

Testing Nucleoside Analogues as Inhibitors of *Bacillus anthracis* Spore Germination *In Vitro* and in Macrophage Cell Culture[∇]

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Bacillus anthracis, the etiological agent of anthrax, has a dormant stage in its life cycle known as the endospore. When conditions become favorable, spores germinate and transform into vegetative bacteria. In inhalational anthrax, the most fatal manifestation of the disease, spores enter the organism through the respiratory tract and germinate in phagosomes of alveolar macrophages. Germinated cells can then produce toxins and establish infection. Thus, germination is a crucial step for the initiation of pathogenesis. *B. anthracis* spore germination is activated by a wide variety of amino acids and purine nucleosides. Inosine and L-alanine are the two most potent nutrient germinants *in vitro*. Recent studies have shown that germination can be hindered by isomers or structural analogues of germinants. 6-Thioguanosine (6-TG), a guanosine analogue, is able to inhibit germination and prevent *B. anthracis* toxin-mediated necrosis in murine macrophages. In this study, we screened 46 different nucleoside analogues as activators or inhibitors of *B. anthracis* spore germination *in vitro*. These compounds were also tested for their ability to protect the macrophage cell line J774a.1 from *B. anthracis* cytotoxicity. Structure-activity relationship analysis of activators and inhibitors clarified the binding mechanisms of nucleosides to *B. anthracis* spores. In contrast, no structure-activity relationships were apparent for compounds that protected macrophages from *B. anthracis*-mediated killing. However, multiple inhibitors additively protected macrophages from *B. anthracis*.

Anthrax is an often-fatal, acute infectious disease caused by the rod-shaped, Gram-positive, endospore-forming bacterium *Bacillus anthracis* (17). The highly resistant *Bacillus anthracis* spores remain dormant in soil, occasionally entering mammals through skin abrasions, ingestion, or inhalation (27). The severity of *B. anthracis* pathogenicity is dependent on the uptake route. In inhalational anthrax, the most deadly form of the disease, spores enter the host organism through the respiratory tract (27). Once inside the lungs, spores are phagocytosed by alveolar macrophages and germinate inside the phagosome (15, 16). The germinated spores become metabolically active and secrete anthrax toxins, the bacterium's primary virulence factors (3, 4, 6). Vegetative bacilli rapidly proliferate within the lymphatic system and disseminate throughout the bloodstream (18). The continuous secretion of toxins leads to fatal septicemia.

Although spore germination is a critical step in the establishment of anthrax infection (18), very little is known about the signaling pathways involved in spore germination (28, 32). The first step in the germination process is most commonly the binding of metabolites by germination (Ger) receptors (8, 23, 38). These receptors are membrane proteins mostly encoded by tricistronic operons. Up to seven Ger receptors have been characterized in *B. anthracis* (13). Combinations of Ger receptors may be involved in different interacting pathways for germination (13, 30). Usually a purine and an amino acid are required for the efficient

germination *in vitro* of *B. anthracis* spores (2, 23, 37). Once germination is activated, a series of degradative events break up spore-specific structures and proteins (24, 29, 34). Germination is followed by a period of outgrowth, during which actively dividing cells are regenerated (19, 20, 22).

It has been observed that *Bacillus* and *Clostridium* spore germination can be blocked by alcohols (11, 36), ion channel blockers (26), protease inhibitors (9), sulfhydryl reagents (14), and other miscellaneous compounds (10). Most of these studies targeted specific germination pathways in different organisms and are not directly comparable. A more recent study tested the activities of subsets of the different types of compounds against *Bacillus subtilis* and *Bacillus megaterium* germination (10). Research from many groups, including ours, has shown that nucleoside and amino acid analogues act as competitive inhibitors of *B. anthracis* spore germination *in vitro* (2, 21, 25). Of these inhibitors, D-alanine (D-Ala) and D-histidine (amino acid analogues) and 6-thioguanosine (6-TG; a nucleoside analogue) were shown to also protect macrophages from *B. anthracis*-mediated necrosis (2, 21, 25).

In this study, we screened 46 different nucleoside analogues for their abilities to inhibit *B. anthracis* spore germination *in vitro* and in macrophage cultures. Structure-activity relationship analysis allowed identification of epitopes necessary for nucleoside recognition by *B. anthracis* spores. However, we found no correlation between *in vitro* germination inhibition and the ability of nucleosides to protect macrophages from *B. anthracis* cytotoxicity. We also showed that a nucleoside analogue (6-TG) and an amino acid analogue (D-Ala) combined to increase macrophage protection from *B. anthracis* cytotoxicity.

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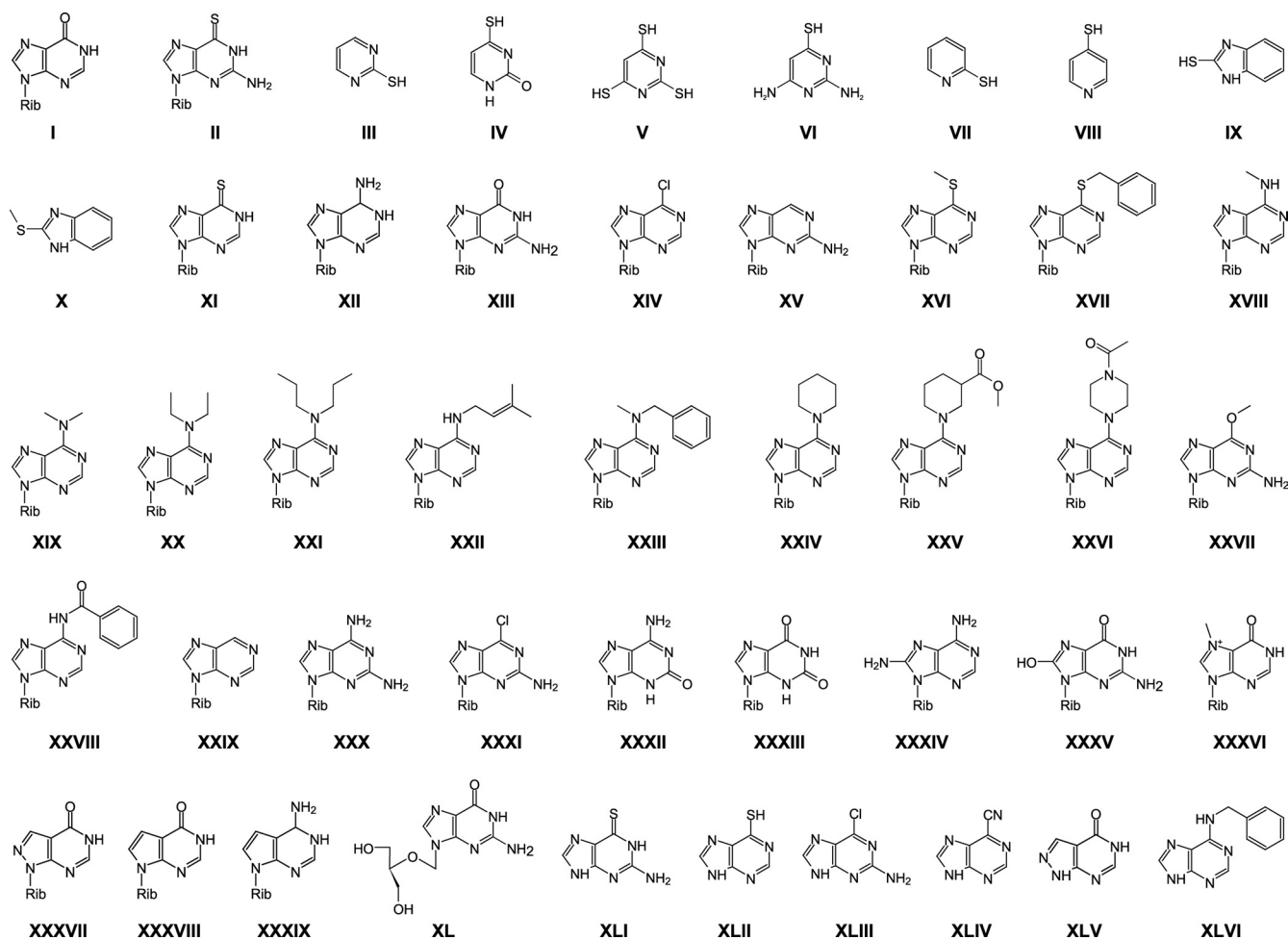


FIG. 1. Compounds tested as *B. anthracis* spore germination inhibitors *in vitro* and in cell culture (with the compound number shown in roman numerals in parentheses): INO (I), 6-TG (II), 2-mercaptopyrimidine (III), 2-thiouracil (2-TU; IV), trithiocyanuric acid (TTCA; V), 2,4-diamino-6-mercaptopyrimidine (DAMPy; VI), 2-mercaptopyridine (VII), 4-mercaptopyridine (VIII), 2-mercaptobenzimidazole (2-MBI; IX), 2-methylmercaptobenzimidazole (2-MMBI; X), 6-TI (XI), ADE (XII), GUA (XIII), 6-CPR (XIV), 2-APR (XV), 6-MMPR (XVI), 6-BTI (XVII), 6-methylaminopurine riboside (6-MAPR; XVIII), 6-*N*-dimethyladenosine (6-DMA; XIX), 6-DEA (XX), 6-DPA (XXI), 6-DMAA (XXII), 6-BMA (XXIII), 6-PPA (XXIV), 6-ECA (XXV), 6-PPER (XXVI), 6-OMG (XXVII), 6-NBA (XXVIII), NEB (XXIX), 2-AADE (XXX), 6-CG (XXXI), ISO (XXXII), XAN (XXXIII), 8-AADE (XXXIV), 8-HG (XXXV), 7-MI (XXXVI), APR (XXXVII), IH (XXXVIII), TU (XXXIX), GCV (XL), 6-Tg (XLI), 6-Mp (XLII), 6-Cg (XLIII), 6-cyanopurine (6-CYp; XLIV), allopurinol (Ap, XLV), 6-BAp (XLVI).

MATERIALS AND METHODS

Cell lines, reagents, and equipment. Murine macrophage J774A.1 cells were a generous gift from Jürgen Brojatsch (Albert Einstein College of Medicine, NY). *B. anthracis* Sterne 34F2 strain was a generous gift from Arturo Casadevall (Albert Einstein College of Medicine, NY). Immunicillin H (IH; compound XXXVIII) was a generous gift from Vern Schramm (Albert Einstein College of Medicine, NY). Nucleoside analogues of 6-benzylthioinosine (6-BTI; compound XVII), 6-*N*-diethyladenosine (6-DEA; XX), 6-*N*-dipropyladenosine (6-DPA; XXI), 6-*N*-benzylmethyladenosine (6-BMA; XXIII), 6-*N*-piperidineadenosine (6-PPA; XXIV), 6-*N*-((3-ethylacetyl)cyclohexyl) adenosine (6-ECA; XXV), and ribofuranosyl-((9*H*-purin-6-yl)piperazin-1-yl)ethanone (6-PPER; XXVI) were synthesized by Kyungae Lee from NSRB. Other compounds were purchased from Sigma-Aldrich Corporation (St. Louis, MO) or Berry & Associates Inc. (Brea, CA) (Fig. 1). *In vitro* spore germination and macrophage viability were monitored in a Tecan Infinite M200 multimode microplate reader.

Synthesis of 6-BTI (compound XVII), 6-DEA (XX), 6-DPA (XXI), 6-BMA (XXIII), 6-PPA (XXIV), 6-ECA (XXV), and 6-PPER (XXVI). N-6- and S-6-substituted purine ribosides were synthesized by substituting 6-chloropurine riboside with various amines or thiols in ethanol or dimethylformamide (DMF), as previously reported (35). In brief, a mixture of 6-chloropurine riboside (1 eq), the corresponding amine or thiol (3 eq), and triethylamine (72 μ l; 0.51 mmol) in

ethanol or DMF was heated at 60 to 90°C for 18 h and cooled to room temperature. After evaporation of the solvent, the residue was dissolved in methanol (1 ml). Upon standing overnight, crystals of the desired product were formed, and these were collected by filtration (50 to 88% yield). Compounds were characterized by ¹H nuclear magnetic resonance and mass spectrometry.

***B. anthracis* spore preparation.** *B. anthracis* cells were plated in nutrient agar (EMD Chemicals Inc.) and incubated at 37°C to yield single cell clones. Individual *B. anthracis* colonies were grown in nutrient broth and replated to obtain bacterial lawns. Plates were incubated for 5 days at 37°C. The resulting bacterial lawns were collected by flooding with ice-cold deionized water. Spores were pelleted by centrifugation and resuspended in fresh deionized water. After two washing steps, spores were separated from vegetative and partially sporulated cells by centrifugation through a 20%-to-50% HistoDenz gradient (1). Spores were resuspended in water and washed three times before storage at 4°C. Spores in all preparations were more than 95% pure as determined by microscopic observation of Schaeffer-Fulton-stained aliquots. Spore viability was assessed by heat treatment followed by serial dilution plating in nutrient agar. Spore viability was retested every 15 to 20 days.

Activation of *B. anthracis* spore germination. *B. anthracis* spore germination was monitored spectrophotometrically whereby the loss in light diffraction following addition of a germinant was reflected by a decreased optical density at 580

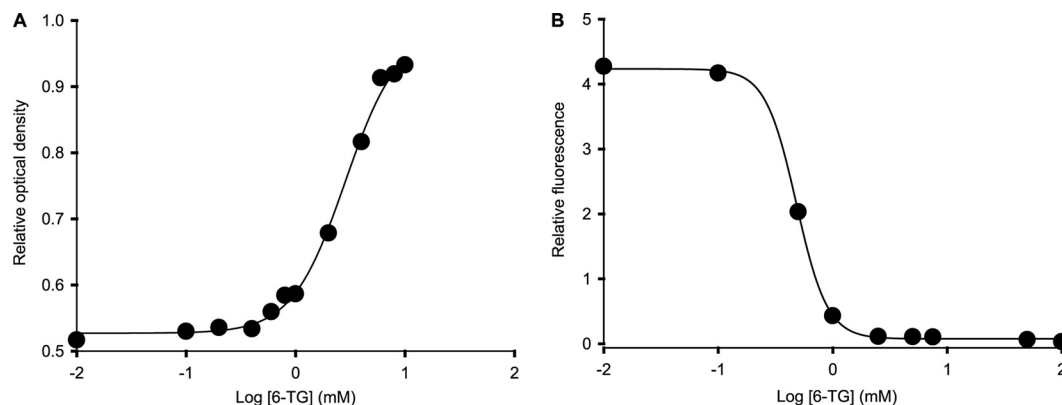


FIG. 2. (A) *B. anthracis* spores were treated with different concentrations of 6-TG, and germination was monitored by decreases in the optical density at 580 nm. The IC_{50} for 6-TG (1 mM) was determined based on these data. (B) Macrophages were treated with *B. anthracis* spores and different concentrations of 6-TG. Macrophage killing was monitored based on increases of PI fluorescence intensity.

nm (OD_{580}). All germination experiments were carried out in a Tecan Infinite M200 multimode microplate reader in UV-visible mode with its monochromator set at 580 nm. The final volume of each reaction mixture was 0.2 ml. Experiments were carried out in triplicate on at least two different days with two different spore preparations. Standard deviations of germination rates were calculated from these six independent assays. Spores were heat activated at 70°C for 30 min before resuspension in germination buffer (50 mM Tris-HCl [pH 7.5], 10 mM NaCl) to an OD_{580} of 1. The spore suspension was monitored for autogermination based on the OD_{580} after 1 h. No autogermination was detected in any sample used. Lack of autogermination was confirmed by phase-contrast microscopy on selected samples. Spore suspensions were individually supplemented with 250 μ M concentrations of compounds I to XLVI and 40 μ M L-alanine to test for compounds that activate *B. anthracis* spore germination. Germination was monitored by following the decrease in OD_{580} for 60 min. Optical densities were normalized by dividing each data point by the absorbance at the time of analogue addition and were reported as the relative OD_{580} . Germination rates were calculated from the initial linear region of the germination curves (2). Germination rates were set to 100% for spores treated with 250 μ M inosine and 40 μ M L-alanine. These inosine and L-alanine concentrations represent the lowest concentrations that induce the maximal germination rate. Percent germination for other nucleosides was calculated as the fraction of germination rate compared to these conditions. Germination was confirmed in selected samples by Schaeffer-Fulton staining.

Inhibition of *B. anthracis* spore germination. To test for antagonists of *B. anthracis* spore germination, spore aliquots were individually supplemented with various concentrations of compounds II to XLVI and incubated for 15 min at room temperature. Spore-analogue mixtures were then treated with 250 μ M inosine and 40 μ M L-alanine to induce germination. As above, optical densities were normalized by dividing each data point by the absorbance at the time of inosine addition and were reported as the relative OD_{580} . Germination rates were calculated from the initial linear region of the germination curves, as described above. Germination inhibition was assessed as the reduction of germination rate with increased analogue concentration. Inhibited germination rates were plotted against inhibitor concentrations to calculate 50% inhibitory concentration (IC_{50}) values using SigmaPlot v.11 software.

Macrophage viability assay. Macrophage J774A.1 cells were cultured using Dulbecco modified Eagle's medium (DMEM) without L-glutamine (Cellgro). The medium was supplemented with 10% fetal bovine serum (FBS; HyClone), 2 mM Glutamax (Invitrogen Corp.), and 100 U of penicillin-streptomycin. The cells were incubated at 37°C and 5% CO_2 until cultures reached approximately 80% confluence. Cells were subcultured three times before use in cell killing assays. Near-confluent macrophages were washed twice with ice-cold phosphate-buffered saline (PBS) and then detached by treating for 15 min with PBS supplemented with 5 mM EDTA. Detached cells were resuspended in DMEM without FBS or antibiotics and seeded at 8×10^5 cells/ml on 96-well transparent plates. After the cells had adhered, compounds I to XLVI were individually added at different final concentrations. The multiwell plate was incubated for 30 min at 37°C. Subsequently, *B. anthracis* spores, suspended in Hanks balanced saline solution (HBSS; HyClone, Logan, UT), were added to macrophages at a multiplicity of infection of 5. To control for compound toxicity, some cells were

treated with analogues but not infected with *B. anthracis* spores. Infected macrophages were incubated at 37°C and 5% CO_2 for 75 min. To remove nonphagocytosed spores, all wells were washed twice with 200 μ l HBSS and then filled with 100 μ l of fresh medium containing identical analogue concentrations. At 3 h postinfection, cells were treated with 20 μ M propidium iodide (PI). Cell necrosis was assessed by increases in PI fluorescence. Fluorescence was measured every hour for up to 7 h postinfection by following PI fluorescence at 617 nm. Relative fluorescence was calculated as the quotient of the difference of each data point and the fluorescence value at the time of PI addition, divided by the fluorescence value at the time of PI addition. Relative fluorescence intensities were plotted against inhibitor concentrations to calculate IC_{50} values by using SigmaPlot v.11 software.

Testing for compounds that enhance macrophage killing. Macrophages were individually treated with potential enhancers to a 100 μ M final concentration. *B. anthracis* spores were then added to each sample. PI fluorescence was determined 5 h postinfection. Macrophage killing enhancement was calculated by dividing the PI fluorescence intensity observed from infected macrophages treated with specific compounds by the PI fluorescence intensity obtained from infected untreated macrophages. GraphPad online calculators were used to run Student's *t* test and to obtain *P* values.

Treatment with combinations of 6-thioguanosine and D-alanine. Macrophages were treated with 1 mM D-Ala, 50 μ M D-Ala, 0.1 mM 6-TG, 0.1 μ M 6-TG, or 50 μ M D-Ala–0.1 μ M 6-TG. *B. anthracis* spores were then added to each sample. Macrophage killing was determined 5 h postinfection based on the increase of PI fluorescence intensity, as described above. GraphPad online calculators were used to run Student's *t* test and to obtain *P* values.

RESULTS AND DISCUSSION

Effects of 6-TG. Our previous studies showed that purine analogues can inhibit inosine (INO; compound I)–L-alanine–triggered *Bacillus anthracis* spore germination *in vitro* (2). Furthermore, one compound, 6-TG (compound II), was also able to inhibit intracellular spore germination and thus protect murine RAW264.7 macrophages from *B. anthracis* cytotoxicity (2). As previously shown, 6-TG is a modest inhibitor of spore germination *in vitro*, with an IC_{50} of 1 mM (Fig. 2A). This inhibition was not due to the reducing properties of the thiol group, as compounds III to X, with free sulfhydryls, were unable to inhibit spore germination. The inability of compounds III to X to affect *B. anthracis* spore germination also suggest that the purine ring is necessary for recognition by the germination machinery. 6-TG was also able to protect J774a.1 macrophages from *B. anthracis* cytotoxicity, with an IC_{50} of 3.5 μ M (Fig. 2B). Thus, 6-TG is approximately 300-fold more

TABLE 1. Antagonists of *B. anthracis* spore germination *in vitro*^a

| Antagonist | Acronym | IC ₅₀ (mM) |
|--|---------|-----------------------|
| 6-Thioguanosine | 6-TG | 1.00 |
| 6-Chloroguanosine | 6-CG | 0.89 |
| 6-Benzylthioinosine | 6-BTI | 0.05 |
| 6-Methylmercaptapurine riboside | 6-MMPR | 0.32 |
| 6-O-Methylguanosine | 6-OMG | 0.35 |
| 2-Aminoadenosine | 2-AADE | 0.70 |
| Xanthosine | XAN | 7.19 |
| 6-Methylaminopurine riboside | 6-MAPR | 0.19 |
| 2-Aminopurine riboside | 2-APR | 0.38 |
| Ribofuranosyl-((9H-purin-6-yl) piperazin-1-yl)ethanone | 6-PPER | 0.05 |
| 6-N-Benzylmethyladenosine | 6-BMA | 0.12 |
| 6-N-Benzylaminopurine | 6-BAP | 1.55 |
| 6-(γ,γ -Dimethylallyl amino)purine riboside | 6-DMAA | 0.36 |
| 6-N-Benzoyladeniosine | 6-NBA | 1.14 |
| 6-N-Piperidineadenosine | 6-PPA | 1.09 |
| 6-N-Diethyladenosine | 6-DEA | 0.31 |
| 6-N-Dipropyladenosine | 6-DPA | 0.76 |
| 6-N-((3-Ethylacetyl)cyclohexyl) adenosine | 6-ECA | 0.70 |
| D-Alanine | D-Ala | 0.02 |

^a Spore suspensions were individually supplemented with various concentrations of compounds II to XLVI and then treated with 250 μ M inosine and 40 μ M L-alanine. Compounds that did not inhibit germination are not shown in this table.

effective at preventing spore germination intracellularly than *in vitro*. The reason for the discrepancies in IC₅₀ values is not clear. *In vitro* spore germination assays require high spore concentrations to obtain reliable optical density changes. As a result, millimolar concentrations of inosine and L-alanine are needed to activate germination. In contrast, *B. anthracis* spores will encounter low inosine concentrations in the macrophage intracellular milieu (7). Low intracellular inosine concentrations might be the reason for the higher efficiency of 6-TG at blocking germination of *B. anthracis* spores in macrophages. It is conceivable that inosine is the principal germinant inside cells and that 6-TG prevents germination by competing with endogenous inosine.

***B. anthracis* spore germination *in vitro*.** Of the 46 compounds screened as agonists and antagonists of *B. anthracis* spore germination, 5 were able to act as cogerminants with L-alanine, and 18 were able to inhibit germination of *B. anthracis* spores treated with inosine and L-alanine. None of the pyrimidine analogues (compounds III to VIII) was able to activate *B. anthracis* spore germination *in vitro*.

Position 6 of the purine ring seems to have a significant role in the interaction of germinants with the *B. anthracis* spore germination machinery. INO, 6-thioinosine (6-TI; compound XI), adenosine (ADE; compound XII), guanosine (GUA; compound XIII), and 6-chloropurine riboside (6-CPR; compound XIV) are germinants for *B. anthracis* spores. All five compounds have 6-exocyclic groups with unsubstituted electronegative atoms directly bound to C-6 of the purine ring.

Electronegative groups are also present in every inhibitor of *B. anthracis* spore germination *in vitro*, with the exception of 2-amino riboside (2-APR; compound XV) (Table 1). However, nucleoside inhibitors normally possess aliphatic or aromatic chains linked to an electronegative atom. For example, 6-TI (compound XI), with a nonsubstituted 6-thiol (-SH), was a

weak germinant *in vitro*. In contrast, 6-mercaptapurine riboside (6-MMPR; compound XVI) and 6-BTI (XVII) are *B. anthracis* spore germination inhibitors, with IC₅₀s of 318 μ M and 50 μ M, respectively. A similar correlation was observed across other 6-substituted germination inhibitors (Table 1). A characteristic pattern of C-6-X-R (where X is oxygen, sulfur, or nitrogen and R is a hydrophobic group) is observed for inhibitors of *B. anthracis* spore germination. Hence, while non-substituted compounds act as germinants, addition of a hydrophobic side chain to the 6-exocyclic heteroatom is sufficient to convert a germinant structure into an inhibitor (Fig. 1, compounds XVI to XXVIII). However, there was no correlation between side chain physical properties and strength of inhibition. As an example, 2-APR (compound XV), 6-MMPR (XVI), 6-DEA (XX), 6-(γ,γ -dimethylallyl amino)purine riboside (DMAA; XXII), and 6-O-methylguanosine (6-OMG; XXVII) showed similar IC₅₀ values (Table 1). This suggests that binding of inhibitors in *B. anthracis* spores is mostly driven by nonspecific hydrophobic interactions. A similar pattern has been previously observed for the inhibition of *Bacillus cereus* spore germination *in vitro* (12).

In vitro assays showed that the presence of a 2-amino exocyclic group in the purine ring also contributes to spore germination inhibition. This is exemplified by 6-TI (compound XI), a 6-TG (compound II) analogue that lacks the 2-amino group and does not inhibit, but activates, spore germination *in vitro*. Similarly, nebularine (NEB; compound XXIX), a non-substituted purine riboside, does not affect *B. anthracis* spore germination, while 2-APR (compound XV), with an exocyclic amino group at position 2 of the purine ring, inhibits spore germination with an IC₅₀ of 370 μ M (Table 1). Furthermore, ADE (XII), with an amino group at the 6-position, is a good germinant *in vitro*. Meanwhile, 2-aminoadenosine (2-AADE; XXX), which has amino groups at both the 2- and 6-positions, is a germination inhibitor, with an IC₅₀ of 700 μ M. Similarly, 6-chloroguanosine (6-CG; XXXI) is also an inhibitor, with an IC₅₀ of 893 μ M. In contrast, isoguanosine (ISO; XXXII), a guanosine positional isomer, and xanthosine (XAN; XXXIII), with carbonyl groups at the 2-positions, are inactive in spore germination assays.

8-AADE (compound XXIV) is a positional isomer of 2-AADE (XXX) and has similar physical and chemical properties. However, 8-AADE did not activate or inhibit spore germination under any conditions tested. Similarly, 8-hydroxyguanosine (8-HG; XXXV) had no effect on *B. anthracis* spore germination. The inability of 8-AADE and 8-HG to influence *B. anthracis* spore germination could be due to steric hindrance at the 8-position.

The inosine derivative 7-methylinosine (7-MI; compound XXXVI) showed no effect on *B. anthracis* spore germination. Similarly, inosine analogues allopurinol riboside (APR; XXVII) and IH (XXXVIII) do not affect spore germination. Furthermore, the adenosine analogue tubercidin (TUB; XXXIX) is inactive in *B. anthracis* spore germination assays. Hence, the presence of an unsubstituted N-7 heterocyclic nitrogen in the purine ring also affects the germination properties of a compound.

Similar to the purine ring, the ribosyl group seems to be required for germination inhibition of *B. anthracis* spores. Ganciclovir (GCV; compound XL), a purine riboside analogue

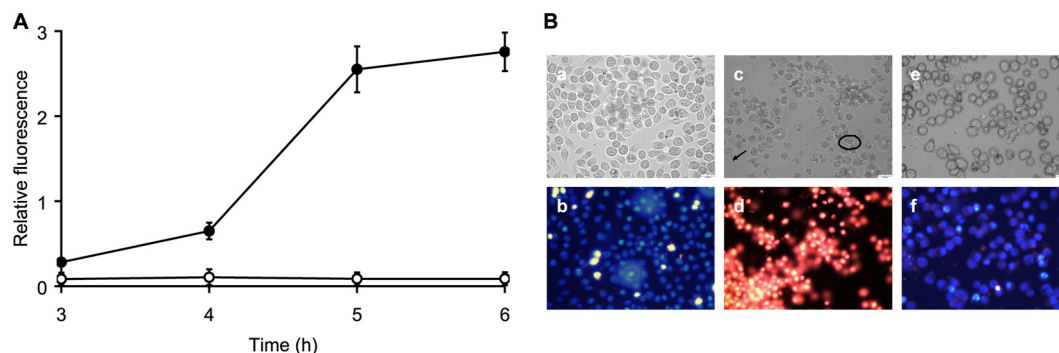


FIG. 3. Kinetics of macrophage killing by *B. anthracis* spores. (A) Macrophages were treated with medium (○) or *B. anthracis* spores (●). Macrophage necrosis was monitored over time by the increase of PI fluorescence intensity. Error bars represent standard deviations of six independent measurements. (B) Macrophages were observed under a microscope in white light (upper panels) or fluorescence (lower panels) modes. Macrophages showed limited cell death 3 h postinfection with *B. anthracis* spores (a and b). In contrast, macrophages showed extensive necrosis at 7 h postinfection with *B. anthracis* spores (c and d). Macrophage-engulfed spores (e.g., black circle in panel c) and vegetative *B. anthracis* cells (e.g., black arrow in panel c) were also seen under these conditions. Macrophages treated with 6-TG were protected from *B. anthracis* cytotoxicity even at 7 h postinfection with *B. anthracis* spores (e and f). No vegetative *B. anthracis* cells were observed.

that lacks the 2'-position, fails to either trigger or hinder *B. anthracis* spore germination. Similarly, purine bases lacking the ribosyl group (compounds XLI to XLVI), with the exception of 6-benzylaminopurine (BAP; XLVI), had no effect on germination *in vitro*. The aromatic side chain at the C-6 position may be responsible for the modest inhibition shown by BAP against the germination of *B. anthracis* spores *in vitro*. Therefore, an entire purine nucleoside frame is required for optimal germination inhibition *in vitro*.

Intracellular *B. anthracis* germination in J774a.1 macrophages. Murine J774a.1 macrophages can readily phagocytose endospores of *B. anthracis*. After infection, spore germination occurs inside the phagolysosome (18). Three hours postinfection, necrosis starts to be apparent, and approximately 20% cell death occurs. Cell death then increases rapidly, reaching almost 100% at 5 h postinfection (Fig. 3A). The timing of cell death is variable, but most cells have entered necrosis at 5 h postinfection. This variability is probably due to nonuniformity in spore intake by macrophages, which leads to heterogeneous cell killing (33).

Due to the heterogeneity of necrosis onset, compounds were tested for their ability to reduce *B. anthracis*-mediated killing at 5 h postinfection. Of the 46 compounds screened, only 8 were able to protect macrophages from *B. anthracis* cytotoxicity (Table 2). No compound showed toxic effects in the absence of *B. anthracis* spores. The protective compounds were either purine nucleoside or purine base derivatives. The purine nucleoside derivatives 6-TG (II), 6-TI (XI), 6-BTI (XVII), 6-CG (XXXI), and APR (XXXVII) protected macrophages, with IC_{50} values ranging from 3.5 μ M (6-TG; II) to near 1 mM (6-BTI; XVII) (Table 2). 6-TG (II) and 6-thioguanine (6-Tg; XLI) were the most efficient analogues and protected macrophages from necrosis even 6 h after infection. The remaining nucleoside analogues started to lose effectiveness at 6 h postinfection. Thus, these compounds did not completely inhibit intracellular spore germination but were able to delay the onset of the cytotoxic effect.

Three purine analogues, 6-Tg (XLI), 6-mercaptopurine (6-Mp; XLII), and 6-chloroguanine (6-Cg; XLIII), were also able to prevent cell necrosis. The protective properties of these

compounds are intriguing, since *in vitro* assays showed no effect on *B. anthracis* spore germination. It is worth noting that these compounds are the corresponding bases for the protective nucleosides 6-TG (II), 6-TI (XI), and 6-CG (XXXI), respectively. Furthermore, the IC_{50} s for nucleosides and free bases were similar in macrophage protection assays (Table 2). Thus, both nucleosides and their free bases are able to protect macrophages from *B. anthracis* cytotoxicity, even though they behave differently in spore germination assays.

Similar to *in vitro* germination assays, the presence of the 2-amino side group of the base favors the action of the compounds in macrophage protection assay. Analogues lacking this group showed decreased macrophage protective effects compared to their substituted counterparts. For example, 6-TG (II) had an IC_{50} value of 3.5 μ M, while the unsubstituted analog, 6-TI (XI), had an IC_{50} value of 35 μ M, a 10-fold decrease in protection ability. Similarly, 6-CG (XXXI) had an IC_{50} value of 189 μ M, while 6-CPR (XIV) was unable to prevent cell killing.

Comparing microscopy observations with viability assay results showed that macrophages in which *B. anthracis* progressed from spores to vegetative cells also showed up as PI-positive necrotic cells. On the other hand, in cell cultures

TABLE 2. Protection of macrophages from *B. anthracis*-mediated killing^a

| Antagonist | Acronym | IC_{50} (mM) |
|----------------------|---------|----------------|
| 6-Thioguanosine | 6-TG | 0.004 |
| 6-Chloroguanosine | 6-CG | 0.19 |
| 6-Thioinosine | 6-TI | 0.04 |
| Allopurinol riboside | APR | 0.65 |
| 6-Benzylthioinosine | 6-BTI | 1.04 |
| 6-Thioguanine | 6-Tg | 0.002 |
| 6-Chloroguanine | 6-Cg | 0.20 |
| 6-Mercaptopurine | 6-Mp | 0.40 |
| D-Alanine | D-Ala | 0.02 |

^a Macrophage cultures were individually supplemented with various concentrations of compounds II to XLVI and then infected with *B. anthracis* spores. Compounds that did not protect macrophages from anthrax cytotoxicity are not shown in this table.

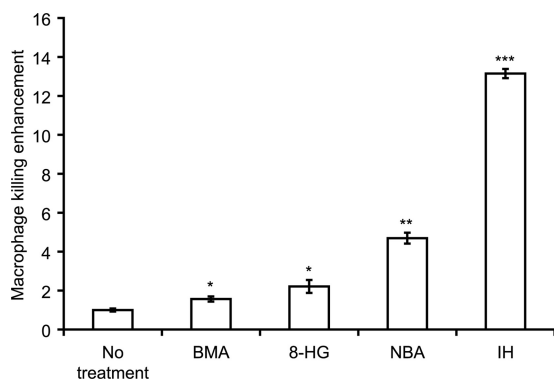


FIG. 4. Compounds that enhance *B. anthracis* cytotoxicity. BMA, 8-HG, NBA, and IH were added to macrophages to a 100 μM final concentration. *B. anthracis* spores were then added to each sample. Macrophage necrosis, based on increased PI fluorescence intensity, was monitored at 5 h postinfection. Macrophage killing enhancement was calculated by normalizing the PI fluorescence intensity determined for each compound treatment by the PI fluorescence intensity for untreated macrophages. Error bars represent standard deviations of six independent measurements. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (all for comparisons to untreated samples).

treated with an efficient inhibitor, spores did not progress to vegetative bacteria and the integrity of mammalian cells was maintained (Fig. 3B). Thus, endospores *per se* do not show toxin-related killing toward macrophages. This suggests that macrophage necrosis was either stopped or slowed when *B. anthracis* spores did not germinate efficiently upon treatment with germination inhibitors.

Compounds that enhance macrophage killing. Cell viability screening allowed the identification of compounds that cooperated with *B. anthracis* spores to increase the rate of macrophage killing (Fig. 4). The compounds that enhanced cell killing more efficiently were 6-BMA (XXIII), 6-benzoyladenine (6-NBA; XXVIII), 8-hydroxyguanosine (8-HG; XXXV), and IH (XXXVIII). This cell killing enhancement was not due to compound toxicity, since these compounds do not induce cell necrosis in the absence of *B. anthracis* spores (data not shown). Surprisingly, some of the compounds that enhanced cell killing were inhibitors *in vitro*. Currently, the mechanism by which these compounds are able to accelerate macrophage killing in the presence of *B. anthracis* spores is not known.

Effect of D-alanine and nucleoside analogue combinations. D-Ala is a natural antigerminant for *B. anthracis* spores (21). This molecule is biosynthesized by an alanine racemase present in the spore and can modulate germination pathways that require L-alanine as a germinant (25). Two research groups have already shown that D-Ala protects macrophages from *B. anthracis* toxin-mediated necrosis by inhibiting spore germination (21, 25). We corroborated the antigerminant ability of D-Ala both *in vitro* (IC_{50} , 16.0 μM) and in the macrophage killing protection assay (IC_{50} , 22.9 μM).

Since both inosine and L-alanine are strong germinants of *B. anthracis* spores, a combination of a nucleoside inhibitor (6-TG; II) and an amino acid inhibitor (D-Ala) could increase effectiveness by simultaneously blocking multiple Ger receptors. Indeed, combinations of 6-TG and D-Ala were

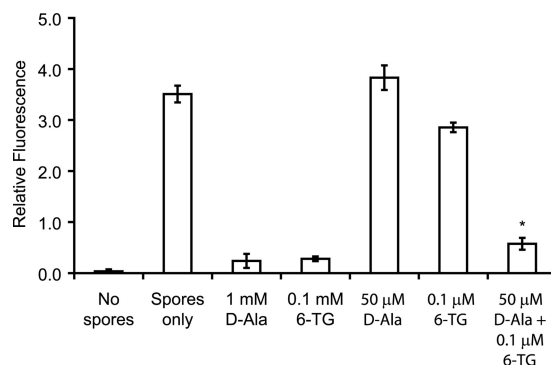


FIG. 5. Macrophage protection by 6-TG and D-Ala combinations. Macrophages were treated with *B. anthracis* spores, and killing was monitored at 5 h postinfection based on increased PI fluorescence intensity. Addition of 1 mM D-Ala or 0.1 mM 6-TG significantly reduced macrophage killing. In contrast, treatment with 50 μM D-Ala or 0.1 μM 6-TG did not prevent cell killing. Simultaneous treatment with 50 μM D-Ala and 0.1 μM 6-TG protected macrophages from *B. anthracis* cytotoxicity. Error bars represent standard deviations of six independent measurements. *, significantly decreased killing in the presence of both 50 μM D-Ala and 0.1 μM 6-TG compared to samples treated with 50 μM D-Ala alone ($P < 0.005$) or samples treated with 0.1 μM 6-TG alone ($P < 0.0001$).

able to protect macrophages from *B. anthracis*-mediated cytotoxicity, even at suboptimal concentrations of both inhibitors (Fig. 5). Similar results were obtained with D-Ala and 6-CG (XXXI) (data not shown).

Conclusions. Spore germination is the first necessary step in the establishment of *B. anthracis* infection (15). Hence, inhibition of *B. anthracis* spore germination could serve as a prophylactic approach to prevent anthrax in exposed personnel (5). Antigermination therapy will delay infection onset and hence allow antibiotic and/or vaccine treatments to be more effective. We had previously tested a limited number of purine nucleosides as inhibitors of *B. anthracis* spore germination (2). In this study, we tested a larger collection of purine nucleosides, purine bases, and pyrimidine bases for their abilities to prevent spore germination *in vitro* and protect macrophages from *B. anthracis*-mediated killing. All compounds (but one) that inhibited spore germination *in vitro* were purine nucleoside derivatives, showing that the sugar moiety is important for inhibitor recognition. Furthermore, hydrophobic-substituted electronegative exocyclic groups at the 6-position and an amine at the 2-position of the purine ring were necessary functional groups for efficient germination inhibition *in vitro*.

Molecular interactions between the spore germination machinery and the nucleoside inhibitors require specific contacts. Hydrogen bonding and/or ionic interactions are probably involved in the recognition of the amino group at the 2-position of purine nucleoside inhibitors. Similarly, hydrogen bonds could also serve to recognize essential sugar hydroxyls and the exocyclic heteroatom at position 6. Hydrophobic and π - π interactions would increase binding affinities for nucleosides with alkyl and aromatic side chains.

The efficiency of spore germination *in vitro* did not correlate with the capacity to protect macrophages from intra-

cellular spore germination and cytotoxicity. Some compounds were able to inhibit spore germination *in vitro* but did not protect macrophages against spore-mediated killing (e.g., 6-MMPR [compound XVI] and 6-OMG [XXVII]). Conversely, other compounds were unable to curtail spore germination *in vitro* but were able to prevent macrophage necrosis (e.g., 6-TI [XI] and APR [XXXVII]). Yet another compound subset was composed of efficient *in vitro* inhibitors that enhanced macrophage killing by *B. anthracis* spores (e.g., 6-BMA [XXIII] and 6-NBA [XXVIII]).

The lack of correlation between *in vitro* and intracellular *B. anthracis* spore germination assay results was not due to drug toxicity. These two assays measured spore germination under different conditions. *In vitro* germination assays are only dependent on the ability of compounds to cross the spore coat and interact with their respective Ger receptor. In contrast, intracellular germination assays are complicated by the additional requirement for compounds to cross both the mammalian cytoplasmic and phagosomal membranes. Furthermore, potential germination inhibitors may have to contend with cellular catabolism, active drug efflux pumps, and other mammalian cellular processes.

It was noticeable that some purine bases that did not hinder germination *in vitro* were effective antigerminants in macrophages. These bases (6-Tg [XLI], 6-Mp [XLII], and 6-Cg [XLIII]) prevented cell killing with efficiencies similar to those of their nucleoside derivatives (Table 2). Previous research has shown that 6-Tg (XLI), 6-Mp (XLII), and probably 6-Cg (XLIII) are transformed into 6-TG (II), 6-TI (XI), and 6-CG (XXXI) by the purine salvage pathway (7, 31). We postulate that this transformation allows the bases to be converted to nucleosides and to block spore germination inside the phagolysosome.

Similarly, two purine nucleosides, 6-TI (XI) and APR (XXXVII), did not affect germination *in vitro* but prevented macrophage necrosis. Thus, there must be a structural change in both 6-TI and APR that interferes with the germination of the *B. anthracis* endospores inside cells. We postulate that the nucleotide metabolism pathways allow these compounds to be converted to active inhibitors in the macrophage cytoplasm (7, 31).

In summary, analyses of *B. anthracis* spore germination activation and of inhibition *in vitro* allow mapping of important contacts necessary for spore binding. However, *in vitro* spore germination assays do not provide a good guide for the behavior of antigerminants inside cells. The multiplicity and variety of germinants and germination pathways *in vivo* make it difficult to find a single "ideal" molecule to block *B. anthracis* spore germination. Even though nucleosides show complex intracellular behavior, combining active nucleoside and amino acid germination inhibitors showed an additive effect in macrophage protection. Hence, simultaneous blocking of multiple germination pathways protects macrophages from *B. anthracis* cytotoxicity, even at suboptimal concentrations of the individual inhibitors.

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