl, aminocarbonyl, and the like. Particularly preferred phenazine compounds include phenazine-N-oxides, including a phenazine 5-oxide, a phenazine-10-oxide, or a phenazine-5,10-dioxide. In certain preferred embodiments, the phenazine compound is a 7,8-disubstituted phenazine compound, including, without limitation, a 7,8-dimethylphenazine or a 7,8-dihalophenazine (e.g., a 7,8-dichlorophenazine, such as 7,8-dichlorophenazine-5,10-dioxide). In certain preferred embodiments, the phenazine is a 1-hydroxyphenazine, more preferably a 1-hydroxyphenazine-5,10-dioxide.

A "drug-resistant microorganism" refers to a microorganism which is resistant to at least one drug which is commonly used for treatment of infections caused by that microorganism, or which is effective against the wild-type microorganism. A "multidrug resistant microorganism" is a microorganism which is resistant to two or more drugs. For example, many bacterial species have acquired resistance to older antimicrobial compounds such as penicillin. Other bacterial strains have acquired resistance to all but a few of the drugs commonly used to combat infection; for example, strains of Enterococcus spp. have been reported which are resistant to vancomycin, a preferred treatment for certain Enterococcus spp. infections. The term "drug-resistant organism", as used herein, refers to any drug-resistant or multidrug resistant organism,
e.g., as described herein. A drug-resistant organism may be resistant to one or more
drugs (depending on the organism) selected from the group consisting of vancomycin
(and related glycopeptides such as teichoplanin and ramoplanin), ampicillin (and
related beta-lactams), aminoglycosides (such as gentamicin, streptomycin, tobramycin,
amikacin, and kanamycin), macrolides (such as erythromycin and chloramphenicol),
tetracycline, metronidazole, cephalosporins, carbapenems, monobactams,
fluoroquinolones, azoles, triazoles, and amphotericin B and the like.

**Phenazine Compounds**

As early as 1860, the production of a blue pigment by the gram-negative bacillus
*Pseudomonas aeruginosa* has been described (Fordos, Compt. Rend. 1860;51:215).
This was determined to be a phenazine molecule called pyocyanin (1-hydroxy-5-methyl-
phenazine). During the ensuing 137 years, the structure of many naturally occurring
phenazines, their physiochemical properties, and their effects on prokaryotic and
eukaryotic cells have been described. More than 50 phenazine compounds have been
described in the scientific literature and even more variously substituted phenazines have
been described in patents (J. Berger, "Microbial phenazines" in Antibiotics,
Chemotherapeutics Scientific, and Antibacterial Agents for Disease Control. M.
phenazines described are produced by microorganisms and these organisms are the sole
source of naturally occurring phenazines. A wide range of organisms produce
phenazines, including *Pseudomonas* spp., *Brevibacterium* spp., *Sorangium* spp.,
*Streptomyces* spp., *Streptosporangium* spp., *Microbispora* spp., and *Nocardia*
cpecies. Some phenazines have been found to possess antimicrobial, antiviral, antifungal,
antiparasitic, and/or antitumor activity. The degree of antimicrobial activity against
selected pathogens is highly variable, depending upon the phenazine structure (Weigele
al., Mikrobiol. Zh (Kiev). 1976;38(1):24-28). However, prior to the present invention,
phenazines have not been tested against recent clinical isolates of pathogens that are
resistant to currently available antibiotics.

**Chemical Characteristics of Phenazines**

All phenazines include the dibenzo-1,4-diazine skeleton. Many substitutions
have been described in the scientific literature or in patents. An important subgroup to
point out are the 5, 10-N, N-dioxides of phenazine and related compounds which have
been found to possess some of the greatest antimicrobial activity. However, other
phenazines, including 1-hydroxyphenazine and pyocyanin, which are simple phenazine
derivatives, also possess significant antimicrobial activity.
Some phenazines are freely soluble in water (e.g., pyocyanin), while others are known for their insolvability in non-polar and polar organic solvents (e.g., iodonin, myxin). The phenazines possess important redox properties which confer upon them the ability to function as acid-base indicators or to interact with the electron transport chain of microorganisms (Bisschop A., et al., European Journal of Biochemistry. 1979; 93:369-374).

Biosynthesis of Phenazines

Many substituted phenazine compounds can be easily extracted from bacterial culture broth, either from the supernatant or as an insoluble precipitate. These biosynthesized phenazines can be purified and, if not possessing antimicrobial activity, modified chemically into a molecule with antibiotic properties (Weigele M., et al. ibid, Gerber N., Biochemistry 1965;4(1):176-180). The synthesis and purification of a wide variety of phenazine compounds is well within the ability of one having ordinary skill in the art using no more than routine experimentation.

The genetics of phenazine biosynthesis have been examined, and the phz R (phenazine regulator) operon has been cloned and sequenced and shown to control phenazine pigment production in response to environmental stimuli (Pierson LS III, Keppenne VD and Wood DW, Journal Bacteriology 1994; 176(13):396-374; and Pierson LSII and Thomashow LS, Mol. Plant. Microbe. Interact 1992;5:330-339).

Antibiotic Effects of Phenazines

It has been known for over 100 years that certain phenazine compounds possess antimicrobial activity. Antibiotic activity has been described against Gram positive and Gram negative bacteria, as well as fungi, yeasts, viruses and parasites (Berger, J., 1982; ibid; Bouchard C.C.R. Acad. Sci. Paris; 1888; 108:713; and Emmerlich R., and Loew O., Zbl. Bakt. 1899; 26:237). Hayes et al. isolated a crude extract of Pseudomonas aeruginosa cultures that included pyocyanin (Hayes et al., Journal of Biological Chemistry 1945). They showed that pyocyanin had antimicrobial activity when administered orally, subcutaneously and intravenously. Other studies from 1924 to 1989 have shown that pyocyanin can be purified from Pseudomonas aeruginosa culture supernatants and that pyocyanin has antibiotic activity against Gram positive and Gram negative bacteria including Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Corynebacterium diphtheriae, and Neisseria gonorrhoeae, Yersinia pestis, and Salmonella typhimurium (Schoentel R., Brit. J. Exp. Pathol., 1941; 22:137-147; Chang PC and Blackwood AC, Can J. Microbiol., 1969; 15:439; Baron, S., Rowe, J., Antimicrobial Agents Chemother, 1981; 20:814-820). Mice have been shown to tolerate 100 mg/kg intraperitoneally of pyocyanin. Pyocyanin is bactericidal. A closely related
degradation product of pyocyanin, 1-hydroxyphenazine (1-HP), also known as hemipyocyanin, has been shown to possess activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Candida albicans*, at concentrations ranging from 10 to 100 µg/ml. Limited toxicity data suggests 1-hydroxyphenazine is less toxic in animals than pyocyanin, where mice tolerated 10 mg intraperitoneal injections and chicken heart fibroblasts did not show toxic effects at 0.1 mg/ml of 1-HP (Gerber, N., J. Org. Chem. 1967; 32:4055; Schoentel, R., Br. J. Exp. Pathol. 1941).

Iodinin was first isolated as a purple pigment in 1938 from a culture of *Brevibacterium iodinum*. This phenazine compound possesses the structure of phenazine-5, 10-N, N-dioxide. Many substituted forms of this basic skeleton have been described, some with significant antimicrobial activity (Weigele, M., Antimicrobial Agents in Chemotherapy, 1971). Iodinin is notoriously insoluble in aqueous solution unless sodium hydroxide or sulfuric acid is added. Iodinin is easily produced from cultures of *Brevibacterium iodinum*, *Streptomyces thiolute*, *Waksmania aerata*, *Nocardia hydrocarbonoxydans*, *Pseudomonas aureofaciens* and *Pseudomonas phenazinium* as an insoluble precipitate. Methylation with dimethyl sulfate in alkali or with diazomethane yields the best known of the phenazine antibiotics, myxin.

Iodinin is itself a weak antibiotic with minimum inhibitory concentrations (MIC) against *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa* and *Candida albicans* greater than 100 µg/ml, although some reviews cite a MIC of 0.08-0.5 µg/ml against *Corynebacterium flimi* (Weigele, M., 1971, Berger, J., 1982). Iodinin has not been used as an antibiotic in animal studies. It has been tested as an antihypertensive at doses of 0.1-300 mg/kg body weight orally in humans, although it has never been brought to market as a treatment for hypertension. The LD50 is reported to be greater than 1 g/kg body weight (Jones, P. and Somani, P, U.S. Patent 3,764,679, October 9, 1973 to Abbott Laboratories).

Myxin has been marketed as an antibiotic, although it is currently no longer produced. Myxin, the 1-methoxy derivative of iodinin, was described in Canada as a product of *Sorangium* sp. (strain 3C) in 1966 (Peterson EA, Gillespie, DC, Cook FD, Can. J. Microbiol. 1966; 12:221-230). It was shown to have a very broad spectrum of action against Gram positive and Gram negative bacteria as well as many fungi and plant pathogens (Peterson EA, et al., 1966, Weigele, M., 1971). Minimum inhibitory concentrations against *Streptococcus agalactiae*, *Staphylococcus aureus* and *E. coli* are less than 4 µg/ml, and against *Candida albicans*, 1.8 µg/ml. Myxin is bactericidal.

Myxin was produced as a copper complex and formulated for topical use in veterinary skin infections. Unfortunately, myxin shows significant toxicity in mice with an LD50 of 40 mg/kg intraperitoneally. 3.75 mg/kg intraperitoneally in mice was not effective.
against bacterial infections. However, a topical cupric complex of mycin was found to be useful in bacterial and fungal pyoderma in animals.

Some 30 derivatives of iodin and myxin have been described, several of which have significant antimicrobial activity, while others do not. Of particular interest are the 1-methyl and 1-methoxy derivatives of phenazine-5,10-N,N-dioxide, which, according to Weigele, M. et al., possess antimicrobial activity equal to or exceeding that of myxin.

Lomofungin (also known as lomondomycin), the 6-formyl trihydro-1-carboxyphenazine derivative, with many highly substituted derivatives, has been described (Bigge, C.F., et al., U.S. Patent 4,657,909, April 14, 1987, Warner-Lambert Company). A wide variety of compounds synthesized using the lomofungin nucleus is described in the above mentioned patent; however, the antimicrobial activity is not especially high, being up to 1000 times less potent than that of the phenazines mentioned previously.

Griseolatein A and B, products of Streptomyces griseoluteus, are also known to have antibiotic activity, although griseolutein B is more potent (Tausig, F., Wolf, FJ, Miller, AK, Antimicrobial Agents and Chemotherapy, 1964:59; Osato, T., Maeda, K., Umezawa, H., J. Antibiot. 1954; 7:15; and Nakamura, S., Maeda, K. and Umezawa, H., J. Antibiot. 1964; 17:33) Griseolatein B has documented inhibitory concentrations against E. coli of 11 μg/ml, S. aureus of 0.2 μg/ml and Salmonella gallinarium of 0.7 μg/ml. Interestingly, griseolatein B has documented efficacy in treating S. aureus infections in mice, with effective doses of 0.95 mg/kg intraperitoneally, 23.5 mg subcutaneously and 850 mg orally. The diacetyl derivative of griseolatein B is 5 times more active against Staphylococcus aureus infections by the oral route than griseolatein B itself.

**Phenazine Derivatives For Treatment of Microbial Infection**

It has now been found that phenazine compounds are active antimicrobial agents against a variety of drug-resistant organisms, and that certain phenazines are also active against wild-type microorganisms. We have tested selected strains of bacteria that commonly cause human infection against several representative phenazine compounds. As described in more detail below (see, e.g., Examples 8 and 9, infra), phenazine compounds of the invention are active antimicrobial compounds against a variety of microbes, including drug-resistant bacteria, fungi, molds, and the like.

The compounds of the invention can also be employed for purposes such as selective gut decontamination. Gut decontamination is useful for preventing microbial infections in immunosuppressed patients; for example, a patient about to receive chemotherapy can be treated with a phenazine compound of the invention to decontaminate the gut, to thereby prevent infection by gut pathogens after the patient has
received chemotherapy. In this embodiment, it is preferred to use a phenazine compound which are not significantly absorbed through the gastrointestinal wall; this increases the effective concentration, and therefore the apparent potency, of the phenazine compound in the gastrointestinal tract.

It would be apparent to one of ordinary skill in the art in light of the teachings herein, that phenazine compounds which are not significantly absorbed through the gastrointestinal wall, but rather are retained at or near the mucosa, would be useful for the treatment of infections due to microorganisms which cause gastrointestinal diseases, including, without limitation, *C. difficile* (diarrhea and colitis) and *H. pylori* (gastric and duodenal ulcers).

In a related embodiment, topical application of a phenazine compound according to the invention, can be used to decontaminate the mouth, throat, and upper respiratory tract to prevent microbial infection. For example, infection by microorganisms resident in the mouth can result in pneumonia in a susceptible individual, especially if the patient is receiving respiratory support from a ventilator.

It has been found by the present inventors that phenazine N-oxides (e.g., phenazine-5-oxides, phenazine-10-oxides, and phenazine-5,10-dioxides) are often more active against microorganisms than the corresponding phenazines. Accordingly, in a preferred embodiment, a phenazine compound of the invention is a phenazine-N-oxide, most preferably a phenazine-5,10-dioxide.

Without wishing to be bound by any theory, it is believed that the phenazine compounds of the invention may exert an antimicrobial activity at least in part due to the ability of phenazine compounds to undergo electron transfer reactions, which result in the formation of toxic active oxygen species at the site of action *in vivo*, with resultant damage to infectious microorganisms (see, e.g., P.W. Crawford et al. *Chem. Biol. Interact.* (1986) 60:67-84). The ability of a phenazine compound to undergoelectroreduction *in vivo* may therefore be correlated with the antimicrobial activity of the phenazine compound (compounds with more positive reduction potentials, and which show reversible reduction waves, are often found to have greater antimicrobial activity).

Accordingly, in certain preferred embodiments, a phenazine compound of the invention can be substituted, e.g., on the phenazine nucleus, with a substituent(s) which increases the ability of the phenazine compound to be reduced *in vivo*. For example, in certain preferred embodiments, a phenazine compound of the invention includes at least one substituent which is capable of stabilizing a radical cation (e.g., of a phenazine) or radical anion (e.g., of a phenazine-5,10-dioxide, see, e.g., M.D. Ryan et al. *J. Pharm. Sci.* (1985) 74:492) resulting from a one-electron reduction of the phenazine nucleus. Without wishing to be bound by theory, it is believed that such a radical anion (e.g., of a phenazine-5,10-dioxide) may be able to transfer electrons in a catalytic manner.
Illustratively, the phenazine nucleus can be substituted, at the 7 and/or 8-positions, with electron-withdrawing substituents (such as, e.g., halogens, including fluoro, chloro, bromo, and iodo), or methyl groups. Other groups which change the electron distribution of the ring structure are also acceptable. Especially preferred embodiments of the invention are phenazine N-oxides which are substituted at the 7 and/or 8 positions, including, without limitation, a 7,8-dimethylphenazine or a 7,8-dihalophenazine (e.g., a 7,8-dichlorophenazine, such as 7,8-dichlorophenazine-5,10-dioxide). It is believed that substituents at the 7 and 8 positions of the phenazine ring system may reduce toxicity of the compounds in vivo (possibly analogous to toxic structure-function relationships of riboflavins) while retaining antimicrobial activity.

In certain preferred embodiments, the phenazine is a 1-hydroxyphenazine, more preferably a 1-hydroxyphenazine-5,10-dioxide. Without wishing to be bound by any theory, it is believed that the 1-hydroxy group of a 1-hydroxyphenazine-10-oxide (or 5,10-dioxide) may interact with the 10-oxide oxygen atom to thereby render the compound more bioactive, possibly by enhancing charge transfer through hydrogen bonding. Similarly, a 6-hydroxy group may interact with the 5-oxide oxygen atom to enhance charge transfer.

Thus, in preferred embodiments, a phenazine compound useful in the methods of the invention can be represented by the structure:

\[
\begin{array}{c}
\text{R}_1 \\
\text{R}_2 \\
\text{(R}_3\text{)}_m \\
\text{N} \\
\text{X} \\
\text{N} \\
\text{X} \\
\text{(R}_4\text{)}_n \\
\end{array}
\]

in which \( R_1 \) and \( R_2 \) are selected independently for each occurrence from the group consisting of hydrogen, halogen (including fluoro, chloro, bromo, or iodo), lower alkyl, aryl (more preferably substituted or unsubstituted phenyl), hydroxy, lower alkoxy (such as methoxy), mercapto, lower alkylthio, nitro, cyano, amino, lower alkylamino, dialkylamino, and trifluoromethyl; \( R_3 \) and \( R_4 \) are selected independently for each occurrence from the group consisting of halogen, lower alkyl, hydroxy, lower alkoxy, mercapto, lower alkylthio, amino, lower alkylamino, dialkylamino, and trifluoromethyl; \( m \) is 0, 1 or 2; and \( n \) is an integer from 0 to 4; and \( X \) is, independently for each occurrence, oxygen or a pair of electrons. In certain preferred embodiments, \( m \) and \( n \) are each 0. In certain embodiments, at least one occurrence of \( X \) is oxygen; in particularly preferred embodiments, both occurrences of \( X \) are oxygen (i.e., the compound is a phenazine-5,10-dioxide). In preferred embodiments, \( n \) is at least 1, and one occurrence of \( R_4 \) is a 1-hydroxy group (i.e., the compound is a 1-hydroxyphenazine (optionally a 1-
hydroxyphenazine-N-oxide)). R₁ and R₂ can be the same or different; in certain embodiments, R₁ and R₂ are the same, and are both methyl or both chlorine. In some embodiments, the organism is a multidrug resistant organism, including a bacterium.

In preferred embodiments, m and n are each 0 (i.e., the phenazine compound can be substituted at the 7 and/or 8-positions, but is otherwise unsubstituted). Exemplary preferred phenazine compounds include 7,8-dichlorophenazine-5,10-dioxide (P5) and 7,8-dimethylphenazine-5,10-dioxide (P6).

It will be understood that a particular phenazine compound can be selected according to several criteria, which will be routine to one of ordinary skill in the art in view of the teachings provided herein. For example, as described above, certain substituents on the phenazine nucleus are preferred to increase the antimicrobial potency of the phenazine compound. In addition, substituents of the phenazine nucleus can be selected to improve the solubility of the compound (e.g., by substitution with hydrophilic substituents such as amino, carboxy, hydroxy, and the like), to improve the biodistribution of the compound, and the like. One of ordinary skill in the art will be able to select an appropriate phenazine compound using no more than routine experimentation in light of the teachings herein.

Moreover, the efficacy of a particular phenazine compound can be determined, e.g., by routine screening, e.g., such as described infra. An effective antimicrobial compound can therefore be readily selected for use against a selected microorganism of interest. Activity of certain known antibiotics is related to the ability of the antibiotic to form metal complexes with metal ions; accordingly, in certain embodiments, a phenazine compound of the invention is capable of forming a metal complex (e.g., with a metal center such as calcium, magnesium, zinc, iron, manganese, or copper) and/or is administered to a subject in the form of a metal complex.

It has been found that the phenazine compounds of the invention are active against bacteria (both Gram-positive and Gram-negative), including drug-resistant strains of bacteria. It is believed that the compounds of the invention are also active against fungi, yeasts, parasites, and the like. Compounds of the invention exhibit both bacteriostatic activity (i.e., inhibit the growth of a bacterium without killing the bacterium) and bactericidal activity (i.e., killing the bacterial cell). In a preferred embodiment, a phenazine compound of the invention is administered to a subject to have a bactericidal effect on the microorganism.

It has been found in preliminary experiments that phenazine compounds described herein are capable of inhibiting the growth of a human tumor cell line in cell culture. Accordingly, the phenazine compounds described herein are useful for inhibiting the growth of tumors and tumor cells, either in vitro (ex vivo) or in vivo. In one embodiment, the invention provides a method for treating a subject having a tumor.
The method includes administering to the subject an effective (preferably non-toxic) anti-tumor amount of a phenazine compound, such that the tumor is treated.

Compounds of the invention can be obtained by methods well known to the skilled artisan. Certain phenazine compounds are commercially available, or can be obtained from cultures as described above. Additional compounds can be prepared by well known techniques of organic synthesis (see, e.g., Examples 1-3, infra).

Pharmaceutical Compositions

In another aspect, the present invention provides pharmaceutically acceptable compositions which comprise a therapeutically-effective amount of one or more of the phenazine compounds described above, optionally formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. As described in detail below, the pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; or (4) intravaginal or intrarectal administration, for example, as a pessary, cream or foam.

The phrase "therapeutically-effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the present invention which is effective for producing some desired therapeutic effect by treating (i.e., preventing or ameliorating) a bacterial, fungal, viral or parasitic infection in a subject, e.g., at a reasonable benefit/risk ratio applicable to any medical treatment.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in suspending, or carrying or transporting a subject phenazine compound, e.g., from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and
sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its
derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose
acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as
cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil,
safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as
propylene glycol; (11) polyols, such as glycine, sorbitol, mannitol and polyethylene
glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering
agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16)
pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20)
phosphate buffer solutions; and (21) other non-toxic compatible substances employed in
pharmaceutical formulations.

As set out above, certain embodiments of the present compounds can contain a
basic functional group, such as amino or alkylamino, and are, thus, capable of forming
pharmaceutically-acceptable salts with pharmaceutically-acceptable acids. The term
"pharmaceutically-acceptable salts" in this respect, refers to the relatively non-toxic,
inorganic and organic acid addition salts of compounds of the present invention. These
salts can be prepared in situ during the final isolation and purification of the compounds
of the invention, or by separately reacting a purified compound of the invention in its
free base form with a suitable organic or inorganic acid, and isolating the salt thus
formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate,
phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate,
phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphylate, mesylate,
glucoheptonate, lactobionate, and laurylsulphonate salts and the like. (See, e.g., Berge et

In other cases, the compounds of the present invention may contain one or more
acidic functional groups and, thus, are capable of forming pharmaceutically-acceptable
salts with pharmaceutically-acceptable bases. The term "pharmaceutically-acceptable
salts" in these instances refers to the relatively non-toxic, inorganic and organic base
addition salts of compounds of the present invention. These salts can likewise be
prepared in situ during the final isolation and purification of the compounds, or by
separately reacting the purified compound in its free acid form with a suitable base, such
as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal
cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary
or tertiary amine. Representative alkali or alkaline earth salts include the lithium,
sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts include
ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine
and the like. (See, for example, Berge et al., supra)
Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations of the present invention include those suitable for oral, nasal, topical, transdermal, buccal, sublingual, rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 1 per cent to about ninety-nine percent of active ingredient, preferably from about 5 per cent to about 70 per cent, most preferably from about 10 per cent to about 30 per cent.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the invention suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present invention as an active ingredient. A compound of the present invention may also be administered as a bolus, electuary or paste.
In solid dosage forms of the invention for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginites, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or other milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets, and other solid dosage forms of the pharmaceutical compositions of the present invention, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.
The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration of the compounds of the invention include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metaphosphate, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations of the pharmaceutical compositions of the invention for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the invention with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound.

Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams, troches, vaginal tablets or spray formulations containing such carriers as are known in the art to be appropriate.

Dosage forms for the topical or transdermal administration of a compound of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

The ointments, pastes, creams and gels may contain, in addition to an active compound of this invention, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.
Powders and sprays can contain, in addition to a compound of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of a compound of the present invention to the body. Such dosage forms can be made by dissolving or dispersing the phenazine compound in the proper medium. Absorption enhancers can also be used to increase the flux of the subject phenazine across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the phenazine in a polymer matrix or gel.

Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this invention.

Pharmaceutical compositions of this invention suitable for parenteral administration comprise one or more compounds of the invention in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material.
having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissue.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

The preparations of the present invention may be given to humans or other animals for therapy by any suitable route of administration, including orally, parenterally, topically, vaginally or rectally. They are of course given by forms suitable for each administration route. For example, when they are administered orally they are formulated, e.g., in tablets or capsule form; for parenteral administration, the formulation is suitable for injection or infusion; for topical administration, including buccally and sublingually, in the form of a powder, drop, lotion or ointment; nasal administration in spray form; and for vaginal and rectal administration the composition including a phenazine compound can be administered in the form of suppositories. Oral or topical administration is preferred.

The phrases "parenteral administration" and "administered parenterally" as used herein mean modes of administration other than enteral and topical administration, usually by injection, and include, without limitation, intravenous, intramuscular, intradermal, intraperitoneal, transtracheal, intraorbital, intraocular, periorcular, intracardiac, subcutaneous, subcuticular, intrarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical
compositions of the present invention, are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular compound of the present invention employed, or the derivative (e.g., ester, salt or amide) thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In general, a suitable daily dose of a compound of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, doses of the compounds of this invention for a patient, when used for the indicated effects, will range from about 0.0001 to about 100 mg per kilogram of body weight per day, more preferably from about 0.01 to about 50 mg per kg per day, and still more preferably from about 0.1 to about 40 mg per kg per day.

If desired, the effective daily dose of the active compound may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical composition.

Exemplification

Illustrative Preparation of Compounds

The following compounds were obtained from commercial suppliers or prepared as described herein: 1-hydroxy-5-methylphenazine (Pyocyanin, P1);
1-hydroxyphenazine (P2); phenazine methosulfate (P3); 1-hydroxyphenazine-5,10-dioxide (P4); 7,8-dichloro-1-hydroxyphenazine-5,10-dioxide (P5); and 7,8-Dimethyl-1-hydroxyphenazine-5,10-dioxide (P6).

P1, P2 and P3 are commercially available:

P1, 1-hydroxy-5-methylphenazine, was obtained from Colour Your Enzyme (Bath, Ontario, Canada); P2, 1-hydroxyphenazine, was obtained from TCI America (Portland, Oregon, U.S.A.); and P3, phenazine methosulfate, was obtained from Aldrich Chemical Co, Milwaukee, WI.

As described in detail below, P4 was synthesized by the method of Issidorides et al [1]. In the synthesis of P5, 5,6-dichlorobenzofurazan oxide and its condensation with 1,2-cyclohexanedione was accomplished by a modification of the method of Issidorides et al [1]. In the synthesis of P6, the condensation of 5,6-dimethylbenzofurazan oxide with 1,2-cyclohexanedione was accomplished by a modification of the method of Issidorides et al [1].

Example 1

Preparation of 1-hydroxyphenazine-5,10-dioxide (P4)

10.2 g of benzofurazan oxide, dissolved in 90 mL diethylamine, were added dropwise to 4.2 g of 1,2-cyclohexanedione dissolved in a 10 mL diethylamine in a 250 mL two-neck round bottom flask. The reaction mixture was stirred by a magnetic stirrer and the flask was kept in an ice bath. After the addition of the benzofurazan oxide was completed, the mixture was stirred for 30 min in the ice bath, followed by additional stirring for 1 hr at room temperature. The mixture was transferred into a 500 mL round bottom flask, diluted with about 100 g crushed ice and acidified by dropwise addition of glacial acetic acid. The brown-red solid obtained was filtered, washed with water and dried in a vacuum oven at 50°C for 2-3 hr.

5.0 g of the crude product was dissolved in a minimum amount of benzene (50-80 mL) and oxidized by addition of a warm benzene solution of 3-chloroperbenzoic acid (5.0 g in 50 mL benzene) to it. The reaction mixture was stirred at room temperature for 12 hr and transferred to a 500 mL separation funnel. The benzene solution was extracted three times with a total of 100 mL of 10% aqueous Na2SO3 in order to reduce 3-chloroperbenzoic acid to 3-chlorobenzoic acid. Six additional extractions were carried out with a total of 200 mL of 10% of aqueous NaHCO3 in order to make 3-chlorobenzoic acid water soluble and thus remove it from the benzene layer. The benzene layer was dried with 10 g of anhydrous Na2SO4 or MgSO4 for 10 min. Evaporation of the dried benzene solution gave a dark red solid. The solid was dissolved in 200-250 mL of 10% aqueous NaOH with stirring and heating. The resulting blue solution was acidified with glacial acetic acid giving a bright red solid.
product. The solid was filtered and dried in a vacuum oven at 40°C for 4–5 hr.
Recrystallization from methanol–chloroform yielded about 0.9 g (12% yield) of the pure
product. Dark red needles melted at 182–184°C, TLC confirmed the homogeneity of the
product and FT–IR. $^1$H NMR and UV–VIS spectra were consistent with reported
spectra [1]:

IR $\nu_{max}$ 3400, 3100, 1590, 1550, 1470, 1400, 1380, 1345, 1320, 1300, 1260,
1245, 1095, 1060, 1040, 880, 835, 800, 770, 750, 655 cm$^{-1}$. $^1$H NMR 7.0 9 (m, 2H)
7.7 (m, 4H), 8.3 (m, 2H)

Example 2
Preparation of 7,8–dichloro–1–hydroxyphenazine–5,10–dioxide (P5)

a. Synthesis of 5,6–dichlorobenzofurazan oxide

5,6–dichlorobenzofurazan oxide was synthesized by the method used by Green
and Rowe [2] to synthesize some benzofurazan oxides by a hypochlorite oxidation of
o–nitroaniline derivatives. Two other methods [3,4], which were adapted from this 1912
procedure of Green and Rowe, are reported for synthesis of several other derivatives of
benzofurazan oxides.

A mixture of 10.5 g of KOH and 125 mL ethanol was stirred and heated on a
water bath until the solid dissolved. 1 g of 4,5–dichloro–o–nitroaniline was dissolved in
25 mL alcoholic KOH in a round bottom flask. The resulting red solution was stirred
vigorously and cooled to 0°C in an ice bath; 25–30 mL of 5% sodium hypochlorite
solution was then added dropwise until the red colour had disappeared. The flocculent
pale yellow precipitate was collected by filtration using a Büchner funnel. The crude
product was washed with 15 mL water and dried in a vacuum oven at room temperature.
0.8 g of crude product was recrystallized from diluted ethanol, giving pale yellow
crystals with a sharp melting point at 110°C.

b. Condensation of 4,5–dichlorobenzofurazan oxide with 1,2–cyclohexanediene

This condensation was carried out by a modification of the method of Issidorides
et al [1]. 0.6 g of 1,2–cyclohexanediene was added in a 100 mL two neck round bottom
flask and dissolved in 3 mL triethylamine. The mixture was cooled in ice, stirred
vigorously, and a solution of 1.95 g 5,6–dichlorobenzofurazan oxide dissolved in 30 mL
triethylamine was added dropwise. After the addition was complete, the reaction
mixture was stirred for 10 hr in ice bath followed for additional 8 hr at room
temperature. The mixture was diluted with 10 g crushed ice and acidified by dropwise
addition of 25 mL glacial acetic acid. The brown–red solid was filtered, washed with
water and dried in vacuum oven at 50°C for 5 hr. TLC indicated purple and olive green products.

0.35 g of crude product was dissolved in benzene and oxidized by addition of a warm solution of 0.70 g 3-chloroperbenzoic acid in 8 mL of benzene to it. The reaction mixture was refluxed for 40 min. The solution was extracted with 10% aqueous Na₂SO₄ followed by a extraction with 10% aqueous NAHCO₃. Dried benzene solution was evaporated giving dark red-purple solid. Recrystallization yielded the pure product with melting point of 207–209°C. FT–IR, NMR and UV–VIS spectra are in agreement with the reported ones [1]:

IR ν max 3400, 3085, 1620, 1580, 1540, 1490, 1450 1420, 1395, 1355, 1340, 1240, 1225, 1205, 1180, 1140, 1110, 1095, 1060, 1040, 910, 900, 830, 785, 735, 720, 665, 635 cm⁻¹. ¹H NMR 7.5 (m)

Example 3

Preparation of 7,8-dimethyl-1-hydroxyphenazine-5,10-dioxide (P6)

a. Synthesis of 5,6-dimethylbenzofurazan oxide

2.0 g of 4,5-dimethyl-o-nitroaniline was dissolved in 70 mL alcoholic KOH. Red solution was cooled to 0°C in ice bath and intensively stirred; 30 mL of 5% sodium hypochlorite solution was added slowly until the red colour had disappeared. The yellow precipitate was filtered, washed with water and dried at room temperature. 1.5 g of crude product was recrystallized giving yellow crystals with melting point of 126°C.

b. Condensation of 4,5-dimethylbenzofurazan oxide with 1,2-cyclohexanedione

This condensation was carried out by a modification of the method of Issidorides et al. [1].

5.6 g of 4,5-dimethylbenzofurazan oxide was dissolved in 110 mL diethylamine and added dropwise to 2.0 g of 1,2-cyclohexanedione during vigorous stirring. After the addition of the solution was completed, the mixture was stirred for an additional 3 hr at room temperature. The mixture was diluted with crushed ice and acidified with glacial acetic acid. The brown-red solid was filtered, washed with water and dried. TLC indicated presence of red and olive green products.

1.7 g of the crude product was dissolved in a minimal amount of benzene and oxidized by dropwise addition of a warm benzene solution of 2.7 g 3-chloroperbenzoic acid (in a minimal amount of benzene). The reaction mixture was refluxed for 24 hr. The solution was first extracted with 10% aqueous Na₂SO₃, followed by extraction with 10% aqueous NaHCO₃. Evaporation of the dried benzene solution produced a dark red solid which was treated with 10% aqueous NaOH. The solution was acidified with
glacial acetic acid, producing 0.8 g red solid. Recrystallization of the crude product yielded the pure product having a melting point of 179–180°C. FT–IR, $^1$H NMR, and UV–VIS spectra were in agreement with reported spectra [1]:

IR $\nu_{\text{max}}$ 3400, 3100, 2900, 1620, 1550, 1500, 1475, 1410, 1385, 1340, 1280, 1265, 1180, 1135, 1100, 1060, 1040, 1010, 915, 805, 750, 680 cm$^{-1}$. $^1$H NMR 2.4 (s, 6H), 7.5 (m, 6H)

References


Example 4

**Susceptibility Testing of Aerobic Bacteria**

Susceptibility testing for aerobic bacteria was carried out using either macrobroth dilution or microbroth dilution methods. It will be appreciated that aper dilution or disk diffusion methods can also be utilized.

The drug to be tested was prepared in absolute ethanol and diluted such that the final working concentration of ethanol was less than 5% of the final working volume, so as to minimize any effects of the ethanol on the microbial assay. The antibiotic was diluted using cation-supplemented Mueller-Hinton broth (BBL, Becton Dickinson Diagnostics, Sparks, MD). Two-fold serial dilutions of the antibiotic were prepared in the concentrations indicated in Tables 1 and 2, below. The antibiotic dilutions were prepared immediately prior to the assay and not stored at their dilutions.

**Preparation of Inoculum**

A final inoculum of $5 \times 10^5$ colony-forming units (CFU) per mL was used. Isolates were inoculated into Mueller-Hinton broth and incubated until turbid and the turbidity adjusted to match a 0.5 McFarland standard. Alternatively, 4-5 isolated colonies from overnight growth on Tryptic soy agar or Tryptic soy blood agar plates were directly suspended into broth to match a turbidity of 0.5 McFarland standard. This latter method was used for testing *Staphylococcus aureus* and *Enterococcus* species. A portion of suspension was diluted 1:100 with broth and 1 mL of this final dilution was
added to each tube containing 1 mL of the prediluted drug so that a final inoculum of 5 x 10^5 CFU/mL was achieved. Tubes without antibiotics were likewise inoculated as growth controls and uninoculated broth was used as a sterility control.

A sample of a final inoculum was routinely serially diluted and plated in duplicate to determine colony counts to verify accuracy of the inoculum.

All inoculated tubes were incubated in room air at 35°C for 6-20 hours and this was extended to 24 hours for vancomycin-resistant Enterococci and methicillin-resistant Staphylococci.

For all MIC testing, appropriate ATCC control organisms were run in parallel as indicated below. The end points were sharp and there were no trailing end points detected. No skipped tubes were ever detected. The lowest concentration that completely inhibited visible growth with the organism by the unaided eye was recorded as the minimum inhibitory concentration.

Microbroth Dilution Method for Aerobic Organisms

Stock antimicrobial solutions are prepared as indicated above. Between 0.05 or 0.1 mL are dispensed to the wells of the microbroth dilution tray. These trays are stored in plastic at -70°C.

Inoculation

A final desired inoculum of 5 x 10^5 CFU/mL is used. The inoculum is prepared similarly to above by either direct colonies or from colonies grown in broth prior to inoculation. A 0.5 McFarland suspension of the test organism is diluted 1:10 in broth and between 0.001 and 0.005 mL is inoculated into wells containing 0.1 mL of broth.

When a 0.05 mL of broth per well system is used, 0.05 mL of inoculum is used, which is prepared by 1:100 dilution of a 0.5 McFarland suspension. The inoculum density is routinely checked by plate counting and an aliquot of the inoculum is routinely plated out to check for purity. Wells not containing antimicrobial agents are used as growth controls.

Plates are covered with plastic tape or sealed in a plastic bag and incubated at 35°C for 6-24 hours.

Quality control strains as mentioned above are used. The end point MIC is the same as for macrobroth dilution. Growth is determined by comparison with that in the growth control well and it is indicated by turbidity throughout the well or by a button, single or multiple, in the well bottom.

Quality control strains used are Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Enterococcus faecalis ATCC 29212, and Staphylococcus aureus ATCC 29213, and E. coli ATCC 25922.
Determination of Minimum Bactericidal Concentration

After the minimum inhibitory concentration was determined, the minimum bactericidal concentration (MBC) was determined by subculturing tubes which had no visible growth, or in the case of microtiter plates, wells that have no visible growth. In the case of macrobroth MBCs, after 20 hours of incubation the tubes were re-vortexed and incubated for an additional 4 hours and then 100 µL of the broth was removed and spread across agar plates with a bent glass rod, in duplicate.

In the case of microbroth MBC determination, 100 µL is aspirated and dispensed to the surface of agar plates and spread evenly using a bent glass rod.

The MBC is defined as the lowest concentration of antibiotic that reduces the inoculum by 99.9% within 24 hours.

References


Example 5

Susceptibility Testing of Anaerobic Bacteria

The antibiotics are prepared as discussed above.

Inoculum Preparation

The inoculum of anaerobic organisms is prepared by suspending colonies taken from a 24-72 hour anaerobic blood agar plate directly to Thioglycolate broth or Brucella broth to a density of a 0.5 McFarland standard. Alternatively, initial suspension is prepared by inoculating 5 colonies into enriched Thioglycolate medium and incubating for 4-6 hours or occasionally overnight for slow-growing organisms. This suspension is then diluted to a density of a 0.5 McFarland standard.

Media

The media for agar dilution anaerobic susceptibility testing is Wilkins-Chalgren agar or Brucella agar base. Where appropriate, Schaedler’s, Wilkins or Brain-Heart
infusion broth are used. The broths are supplemented with vitamin K1, 1 µg/mL and Hemin 5 µg/mL. Defibrinated sheep blood or lysed sheep blood is also used where appropriate to supplement Wilkins-Chalgren agar and Brucella agar base. Both of these supplements are used at a final concentration of 5%. Where required, 1% Tween 80 for Gram positive cocci, and rabbit serum or 2-3% lysed horse blood for pigmented organisms, are used. The plates are not stored for more than 7 days prior to use.

**Incubation Conditions**

Anaerobic jars with disposable hydrogen-carbon dioxide generators and palladium coated catalyst pellets are routinely used. The incubation atmosphere contains 4-7% carbon dioxide. Incubation is at 35°C for 48 hours. Control plates, microdilution trays or broth macrodilution tubes are divided in duplicate, where one is incubated anaerobically as a growth control and the other incubated aerobically as a contaminant control.

**Agar Dilutions for Susceptibility Testing of Anaerobes**

In agar dilution testing the final inoculum is $10^5$ CFU/spot.

**Broth Microdilution Anaerobic Susceptibility Testing**

Microdilution trays are prepared and frozen, and frozen trays are held at 70°C for up to 6 months prior to use. The final volume per well is not less than 100 µL.

The antibiotics are prepared as described above. The inocula are prepared similarly as for agar dilution with the final inoculation in each well of $10^6$ CFU/mL.

The microdilution trays are pre-reduced by holding them in an anaerobic environment for 2-4 hours prior to inoculation.

Control wells include a well with broth but no drug as a growth control and an uninoculated well as a sterility control.

**Interpretation of Results**

For anaerobic agar dilution testing, the end point is read as the concentration at which there is the most marked change from the growth control. This change is manifested as no growth, or as tiny colonies or a slight haze, or as significantly diminished growth compared to that of the control. In broth microdilution, MIC anaerobic determination trays are examined with indirect transmitted light. That concentration at which the most significant reduction in growth is observed is chosen as the end point. This is either complete inhibition of growth or tiny, gradually diminishing button of growth.
The following quality control strains are used for anaerobic susceptibility testing: 
*Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, and 
*Eubacterium lentum* ATCC 43055.

References for Aerobic (Example 4) and Anaerobic (Example 5) Susceptibility Testing

1. National Committee for Laboratory Clinical Standards. 1993. Performance 
   Standards for Antimicrobial Disk Susceptibility Tests. Approved Standards for 
   Villanova, PA.

   antimicrobial susceptibility tests for bacteria that grow aerobically. Approved 
   Standard M7-A3. National Committee for Clinical Laboratory Standards, 
   Villanova, PA.

   production lots of dehydrated Mueller-Hinton agar. Tentative standard M6-T. 
   National Committee for Clinical Laboratory Standards, Villanova, PA.

   *vitro* susceptibility testing criteria and quality control parameters. NCCLS 
   document M23-A. National Committee for Clinical Laboratory Standards, 
   Villanova, PA.

   antimicrobial susceptibility testing of anaerobic bacteria, 3rd Edition. Approved 
   standard M11-A3. National Committee for Clinical Laboratory Standards, 
   Villanova, PA.

Example 6

Methodology for Testing Susceptibility of Mycobacteria spp.

Source of inoculum: The source inoculum for mycobacterial susceptibility 

30 testing is derived from a mycobacterial subculture using an indirect method. Inoculum 

is grown on Middlebrook 7H10 or 7H11 agar or another egg-based medium, but the 

sample is taken less than 4 weeks after growth on the solid media. Turbid growth in a 

liquid medium such as Middlebrook 7H9 broth or in BACTEC 12B media is also used, 

as appropriate.

Media used

Middlebrook 7H10 agar supplemented with oleic acid-albumin-dextrose-catalase 

(OADC) is used as a standard medium for susceptibility testing of slow growing 

mycobacteria by the proportion method. Where required to support growth of more
fastidious strains, Middlebrook 7H11 medium is substituted. The BACTEC method (BBL, Becton Dickinson Diagnostics, Sparks, MD) is used preferentially for all susceptibility testing against mycobacteria. This uses an enriched Middlebrook 7H9 broth containing 4 µCi of C-14 labelled palmitic acid per vial (also known as BACTEC 7B media).

The antibiotics are prepared as mentioned above. Generally, growth from a BACTEC 12B model of the test organism is used where it reached a growth index of 500 or higher. After mixing to break up clumps, turbidity is adjusted to match a McFarland standard of 0.5 and a 1:100 dilution of the final inoculum is used as a control. Dilutions are prepared in distilled water or BACTEC diluting fluid but not broth. Purity is determined by plating the inoculum to blood agar and 7H10 plates.

*Incubation*

BACTEC 12B vials are pre-run in a BACTEC 460 instrument in order to establish a gas phase of 5% CO₂ in air in the headspace of the vial. Any vial with initial growth index greater than or equal to 20 is rejected. Each pre-run vial is inoculated with 0.1 mL of the adjusted suspension of the test or quality control isolate and the vials are incubated at 37°C in the dark.

The BACTEC vials are read on the BACTEC instrument at 24 hour intervals for a minimum of 4 days and until the control vial reached a growth index of greater than or equal to 30. When the growth index of the control is greater than or equal to 30 after a minimum of 4 days, the difference in the growth indices from one date to the next are examined. If the difference in growth index of the control is greater than the difference in the growth index of the drug, the organism is considered susceptible to the test drug. If the difference in the growth index of the control is less than the difference in the growth index of the drug, it is classified to be resistant. If the growth index is greater than or equal to 500, and then is 500 on the next reading, the isolate is considered resistant to that drug regardless of the change in growth index. If the change in differences in growth index between the control and the test drug are close and within 10% of each other, the trials are repeated.

*Quality Control*

Referenced strains of known susceptibility pattern are tested with each new batch of drug and BACTEC 12B media. Quality control tests are performed with each test isolate. *M. tuberculosis* ATCC27294 (H37Rb) strain is used as a control.
Example 7

Susceptibility Testing of Yeasts and Fungi

Test Medium

A completely defined synthetic medium such as RPMI 1640 with L-glutamate containing a pH indicator without sodium bicarbonate is the medium of choice. It is buffered to a pH of 7.0 at 25°C. The buffer used is morpholinepropanesulfonic acid (MOPS) at a final molarity of 0.165 for pH 7.0.

Stock Drug Solutions

Solutions of antibiotic to be tested are prepared at 10 times the highest concentration to be tested. All test solutions are freshly prepared.

Preparation of Inocula

Spectrophotographic methods are used to prepare the inocula. The test organisms are grown on Sabouraud agar at 35°C and subcultured twice, thus ensuring viability and purity. The inoculum suspension is created by selecting 5 isolated colonies of at least 1 mm in diameter and suspending them in 5 mL of sterile 0.85% saline.

Turbidity is measured at 530 nanometers and the culture is adjusted with saline to provide a 0.5 McFarland barium sulphate standard equivalent. This resulted in a suspension of 1-5 x 10^6 organisms per mL, which is further diluted 2000 times with RPMI media, giving a final test inoculum of 0.5-2.5 x 10^3 organisms per mL.

A 0.5 mL sample of the prepared antibiotic solution is added to 4.5 mL of medium; for each of compounds P1-P6 a 1:10 dilution is similarly prepared. A two-fold drug dilution scheme is used. The 10 times strength antibiotic solutions are dispensed in 100 µL volumes in round-bottom, polystyrene, sterile tubes and inoculated by adding 900 µL of the corresponding diluted fungus or yeast inoculum. The growth control tube received 900 µL of the inoculated suspension and 100 µL of the drug-free medium. A quality control organism is tested in the same manner and is included each time a test isolate is tested. 1 mL of uninoculated drug-free media is included as a sterility control.

The tubes are incubated at 35°C for 50 hours in ambient air and observed for the presence or absence of visible turbidity or growth. Where appropriate, incubation time is extended to 75 hours.

The minimum inhibitory concentration endpoint was determined visually by examining for turbidity and the minimum inhibitory concentration determined to be the lowest concentration of the test drug that substantially inhibited the growth of the organism. In general, this was the lowest concentration preventing any visible growth.
Alternatively, by visual examination, the MIC was defined as the lowest drug concentration reducing growth by 80% relative to the growth control.

Control organisms tested are *Candida albicans* ATCC90028 and *Candida albicans* ATCC90029, *Candida parapsilosis* ATCC90018, *Cryptococcus neoformans* ATCC90112 and ATCC90113, and *Torulopsis glabrata* ATCC90030. *Candida parapsilosis* ATCC22019 and *Candida krusei* ATCC6258 are also used as appropriate as reference strains.

A microbroth dilution method similar to the above but scaled down to microbroth dilutions is also used where convenient.

For testing filamentous fungi, the above method is altered as follows. The inoculum is prepared from growth on potato dextrose agar after 7 days at 35°C. A conidial suspension in 2 mL of sterile 85% saline is prepared and the turbidity of the suspension measured using a spectrophotometer at 530 nanometers and adjusted to provide a conidial suspension of 0.5-5.0 x 10^4 CFU/mL.

Daily examination of the tubes for growth is carried out, and when visible growth is seen, each tube is vortexed for 10 seconds to allow for the detection of small amounts of growth. Growth in tubes is compared to the sterile control and scored as either showing no growth, 75% reduction in growth, 50% reduction in growth, 25% reduction in growth, or no reduction in growth. The minimum inhibitory concentration for fungi (molds) is determined as the lowest drug concentration which inhibited 75% or more of the growth compared to the growth control.

Reference


Example 8

*Testing of Phenazine Compounds*

Several compounds were tested to determine the minimum inhibitory concentration against a variety of bacteria. The results are shown in Table 1.
Table 1:
Minimum Inhibitory Concentration (MIC) & Minimum Bactericidal Concentration (MBC) of Selected Bacterial Pathogens to 4 Phenazine Compounds

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Pyocyanin (P1) μg/ml</th>
<th>1-hydroxyphenazine (P2) μg/ml</th>
<th>Phenazine methosulfate (P3) μg/ml</th>
<th>Phenazine N, N-dioxide μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> 29213</td>
<td>18.8/37.5</td>
<td>6-12.5/&gt;60</td>
<td>37.5/&gt;300</td>
<td>&gt;166</td>
</tr>
<tr>
<td><em>S. aureus</em> 25923</td>
<td>30/150</td>
<td>15/&gt;60</td>
<td>37.5/&gt;300</td>
<td>94</td>
</tr>
<tr>
<td><em>S. aureus</em> (approximately 25 clinical strains)</td>
<td>30/nd</td>
<td>15/nd</td>
<td>60/nd</td>
<td>nd</td>
</tr>
<tr>
<td><em>E. coli</em> 25922</td>
<td>37.5/75</td>
<td>&gt;60/&gt;60</td>
<td>18.8/75</td>
<td>188</td>
</tr>
<tr>
<td><em>E. faecalis</em> 29212</td>
<td>25/&gt;300</td>
<td>25/&gt;60</td>
<td>75/&gt;300</td>
<td>&gt;125</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> 27853</td>
<td>&gt;300</td>
<td>&gt;60/&gt;60</td>
<td>300/&gt;300</td>
<td>188</td>
</tr>
<tr>
<td>Methicillin-resistant <em>S. aureus</em> (MRSA)</td>
<td>12/25</td>
<td>≤12.5/≤25</td>
<td>12.5-25/nd</td>
<td>≥166</td>
</tr>
<tr>
<td>Methicillin-resistant <em>S. epidermidis</em> (MRSE)</td>
<td>12/25</td>
<td>≤12.5/≤12.5</td>
<td>≤12.5/nd</td>
<td>166</td>
</tr>
<tr>
<td>Vancomycin-resistant <em>Enterococcus</em> (VRE)</td>
<td>≤6/50</td>
<td>12.5/25</td>
<td>50-100/≥200</td>
<td>nd</td>
</tr>
</tbody>
</table>

5 Results are presented as MIC/MBC
nd= No data

It can be seen from Table 1 that 1-hydroxyphenazine appears to be effective against several microorganisms, with an MIC against *S. aureus* of 6-12 μg/ml and an MIC against *Enterococci* of 12.5-25 μg/ml. 1-hydroxyphenazine is clearly bactericidal against *S. aureus*, as well as MRSA and multidrug-resistant *S. epidermidis*. Most significantly, 1-hydroxyphenazine is very active against vancomycin-resistant *Enterococci* (VRE) and MRSA, with an MIC of 12.5 μg/ml and a minimum bactericidal concentration of 25 μg/ml. These are 2 organisms which are very difficult to treat.
because of their resistance to beta-lactams, and in the case of VRE, resistance to vancomycin and related glycopeptides. MRSA, MRSE and VRE are increasing in frequency and therefore any drug with activity against these pathogens could see widespread applicability in human and veterinary medicine.

1-methoxyphenazine was tested in this assay and was found to be a relatively poor antimicrobial compound.

**Example 9**

Compounds P1-P6 were tested against a sample panel of microorganisms to determine the effect of additional substituents on the phenazine nucleus on the antibacterial spectrum.

The results are shown in Table 2

**Table 2:**

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>P1 µg/ml</th>
<th>P2 µg/ml</th>
<th>P3 µg/ml</th>
<th>P4 µg/ml</th>
<th>P5 µg/ml</th>
<th>P6 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus (methicillin-susceptible)</td>
<td>18-37</td>
<td>6-15</td>
<td>25</td>
<td>1.6</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>S. aureus (methicillin-resistant)</td>
<td>12</td>
<td>≤12</td>
<td>25</td>
<td>1.6-3.1</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>12</td>
<td>≤6-12</td>
<td>12.5</td>
<td>1.3</td>
<td>&lt;0.8</td>
<td>ND</td>
</tr>
<tr>
<td>E. faecalis (vancomycin-susceptible)</td>
<td>25</td>
<td>25</td>
<td>75-100</td>
<td>1.6</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>E. faecalis and E. faecium (vancomycin-resistant)</td>
<td>≤6-25</td>
<td>12-50</td>
<td>50-100</td>
<td>1.6-3.1</td>
<td>3.1</td>
<td>ND</td>
</tr>
<tr>
<td>P. aeruginosa 27853</td>
<td>&gt;300</td>
<td>&gt;60</td>
<td>300</td>
<td>&gt;100</td>
<td>12.5-25</td>
<td>12.5</td>
</tr>
<tr>
<td>E. coli</td>
<td>37.5</td>
<td>&gt;60</td>
<td>18.7</td>
<td>&lt;3.1</td>
<td>12.5</td>
<td>12.5</td>
</tr>
</tbody>
</table>

ND=No data
It can be seen from the data in Figure 2 that compounds P5 (7,8-dichloro-1-hydroxyphenazine-5,10-dioxide) and P6 (7,8-dimethyl-1-hydroxyphenazine-5,10-dioxide) are especially effective against the tested bacterial strains, and that in several cases P5 and P6 are as effective against drug-resistant strains as against the non-resistant wild-type organisms. Accordingly, P5 and P6 (or equivalents thereof, including 7,8-disubstituted phenazines) are particularly preferred compounds.

Example 10

Acute Toxicity Studies

Six mice were given compound P4, mixed with a polyethylene glycol carrier, in doses of 100 mg/kg, 500 mg/kg and 1000 mg/kg orally (two mice per group). The mice were observed for 48 hours. No acute toxicity was observed.

Another group of six mice were administered compound P4 mixed with polyethylene glycol in doses of 100 mg/kg, 500 mg/kg, and 1000 mg/kg by intraperitoneal injection (two mice per group). The mice were observed for 48 hours. No significant toxicity was observed at 100 mg/kg, but some toxicity was seen at 500 and 1000 mg/kg.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

The contents of all cited references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application (including the Background Section) are hereby expressly incorporated by reference.

Other embodiments are within the following claims.

What is claimed is:
1. A method for treating an infection due to a multidrug-resistant microorganism in a subject, the method comprising the step of administering to a subject in need thereof an effective amount of a phenazine compound, or a pharmaceutically-acceptable salt thereof, such that the infection is treated.

2. The method of claim 1, wherein the phenazine compound is a phenazine N-oxide.

3. The method of claim 2, wherein the phenazine N-oxide is a phenazine 5,10-dioxide.

4. The method of claim 3, wherein the phenazine N-oxide is a 7,8-disubstituted phenazine 5,10-dioxide.

5. The method of claim 1, wherein the multidrug resistant microorganism is a bacterium.


7. A method for treating an infection due to a microorganism in a subject, the method comprising the step of administering to a subject in need thereof an effective amount of a phenazine compound, or a pharmaceutically-acceptable salt thereof, such that the infection is treated, wherein the microorganism is selected from the group consisting of *Hemophilus influenzae*, *Hemophilus parainfluenzae*, *Hemophilus aphrophilus*, *Campylobacter* spp., *Helicobacter pylori*, *Vibrio* spp., *Streptococci* of the viridans group, *Cryptococcus* spp., *Histoplasma* spp., *Candida* spp., *Torulopsis* spp., *Blastomyces* spp., *Coccidioides* spp., *Nocardia* spp., *Actinomyces* spp., and *Aspergillus* spp., coagulase-negative *Staphylococcus* spp., and *Corynebacterium jeikeium*.

8. A method for treating an infection due to a microorganism in a subject, the method comprising the step of administering to a subject in need thereof an effective amount of a phenazine compound, or a pharmaceutically-acceptable salt thereof, such
that the infection is treated, wherein the phenazine compound is represented by the formula:

\[
\begin{align*}
& \text{R}_1 \quad (R_3)_m \quad X \\
& \text{R}_2 \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{N} \quad (R_4)_n \\
& \text{X}
\end{align*}
\]

in which

5. \( R_1 \) and \( R_2 \) are selected independently for each occurrence from the group consisting of hydrogen, halogen, lower alkyl, phenyl, hydroxy, lower alkoxy, mercapto, lower alkylthio, nitro, cyano, amino, lower alkylamino, dialkylamino, and trifluoromethyl;

10. \( R_3 \) and \( R_4 \) are selected independently for each occurrence from the group consisting of halogen, lower alkyl, hydroxy, lower alkoxy, mercapto, lower alkylthio, amino, lower alkylamino, dialkylamino, and trifluoromethyl;

15. \( m \) is 0, 1 or 2;

\( n \) is an integer from 0 to 4; and

\( X \) is, independently for each occurrence, oxygen or a pair of electrons.

9. The method of claim 8, wherein \( X \) is, for each occurrence, oxygen; and \( m \) and \( n \) are each 0.

10. The method of claim 9, wherein at least one of \( R_1 \) and \( R_2 \) is selected from the group consisting of methyl and halogen.

11. The method of claim 8, wherein the organism is a multidrug resistant organism.

12. The method of claim 11, wherein the organism is a bacterium.

13. The method of claim 11, wherein the organism is a fungus or a yeast.

14. A method for inhibiting the growth of a drug-resistant microorganism, the method comprising contacting the drug-resistant microorganism with an effective amount of a phenazine compound, such that growth of the drug-resistant microorganism is inhibited.
15. The method of claim 14, wherein the microorganism is a bacterium, and the phenazine compound has bacteriostatic activity against the bacterium.

16. The method of claim 14, wherein the microorganism is a bacterium, and the phenazine compound has bactericidal activity against the bacterium.

17. The method of claim 14, wherein the organism is a fungus or a yeast.

18. The method of claim 14, wherein the phenazine compound is a phenazine N-oxide.

19. The method of claim 18, wherein the phenazine compound is a phenazine 5,10-dioxide.

20. A method for targeting a drug-resistant microorganism in a subject for the purpose of treating an infection, the method comprising administering an effective amount of a phenazine compound to a subject in need thereof, such that the drug is targeted to a drug-resistant microorganism known or suspected to be present in the subject and the infection is treated.

21. The method of claim 20, wherein the phenazine compound also targets a non-drug resistant microorganism.

22. The method of claim 20, wherein the organism is a fungus or a yeast.

23. A method for significantly decreasing the infective ability of a drug-resistant microorganism, the method comprising contacting the drug-resistant microorganism with an effective amount of a phenazine compound, such that the infective ability of the drug-resistant microorganism is significantly decreased.

24. A packaged drug, comprising:
   a pharmaceutical composition including a phenazine compound in a container,
   packaged with
   instructions for administering the pharmaceutical composition to a subject for treating an infection due to a drug resistant microorganism in the subject.
25. A method for treating a subject having a tumor, the method comprising administering to the subject an effective anti-tumor amount of a phenazine compound, such that the tumor is treated.