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Novel Broad-Spectrum Bis-(Imidazolinylindole) Derivatives with Potent Antibacterial Activities against Antibiotic-Resistant Strains▽†

Rekha G. Panchal,[1]* Ricky L. Ulrich,[1] Douglas Lane,[2] Michelle M. Butler,[3] Chad Houseweart,[3] Timothy Opperman,[3] John D. Williams,[3] Norton P. Peet,[3] Donald T. Moir,[3] Tam Nguyen,[2] Rick Gussio,[4] Terry Bowlin,[3] and Sina Bavari[1]*

United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, Maryland 21702-5011[1]; Target Structure Based Drug Discovery Group, SAIC-Frederick, Inc., NCI-Frederick, Frederick, Maryland 21702-1201[2]; Microbiotix Inc., One Innovation Dr., Worcester, Massachusetts 01605[3]; and Target Structure Based Drug Discovery Group, Information Technology Branch, Developmental Therapeutic Program, National Cancer Institute, Frederick, Maryland 21702-1201[4]

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Given the limited number of structural classes of clinically available antimicrobial drugs, the discovery of antibacterials with novel chemical scaffolds is an important strategy in the development of effective therapeutics for both naturally occurring and engineered resistant strains of pathogenic bacteria. In this study, several diarylamidine derivatives were evaluated for their ability to protect macrophages from cell death following infection with *Bacillus anthracis*, a gram-positive spore-forming bacterium. Four bis-(imidazolinylindole) compounds were identified with potent antibacterial activity as measured by the protection of macrophages and by the inhibition of bacterial growth in vitro. These compounds were effective against a broad range of gram-positive and gram-negative bacterial species, including several antibiotic-resistant strains. Minor structural variations among the four compounds correlated with differences in their effects on bacterial macromolecular synthesis and mechanisms of resistance. In vivo studies revealed protection by two of the compounds of mice lethally infected with *B. anthracis*, *Staphylococcus aureus*, or *Yersinia pestis*. Taken together, these results indicate that the bis-(imidazolinylindole) compounds represent a new chemotype for the development of therapeutics for both gram-positive and gram-negative bacterial species as well as against antibiotic-resistant infections.

Bacillus anthracis is a serious bioterrorism threat because its spores are stable under extreme conditions in the environment, easily cultured and produced, easily distributed by aerosol (in a powder form), and highly fatal via inhalation, as dramatically demonstrated in 2001 (4, 16, 17). While *B. anthracis* cells are sensitive to several antibiotics (3, 13), naturally occurring or intentionally engineered drug resistance is a concern. Antibiotic resistance is also a growing problem in the clinic (10), and the recent increased prevalence of community-acquired methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) has added to the concern (11, 12). While effective new agents are in the pipeline, they are all new analogs of existing classes of antibiotics (22). The development of new antibiotics against unexploited targets with novel mechanisms of action is a vital part of the solution to these problems because such antibiotics are unlikely to be affected by preexisting target-based resistance alleles.

In order to explore potential new chemotypes of antibacterial agents with a variety of possible mechanisms of action, we developed a cell-based screen for rescue of macrophages from *B. anthracis*-mediated death and applied it to a focused library containing diarylamidine compounds. This class of compounds has been evaluated previously for antimicrobial properties (2), as well as for antiproteolytic, anticoagulant (23), and antiproliferative activity (5). In our study, four bis-(imidazolinylindole) compounds from the diarylamidine library exhibited very potent activity in the macrophage rescue screen. Mechanism of action studies indicated that these compounds are neither nonspecific inhibitors of macromolecular synthesis nor membrane active but are rapidly bactericidal inhibitors of a broad spectrum of bacterial species. Finally, we demonstrate activities of these inhibitors in animal models of infection.

MATERIALS AND METHODS

Small molecule library. A focused library containing ~70 compounds sharing a diarylamidine chemical scaffold was obtained from the National Cancer Institute and used for screening (see Table S1 in the supplemental material).

Bacterial strains. The different bacterial species and strains used in this study include *Bacillus anthracis* (Sterne), *Bacillus anthracis* (Ames), *Bacillus brevis*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus anthracis* (Vollum), *Bacillus subtilis*, *Bacillus pumilus*, *Staphylococcus aureus* NCTC8325, *S. aureus* (Smith strain), MRSA 1094, *Enterococcus faecalis* ATCC 29212, *Mycobacteria smegmatis* ATCC 19420, *M. smegmatis* ATCC 35798, *M. smegmatis* ATCC 700009, *Escherichia coli* J53, *Klebsiella pneumoniae* 5657, *Pseudomonas aeruginosa* PA01, *Yersinia pestis* CO92, *Y. pestis* KIM (Δ*pgm* pCD1$^-$), *Burkholderia mallei* ATCC 3344, *Burkholderia pseudomallei* DD503, *Burkholderia thailandensis*, and *Burkholderia cepacia*.

Cell-based infection assays. J774A.1 macrophages (6×10^5) were infected with *B. anthracis* Sterne spores at a multiplicity of infection (MOI) of 5, in the

* Corresponding author. Mailing address: United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD 21702-5011. Phone for Rekha G. Panchal: (301) 619-4985. Fax: (301) 619-2348. E-mail: rekha.panchal@amedd.army.mil. Phone for Sina Bavari: (301) 619-4246. Fax: (301) 619-2348. E-mail: sina.bavari@amedd.army.mil.

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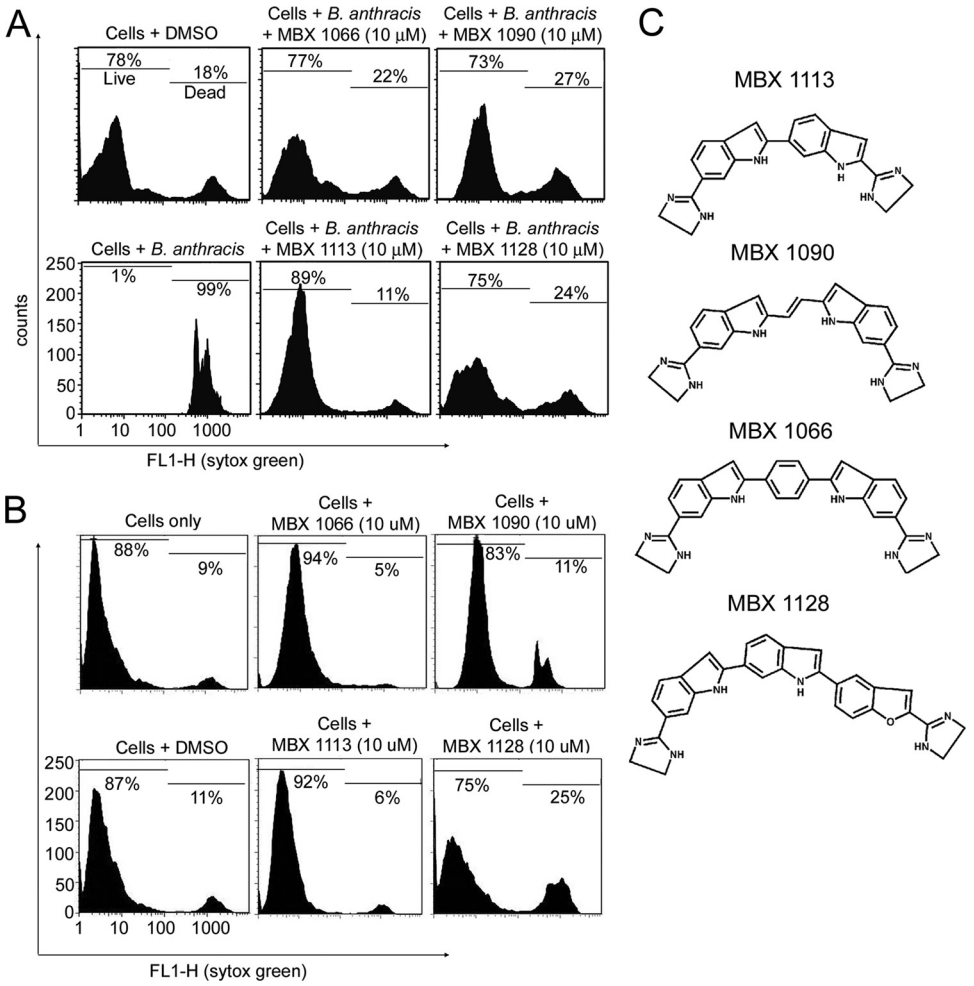

FIG. 1. Small molecules protect macrophages from *B. anthracis*-induced cell death. J774A.1 macrophages were treated with either DMSO (1%) control or compounds (10 μM) and infected with *B. anthracis* spores (MOI, 5) (A) or not infected (B). Cell viability was measured by uptake of Sytox green dye and analyzed by flow cytometry. The percentages of live and dead cells are indicated. (C) Chemical structures of the four most potent antimicrobial compounds.

presence of dimethyl sulfoxide (DMSO; 1%, as control) or test compounds (10 μM). After 4 h incubation at 37°C, bacterial growth was inhibited by the addition of the antibiotics penicillin (100 IU) and streptomycin (100 μg/ml). To determine cell viability, Sytox green dye that is impermeant to live cells was added and incubated for 15 min at 37°C. The cells were centrifuged at 2,000 rpm for 2 min and then washed two times with complete medium containing antibiotics. The cells were then analyzed by flow cytometry.

In vitro inhibition of bacterial growth. MICs were determined by the broth microdilution method (20). *B. anthracis* Sterne spores or bacterial cultures (5×10^5 CFU/ml) in log-phase growth were seeded in 96-well plates and treated with DMSO (1%) or compound at concentrations ranging from 0 to 20 μM. Plates were incubated at 37°C for 16 to 20 h, and cell growth determined by measuring the absorbance at 600 nm.

The minimal bactericidal concentration (MBC) was determined by a modification of the MIC method. Bacteria from MIC wells and 4 dilutions above the MIC were diluted, and bacteria were plated onto sheep blood agar plates. The next day, colonies were counted, and MBCs reflecting a 99.9% reduction in viable counts were determined.

Spore germination assay. Sterne spores (5×10^5 CFU/ml) were germinated in Mueller-Hinton broth in the presence of DMSO (control) or compounds (1× MIC). At time intervals of 0, 15, and 30 min, samples were heated at 70°C for 30 min to kill any germinated spores, and appropriate dilutions were plated onto sheep blood agar plates to quantify remaining viable spores.

Kinetics of bactericidality. To determine the kinetics of cidality, compounds were diluted in Mueller Hinton broth and tested at concentrations equivalent to 4× their respective MICs against *B. anthracis* Sterne spores (5×10^5 CFU/ml) or *B. anthracis* Sterne vegetative bacilli or an attenuated *Yersinia pestis* strain (KIM Δ*pgm* pCD1$^-$) (1×10^6 CFU/ml). The cultures were incubated and sampled at various time points (0, 1, 2, 4, 6, and 24 h), diluted appropriately into fresh medium, and then plated onto drug-free agar plates to determine the number of CFU/ml present in the sample (CFU/ml is number of colonies on the plate multiplied by the dilution factor and adjusted for a volume of 1 ml). An additional experiment was conducted, as described above, using *B. subtilis* BD54, MBX 1066, and the antibiotic ciprofloxacin, an inhibitor of bacterial DNA replication, at concentrations of 5× their MICs. The minimum level of detection in these experiments was 50 CFU/ml. The $\log_{10}$ value of CFU/ml was plotted versus time. Bactericidal activity is defined as a ≥3 log reduction in initial CFU count within 24 h.

Determination of mammalian cytotoxicity. Cytotoxicity of the compounds was measured as described previously, except that HeLa cells were used (9). Cytotoxicity was quantified as the CC_{50}, the concentration of compound that inhibited 50% of conversion of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] to formazan (18). The "selectivity index" is defined as the ratio of the mammalian cell cytotoxicity to the MIC against *B. anthracis* Ames (i.e., CC_{50}/MIC), both measured in the presence of 10% fetal calf serum. The addition of 10% fetal calf serum had no effect on the MICs.

Macromolecular synthesis assays. Compounds were examined at 5× MIC for inhibitory effects on bacterial DNA, RNA, protein, and cell wall biosynthesis in *B. subtilis* BD54, in 96-well polystyrene plates. Incorporation of the following

radiolabeled precursors into macromolecules was measured: [methyl-^{3}H]thymidine for DNA, [5-^{3}H]uridine for RNA, L-[4,5-^{3}H]leucine for protein, and *N*-acetyl-D-[1-^{3}H]glucosamine for cell wall synthesis. At time zero, compound- or pathway-specific control antibiotic (in DMSO) at a concentration of 5× its MIC and radioactive precursors were added to log phase cells. At various times, samples were collected from each 37°C culture, precipitated with an equal volume of ice-cold 20% trichloroacetic acid, collected onto 96-well glass fiber filter plates, dried, and counted for radioactivity. Plots of counts (cpm) incorporated versus incubation times were generated for each compound.

Membrane activity assays. The effect of compounds on bacterial membrane potential of *B. subtilis* BD54 was determined using a fluorescent assay essentially as described by Wu and Hancock (25). Briefly, *B. subtilis* BD54 cultures were grown to exponential phase (optical density at 600 nm, 0.4 to 0.5) in LB media. Cells were harvested, washed, and resuspended in wash buffer (5 mM HEPES [pH 7.4], 20 mM glucose) to an optical density at 600 nm of 0.1. The fluorescent dye $DiSC_3(5)$ was added to the cell suspension (final concentration, 1 μM) and incubated at room temperature for 10 min to allow dye uptake. KCl was added to a final concentration of 100 mM. The cell suspension was transferred to a 96-well assay plate (200 μl/well) containing control and test compounds dissolved in DMSO (final concentration, 2%). A total of eight wells were tested under each condition. Fluorescence intensity (RFU) was measured after 5 min, using a Molecular Devices SpectraMax fluorescence plate reader with an excitation wavelength of 622 nm and an emission wavelength of 670 nm. 2-4-Dinitrophenol (DNP), a protonophore, was used at a concentration of 200 μg/ml as a positive control. The average RFU and standard deviation for eight assay wells were calculated and are presented in Fig. 4F.

The effect of the compounds on mammalian cell membrane integrity was determined by measuring the release of lactate dehydrogenase (LDH) from HeLa cells treated with a concentration range of compounds. Briefly, HeLa cells were grown to confluence in Dulbecco's modified Eagle's medium and were treated for 1 h with 1% DMSO alone (untreated control), various concentrations of test compounds, and a control antibiotic (32× MIC for vancomycin). The final concentration of DMSO in all samples was 1%. LDH activity in the supernatant was measured using the CytoTox ONE homogenous membrane integrity assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Selection for resistant mutants. Eight independent cultures of *S. aureus* NCTC8325 were grown in 96-well assay plates in the presence of several concentrations of each of the compounds (0.125× to 128× MIC). Cultures were recovered from the well with highest compound concentration that exhibited robust growth (>50% of untreated control). This process was repeated for 20 days, and results were displayed as the highest sublethal compound concentration for each culture for each day. Colonies were isolated from apparently resistant cultures and confirmed to be resistant by MIC assays.

Animal studies. Eight- to 10-week-old C57BL/6 mice were used in this study. The dosing regimen was based on the MICs and on initial pilot studies varying dose and administration frequency. For in vivo *B. anthracis* studies, mice (n = 10/group) were challenged via intraperitoneal (i.p.) injection with ~300 CFU of *B. anthracis* Ames. After 6 h postchallenge, mice were treated via i.p. injection with vehicle control, MBX 1066 (5 or 10 mg/kg/injection), or MBX 1090 (0.2, 0.5, or 1.0 mg/kg/injection). Mice were treated every 6 hours for 5 days, and survival was monitored for up to 20 days. Compounds in this study were dissolved at a stock concentration of 50 mg/ml in DMSO and then diluted to an appropriate working concentration in 5% dextrose in water.

To determine the protective effect of the compounds during the late stages of infection, treatment with compound MBX 1066 (10 mg/kg/injection) via i.p. injection was initiated 6, 12, 18, or 24 h postchallenge. Thereafter, mice were treated every 6 hours for 5 days, and survival was monitored for up to 20 days. Compound MBX 1066 was dissolved at a stock concentration of 50 mg/ml in DMSO and then diluted to an appropriate working concentration in 5% dextrose in water.

To test the efficacy of the compounds in a *Y. pestis* infection model, C57BL/6 mice (n = 10/group) were challenged via i.p. injection with ~100 CFU of *Y. pestis* (strain CO92). After 6 h, mice were treated via i.p. injection with MBX 1066 (5 or 10 mg/kg/injection) and treatment continued every 6 hours for 5 days. Survival of the mice was monitored for 15 days.

To investigate the efficacy of the compounds administered via a different route from that of the pathogen challenge, Swiss Webster mice (n = 10/group) were challenged via i.p. injection with 8.3×10^8 CFU of *S. aureus* (Smith strain). After 15 min, mice were treated via intravenous (i.v.) injection with a single dose of MBX 1066 (10 mg/kg), MBX 1090 (10 mg/kg) prepared in 10% dimethyl acetamide-5% dextrose in water (pH 4.0), daptomycin control (10 mg/kg), or vehicle control (10% dimethyl acetamide-5% dextrose in water [pH 4.0]). Survival was monitored for 48 h.

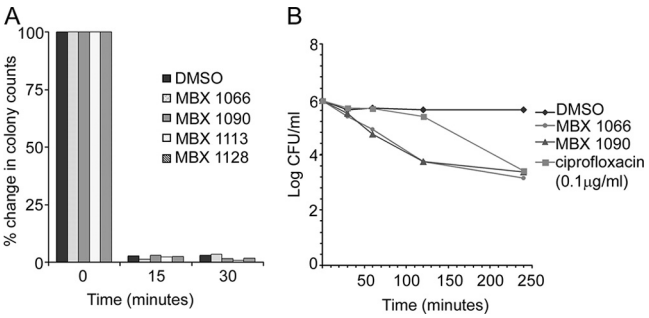

FIG. 2. Identified small molecules do not inhibit spore germination but kill germinated spores. (A) *B. anthracis* Sterne spores were treated with 1% DMSO (control), MBX 1066, MBX 1090, MBX 1113, and MBX 1128 (all compounds at 1× MIC). At the indicated time intervals, samples were heated and cooled, and appropriate dilutions were plated onto blood agar plates. The percentage of change in colony counts was plotted against time. (B) *B. anthracis* Sterne spores were treated with 1% DMSO (control), MBX 1066 (1× MIC), MBX 1090 (1× MIC), or ciprofloxacin (0.1 μg/ml), and at the indicated time intervals, appropriate dilutions of the samples were plated onto blood agar plates. The $\log_{10}$ value of CFU/ml was plotted versus time, as shown.

All research was conducted under an approved protocol and in compliance with the Animal Welfare Act and other federal statutes and regulations related to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facilities in which this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

RESULTS

Small molecules protect macrophages from *B. anthracis*-induced cell death. *B. anthracis*, a gram-positive spore-forming bacterium, produces a virulent lethal toxin that causes death of susceptible macrophages (14). A cell-based *B. anthracis* infection assay was used to test a focused library that contained diarylamidine derivatives (see Table S1 in the supplemental material for chemical structures). Macrophages were infected with *B. anthracis* spores (MOI of 5) in the presence of a DMSO control (1%) or the compounds (10 μM), and cell death was monitored by the uptake of membrane-impermeant Sytox green using flow cytometry. A number of compounds (28% hit rate) protected macrophages to various extents (≥50%) from *B. anthracis*-induced cell death. Representative results for the four most potent compounds (MBX 1066, MBX 1090, MBX 1113, and MBX 1128, also known as NSC 317881, NSC 317880, NSC 330687, NSC 369718, respectively, in Table S1 in the supplemental material) are shown in Fig. 1A and B. These potent inhibitors all share a bis-(imidazolinylindole) chemotype; their chemical structures are shown in Fig. 1C. The observed cellular protection also suggests that the compounds are relatively nontoxic to macrophages at the tested dose. In an independent experiment, in the absence of bacteria, only MBX 1128 caused more macrophage death than the DMSO control, and the effect was modest (Fig. 1B).

In vitro inhibition of bacterial growth. The protection of macrophages from cell death by the identified compounds suggests that they may be targeting bacterial growth or viability, bacterial virulence factors, or host factors vital to the bacteria.

TABLE 1. MICs and MBCs of the four most potent compounds against select gram-positive and gram-negative bacteria and antibiotic-resistant strains of selected species

Strain/species	MIC (MBC) (μg/ml)[b]				
	MBX 1066	MBX 1090	MBX 1113	MBX 1128	Ciprofloxacin
Gram-positive bacteria					
B. anthracis Ames	0.3 (0.7)	0.6 (2.3)	1.1 (2.2)	1.4 (2.8)	ND
B. brevis[a]	0.7	0.6	0.3	0.2	ND
B. licheniformis[a]	0.7	2.3	2.2	1.4	0.1
B. megaterium[a]	0.3	0.6	0.6	0.4	0.2
B. pumilus[a]	<0.1	0.2	0.1	0.4	ND
B. anthracis Vollum[a]	0.3 (ND)	0.6 (1.2)	0.6 (1.1)	0.4 (0.4)	ND
B. subtilis[a]	0.2	0.3	0.6	0.4	0.1
B. anthracis Ames spores	0.2 (0.4)	0.2 (1.6)	0.3 (0.5)	0.2 (1.2)	ND
S. aureus ATCC 25923	0.1	0.9	0.3	0.3	0.3
S. aureus	0.3	1.2	1.1	0.7	ND
E. faecalis[a]	0.2	0.6	2.2	0.7	ND
E. faecalis 29212	0.2	0.3	0.3	0.1	2.5
M. smegmatis 19420	<0.1	0.2	0.2	0.1	0.1
M. smegmatis 35798	<0.1	0.1	<0.1	<0.1	0.2
M. smegmatis 700009	0.1	0.2	0.3	<0.1	0.4
Ciprofloxacin-resistant *B. anthracis* Ames (strain 105-6)	0.2 (0.8)	0.4 (1.6)	0.2 (1.8)	4.9 (>10)	>100
MRSA[a]	0.3	2.3	2.2	2.8	ND
MRSA 1094	0.2	0.6	0.3	0.5	5.0
Vancomycin-resistant *E. faecium* B42762	<0.1	0.3	0.2	0.1	20
Vancomycin-resistant *E. faecalis* ATCC 51575	0.1	0.6	0.5	0.1	0.6
Gram-negative bacteria					
E. coli J53[a]	0.7	0.6	1.1	1.4	<0.1
K. pneumoniae 5657	3.8	1.3	0.4	16	0.2
P. aeruginosa PAO1	7.5	25	25	>80	0.1
Y. pestis	3.4	13	7.4	9.7	ND
B. mallei ATCC 23344	1.7	1.6	1.8	>9.7	ND
B. pseudomallei DD503	1.7	3.1	1.8	>9.7	ND
B. thailandensis[c]	21	19	18	2.8	ND
B. cepacia[a]	>20	19	4.4	22	ND

[a] Clinical isolate.
[b] MBCs are shown in parentheses. ND, not determined.
[c] Environment.

The cells infected with *B. anthracis* in the presence of the identified hit compounds did not show any changes in the pH of the medium, and microscopic examination of these samples showed little to no outgrowth of the bacteria. These results suggest that the compounds are acting as antibacterials. To investigate the antibacterial properties, all compounds from the focused library were tested in vitro for their ability to inhibit growth of *B. anthracis* Sterne spores and vegetative bacilli (see Table S1 in the supplemental material). The four most potent antibacterial compounds identified in the macrophage cell-based rescue assay were also potent bacterial growth inhibitors. Compounds MBX 1066, MBX 1090, MBX 1113, and MBX 1128 displayed MICs ranging from 0.2 to 1 μg/ml on both spores and vegetative bacilli.

Small molecules do not inhibit *B. anthracis* spore germination but rapidly kill bacteria. *B. anthracis* spores germinate within minutes following contact with a suitable medium (24), and their conversion into the vegetative form is essential for anthrax pathogenicity. Spore germination may be detected in vitro by alterations in the spore refractility, heat resistance, and staining. To evaluate the effects of the compound on spore germination, the four most potent compounds were selected for further study. As shown in Fig. 2A, a dramatic reduction in the CFU was seen as early as 15 min following treatment with the compounds, suggesting that the compounds did not affect spore germination.

Since these compounds had no effect on spore germination, but inhibited spore outgrowth, we examined the time-dependent killing of *B. anthracis* Sterne spores during germination and outgrowth. As shown in Fig. 2B, there was a greater than 2 log reduction in the initial CFU count after 4 hours of treatment with compound MBX 1066 or MBX 1090. A similar reduction was observed following treatment with ciprofloxacin (0.1 μg/ml).

Lead compounds exhibit broad-spectrum antibacterial activity. To determine if the compounds were capable of acting against a broad spectrum of gram-positive and gram-negative bacteria, the four most potent inhibitors, MBX 1066, MBX 1090, MBX 1113, and MBX 1128, were tested for their antibacterial activity against a diverse panel of bacterial species and strains. Table 1 demonstrates that these four compounds are active against a range of gram-positive (MICs, <0.1 to 4.9 μg/ml) and gram-negative (MICs, 0.4 to >80 μg/ml) bacteria. The compounds were also tested on selected antibiotic resistant bacteria. As shown in Table 1, all four compounds were active against ciprofloxacin-resistant *B. anthracis* Ames (MICs, 0.2 to 4.9 μg/ml), MRSA (MICs, 0.16 to 2.8 μg/ml), and vancomycin-resistant *Enterococcus* (MICs, <0.1 to 0.6 μg/ml).

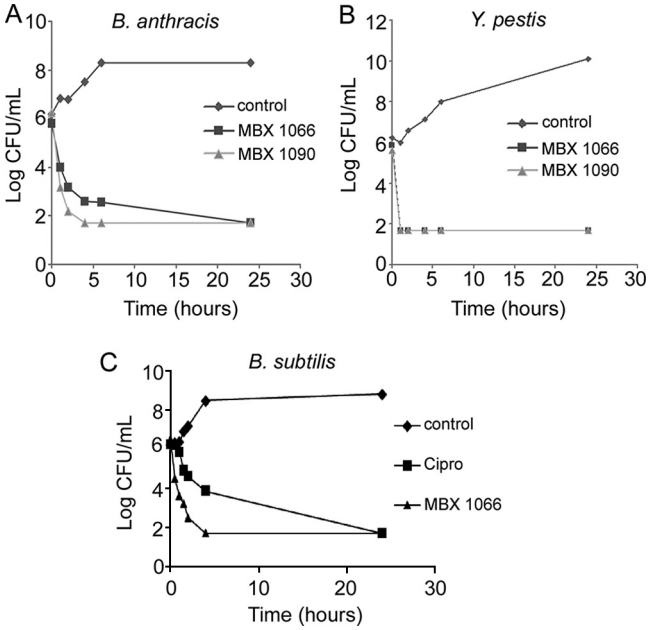

FIG. 3. Rapid cidality of lead compounds. Time kill assay for two MBX compounds tested at 4× their respective MICs against the *B. anthracis* Sterne strain (A) or the *Y. pestis* strain KIM Δ*pgm*, pCD1$^-$ (B). Bacteria were incubated in the presence of compounds at 4× the known MIC and sampled at the indicated time points. The $\log_{10}$ value of CFU/ml was plotted versus time, as shown. (C) *B. subtilis* BD54 was incubated in the presence of ciprofloxacin (Cipro) or MBX 1066 at concentrations of 5× their MICs and sampled at the indicated time points.

Thus, the identified compounds have broad-spectrum potent antibacterial activity and are more effective than many antibiotics in current use.

Minimum bactericidal activities of the most potent antibacterial compounds. To determine if the identified bioactive compounds were bactericidal, the MBCs of MBX 1066, MBX 1090, MBX 1113, and MBX 1128 against several strains of gram-positive bacteria were determined. As shown in Table 1, three of the compounds (MBX 1066, 1090, 1113) demonstrated potent bactericidal (MBCs, 0.8 to 1.8 μg/ml) activity against ciprofloxacin-resistant Ames *B. anthracis*.

To further evaluate the time course of bactericidality of the compounds, *B. anthracis* vegetative bacilli (Sterne strain) and attenuated *Y. pestis* (strain KIM Δ*pgm* pCD1$^-$) (10^6 CFU/ml) were incubated in Mueller-Hinton broth in the presence of MBX 1066 or MBX 1090 at 4× MIC concentrations over a time course of 24 h. As shown in Fig. 3A and B, both MBX 1066 and MBX 1090 were rapidly bactericidal (1 to 4 h) to both *Y. pestis* and *B. anthracis* at concentrations of 4× MIC, generating a reduction in CFU of ≥3 log within 6 h.

Lead compounds exhibit favorable selectivity indices. The cytotoxicity of the compounds was determined using HeLa cells in a 3-day incubation assay. The results, shown in Table 2, confirm that little or no cytotoxicity would be expected at the screening concentration (10 μM or ~6 μg/ml). However, some cytotoxicity is observed in this more stringent assay, with CC_{50} values of <50 μg/ml for all four compounds. When evaluated in relation to the very potent antibacterial activity versus *B. anthracis* Ames, the selectivity indices (CC_{50}/MIC) for three of the compounds are >10, suggesting some preference for bacterial versus mammalian cells in the mode of action of these compounds.

Lead compounds inhibit DNA synthesis. To identify the molecular mechanism of bacterial growth inhibition, MBX 1066, MBX 1090, MBX 1113, and MBX 1128 were examined at 5× MIC for their inhibitory effects on bacterial DNA, RNA, protein, and cell wall biosynthesis in *B. subtilis* BD54. The time course of bacterial killing, growth, and incorporation of radiolabeled precursors into macromolecules of cells incubated in the presence or absence of the compounds MBX 1066 and MBX 1090 is shown in Fig. 3C and Fig. 4A to E. Control antibiotics ciprofloxacin, rifampin (rifampicin), chloramphenicol, and vancomycin displayed potent and specific inhibition of incorporation of radiolabeled precursors into their target macromolecules DNA, RNA, protein, and cell wall, respectively. Cells appeared to double only once in the presence of MBX 1066 and MBX 1090, and both compounds inhibited DNA synthesis (Fig. 4A to E). Compounds MBX 1113 and MBX 1128 also inhibited DNA synthesis but in addition inhibited RNA and cell wall synthesis to about 40% of control values, although protein synthesis was less potently affected (not shown). The somewhat broader inhibitory activity of MBX 1113 and MBX 1128 suggests that they are less specific than are MBX 1066 and MBX 1090.

Lead compounds are not membrane active. The effect of MBX 1066 and MBX 1090 on the membrane potential of *B. subtilis* BD54 was investigated using $DiSC_3(5)$, a membrane potential-sensitive fluorescent dye. As $DiSC_3(5)$ is taken up by cells with intact membrane potential, the fluorescence decreases due to quenching. However, disruption of the membrane potential (or integrity) results in release of $DiSC_3(5)$ into the buffer, which can be detected due to an increase in fluorescence intensity. As shown in Fig. 4F, the untreated cells exhibited low fluorescence signal, whereas cells treated with DNP, a compound that disrupts the membrane potential, produced a significantly higher signal. Significantly, treatment with MBX 1066 or MBX 1090 at concentrations up to 10× MIC did not result in increased fluorescence, indicating that these compounds do not perturb bacterial membranes.

The effect of three of the compounds on mammalian membrane integrity was measured by examining the degree of cell lysis after incubation with the compounds (see Materials and Methods). Exposure of HeLa cells to MBX 1066, MBX 1090, and MBX 1113 at concentrations equivalent to 32× MIC (*S. aureus*, 9.6 μg/ml) did not result in the release of LDH at levels

TABLE 2. Cytotoxicity assessment of compounds against HeLa cells in a 3-day exposure

Compound	CC_{50} (μg/ml) with HeLa cells	*B. anthracis* Ames with MIC (μg/ml) of:	Selectivity index (CC_{50}/MIC)[a]
MBX 1066	33	0.3	110
MBX 1090	10	0.6	17
MBX 1113	3	1.1	2.7
MBX 1128	17	1.4	12

[a] CC_{50} values are divided by MICs for *B. anthracis* Ames in order to determine the selectivity index.

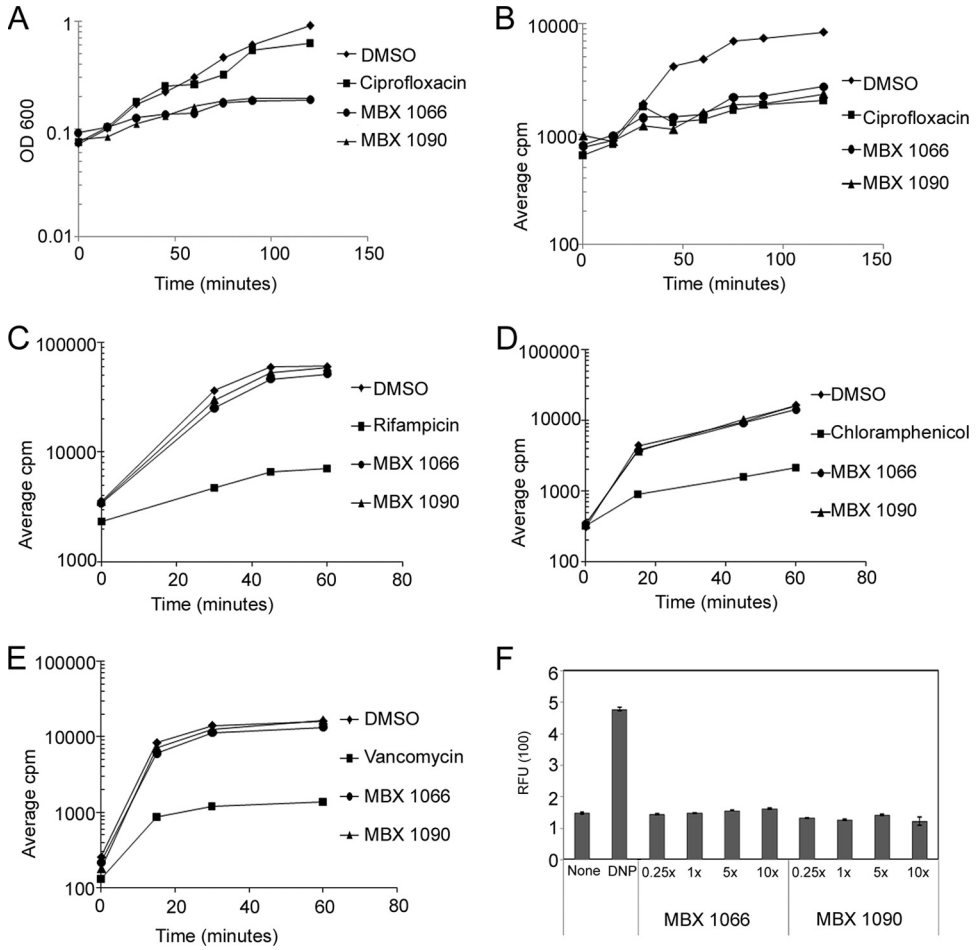

FIG. 4. Mechanism studies of inhibitors MBX 1066 and MBX 1090. (A) Effect of compounds on growth of *B. subtilis* BD54. Assays were performed in the absence or presence of compounds at 5× their MICs. OD600, optical density at 600 nm. (B to E) Effect of compounds on macromolecular biosynthesis in *B. subtilis* BD54. Assays were performed using radiolabeled precursors and *B. subtilis* BD54 as described in Materials and Methods with compounds MBX 1066 and MBX 1090 and appropriate control antibiotics present at 5× their MICs as indicated. Macromolecular pathways and control antibiotics are as follows: DNA synthesis, ciprofloxacin control (B); RNA synthesis, rifampin control (C); protein synthesis, chloramphenicol control (D); cell wall synthesis, vancomycin control (E). (F) Effect of compounds on bacterial membrane permeability. Compounds were incubated with *B. subtilis* BD54 at various concentrations (0.25×, 1×, 5×, and 10× their MICs) in the presence of $DiSC_3(5)$ dye. Control is DNP.

significantly different from those of the control samples treated with no antibiotic or with vancomycin concentrations up to 32× MIC (32 μg/ml) (data not shown). The results of this experiment indicate that neither MBX 1066 nor MBX 1090 disrupts membranes of HeLa cells at high concentrations.

Selection of mutants resistant to bis-(imidazolinylindole) compounds. To assess the capability of cells to become resistant to the antibacterial effects of these compounds, we attempted to identify resistant mutants. Initial attempts to select directly for colonies with spontaneous mutations to resistance on agar plates containing MBX 1066 were unsuccessful. Therefore, mutations with decreased susceptibility to MBX 1066, MBX 1090, and MBX 1113 were selected in liquid media in serial passage experiments. After 20 days of growth in sublethal concentrations of MBX 1066 and MBX 1113, mutants with significant increase in resistance to these compounds (>4× MIC) were not isolated (Fig. 5). In contrast, mutants able to grow in concentrations up to 16× MIC of MBX 1090 appeared within 10 to 12 days of culture.

Individual clones from each of the resistant populations were isolated, and MICs for MBX 1090 and MBX 1066 were determined in order to confirm decreased susceptibility to MBX 1090 and to test for cross-resistance to MBX 1066. MICs for six independent MBX 1090-resistant mutants against MBX 1090 were 16- to 32-fold higher (16 to 32 μg/ml) than for the parent strain (WT), confirming reduced susceptibility of the mutants. Interestingly, these mutants were not cross resistant to MBX 1066, indicating that the resistance mutations are specific for MBX 1090 (data not shown). This result indicates that MBX 1090 and MBX 1066 have distinct mechanisms of resistance.

Lead compounds protect mice in *B. anthracis* and *Y. pestis* infection models. To evaluate their in vivo efficacy, the compounds MBX 1066 and MBX 1090 were tested in *B. anthracis*

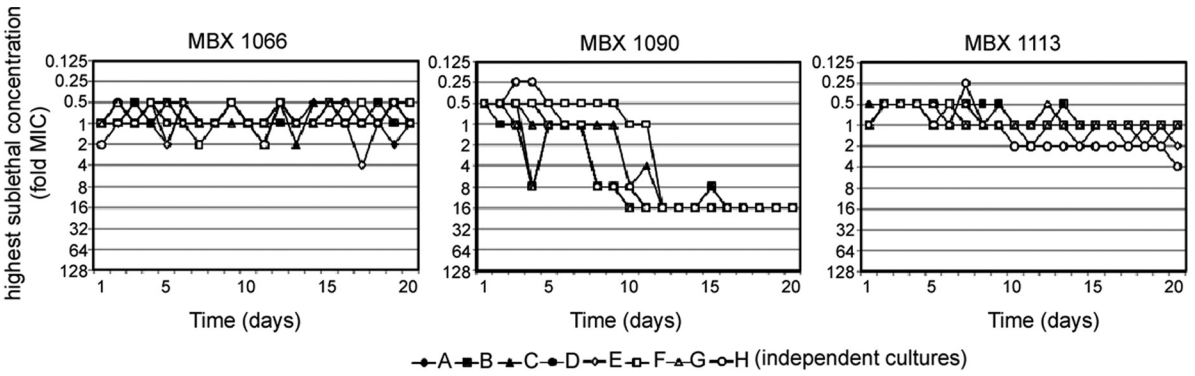

FIG. 5. Serial passage of *S. aureus* NCTC 8325 in MBX 1066, MBX 1090, and MBX 1113 selecting for increased resistance. The highest sublethal concentration of compound (fold MIC) is plotted versus the number of days of serial passage for eight independent cultures (A to H) for each compound.

and *Y. pestis* lethal infection mouse models. In *B. anthracis* studies, the C57BL/6 control mice were highly susceptible to *B. anthracis* infection, with death being observed as early as 48 h following challenge and 100% mortality within 4 to 5 days (Fig. 6A to C). In mice treated with the compound MBX 1066 (Fig. 6A) or MBX 1090 (Fig. 6B), 100% survival was observed at the highest concentration of the compounds tested (10 mg/kg/injection and 1 mg/kg/injection, respectively). To determine the protective effect of compounds at late stages of infection, mice were infected with *B. anthracis* Ames spores, and after 6, 12, 18, and 24 h postinfection, treatment with MBX 1066 was initiated. Protective effects of the compound MBX 1066 were observed as late as 18 to 24 h postinfection (Fig. 6C). In *Y. pestis* studies, a 90% protective effect was observed in the mice treated with the higher dose (10 mg/kg/injection) of the compound MBX 1066 (Fig. 6D). Thus, these studies indicate that MBX 1066 and MBX 1090 exhibit potent in vivo antibacterial activity against representative gram-positive and gram-negative bacteria.

Intravenous administration of the compounds protects mice from *Staphylococcus aureus* infection. To begin to investigate the efficacy of the compounds via an i.v. administration route, Swiss Webster mice were infected via i.p. injection with *S. aureus* (Smith strain), and after 15 min, treatment was initiated. A single i.v. injection of the compounds protected at least 80% of the mice from death due to *S. aureus* infection (Fig. 6E). These results indicate that i.v. administration of the compounds could be considered an alternative administration approach for treatment with these antibacterial compounds.

DISCUSSION

The development of resistance to clinically important antibiotics in bacterial pathogens and potential biowarfare agents poses a major threat to public health (1, 6, 19, 21). Furthermore, it is clear that resistance is more likely when newly introduced antibiotics are chemically similar to those that are already ineffective. Therefore, new antimicrobial compounds, possessing novel scaffolds and unique mechanisms of action, are urgently needed to combat this growing incidence of antibacterial-resistant strains in the clinic (21).

In this study we describe the identification and initial characterization of novel bis-(imidazolinylindole) compounds with potent antibacterial activities. Four potent inhibitors, MBX 1066, MBX 1090, MBX 1113, and MBX 1128, were identified in a cell-based *B. anthracis* infection assay of a focused library of diarylamidines. One member of this set, MBX 1090, has been reported previously to display antibacterial activity (2), but no further investigations of mechanism or activity in animal infection models have been described. In this report, we describe in vivo studies demonstrating that two of the most potent compounds could protect mice following challenge with *B. anthracis*, a gram-positive spore-forming bacterium; *Staphylococcus aureus*, a gram-positive coccus; and *Y. pestis*, a gram-negative ovoid bacillus that is a facultative intracellular organism.

The results described in this study offer some clues regarding the mechanism of action of these compounds. They are potent inhibitors of DNA synthesis (Fig. 4B). While the molecular target of these compounds is not known, the fact that they share some structural features with compounds that bind in the minor grove of duplex DNA (7) suggests that these compounds may inhibit DNA synthesis by binding to DNA. However, the efficacy of MBX 1066 and MBX 1090 in live animal models of infection, together with favorable selectivity indices in vitro, suggests that these compounds prefer bacterial DNA over mammalian DNA. Similar moderate levels of species selectivity have been observed previously for other compounds with a likely DNA binding mode of action, possibly resulting from a preference for AT-rich DNA (8, 15). While preliminary experiments indicate that MBX 1066 and MBX 1090 also prefer AT-rich DNA (unpublished observations), it is unclear whether further optimization of the structures can provide sufficient selectivity for clinical studies.

In contrast to MBX 1066 and MBX 1090, the other two compounds, MBX 1113 and MBX 1128, exhibit some inhibition of RNA and cell wall biosynthesis as well as DNA synthesis. While all four compounds exhibit detectable cytotoxicity in a stringent 3-day incubation with HeLa cells, compared to their antibacterial activity, MBX 1066 and MBX 1090 exhibit higher selectivity indices (CC_{50}/MIC, >15) than do the other two compounds. These observed differences in macromolecular synthesis specificity and selectivity indices could be related to structural differences between these two pairs of compounds.

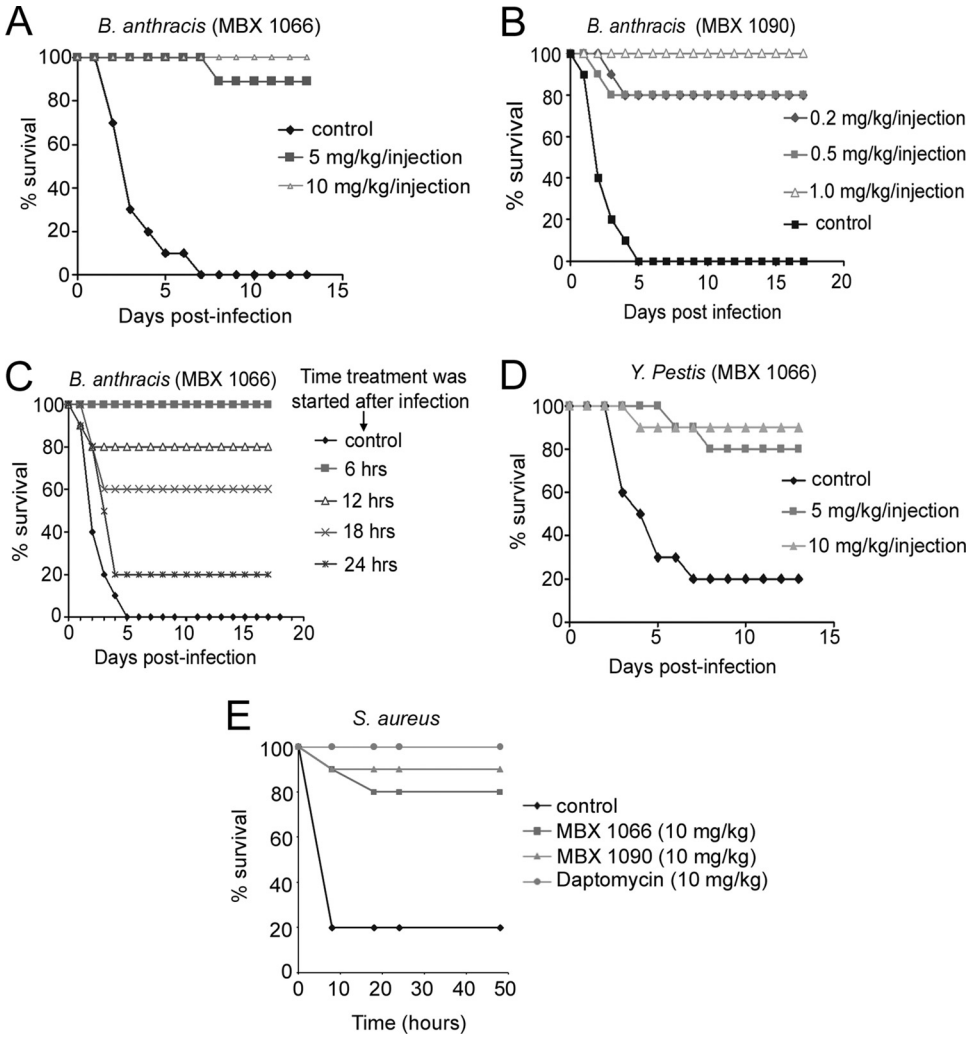

FIG. 6. Identified lead compounds protect mice against *B. anthracis*, *Y. pestis*, and *S. aureus* infection. C57BL/6 mice ($n = 10$) were infected via i.p. route with *B. anthracis* Ames spores (A to C) or *Y. pestis* CO92 strain (D), or Swiss Webster mice ($n = 10$) were infected i.p. with *S. aureus* (Smith) (E). (A) At 6 h postchallenge, the mice were treated with DMSO control (1%) or the indicated concentrations of MBX 1066. (B) At 6 h postchallenge, the mice were treated with DMSO control (1%) or the indicated concentrations of MBX 1090. (C) Treatment with the compound MBX 1066 (10 mg/kg/injection) was initiated at 6, 12, 18, and 24 h postchallenge. (D) At 6 h postchallenge with *Y. pestis*, mice were treated with DMSO control (1%) or the indicated concentrations of MBX 1066. In all studies, treatment with compound was every 6 h for 5 days. (E) At 15 min postchallenge, mice were treated i.v. with the indicated doses of MBX 1066, MBX 1090, daptomycin, or vehicle control. Survival of mice was monitored for 48 h.

The indole groups of MBX 1066 and MBX 1090 face each other in a symmetrical fashion, while they are positioned in a tandem arrangement in the other two compounds. Further studies will be required to determine if this is an important feature for selective antibacterial activity. Attempts to select mutants resistant to three of the compounds (MBX 1066, MBX 1090, and MBX 1113) were successful only for MBX 1090. Furthermore, the MBX 1090-resistant mutants were not cross-resistant to MBX 1066, indicating that these two related compounds do not share this mechanism of resistance. In summary, the indications that MBX 1066 protects mice from lethal infections with *B. anthracis*, *Y. pestis*, and *S. aureus* and does not readily select resistant mutations suggest that it represents a new antibacterial chemotype worthy of further exploration for use against drug-resistant bacterial pathogens.

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