

Identification of an *in Vivo* Inhibitor of *Bacillus anthracis* Spore Germination*

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Germination of *Bacillus anthracis* spores into the vegetative form is an essential step in anthrax pathogenicity. This process can be triggered *in vitro* by the common germinants inosine and alanine. Kinetic analysis of *B. anthracis* spore germination revealed synergy and a sequential mechanism between inosine and alanine binding to their cognate receptors. Because inosine is a critical germinant *in vitro*, we screened inosine analogs for the ability to block *in vitro* germination of *B. anthracis* spores. Seven analogs efficiently blocked this process *in vitro*. This led to the identification of 6-thioguanosine, which also efficiently blocked spore germination in macrophages and prevented killing of these cells mediated by *B. anthracis* spores. 6-Thioguanosine shows potential as an anti-anthrax therapeutic agent.

The 2001 anthrax bioterrorist attacks rekindled interest in the anthrax causative agent, *Bacillus anthracis*. The spores derived from *B. anthracis* survive exposure to extreme temperatures and harsh chemicals (1, 2) and represent an infectious agent with significant potential for biowarfare and bioterrorism. The severity of anthrax pathogenicity is dependent on the uptake route. Following inhalation, the most toxic route, *B. anthracis* spores are phagocytosed by alveolar macrophages in the lungs of infected hosts (3, 4). During the migration of infected macrophages to regional lymph nodes, *B. anthracis* spores germinate into the vegetative form (4), which secretes the anthrax toxins, the primary virulence factors of the bacterium (5–10). The transformation process from a dormant spore to a fully vegetative bacterium is a critical initial step in anthrax pathogenicity (3, 11). Therefore, drugs that prevent germination of phagocytosed spores are attractive candidates for controlling the anthrax disease.

The transformation into vegetative bacteria is a two-step process *in vitro*. Following activation of spores by heavy metals, heat, or hydrostatic pressure, bacterium-specific germinants

trigger germination (12). Different bacterial species use an array of germinants, which include amino acids and nucleosides (13–15). The exact mechanism by which these compounds induce germination is not clearly understood. It has been proposed that germinants trigger signal transduction pathways upon binding to specific receptors (16, 17). Mutational analysis of the *B. anthracis* genome led to the identification of germination operons, which control the germination process (17, 18). Based on these mutational assays, seven germination (*ger*) operons have been identified in *B. anthracis* (19). Moreover, four distinct amino acid- and nucleoside-dependent germination pathways have been described for *B. anthracis* (17). These pathways are purportedly initiated by binding of specific germinants to their corresponding receptors during the initial phase of germination (20). Efficient germination of *B. anthracis* spores *in vitro* requires the presence of purine ribonucleosides and amino acid co-germinants. Although specific amino acids like alanine are able to germinate *B. anthracis* spores at unphysiologically high concentrations, the presence of a second co-germinant, like inosine, can promote germination at lower concentrations (21).

B. anthracis spores have a dense, highly structured morphology and scatter light more strongly than their vegetative form. Upon germination, there is a marked decrease in the optical density at 600 nm. Analysis of optical density has been applied extensively to monitor the germination process. The kinetics of spore germination have been fitted to an exponential equation that describes the changes in optical density as spores germinate (22). Previous reports postulated that spore germination kinetics could be described using a rapid equilibrium approach (23). More recently, Ireland and Hannah (21) demonstrated that *B. anthracis* germination with alanine follows complex kinetics.

It has been observed that *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus megaterium* spore germination can be blocked by alkyl alcohols (24), ion channel blockers (25), protease inhibitors (26, 27), sulfhydryl reagents (28), and a vast array of other compounds (29). Most of these studies targeted specific germination pathways in different organisms and are not directly comparable. A more recent study tested subsets of the different types of compounds against *B. subtilis* and *B. megaterium* germination (29). All of these potential *Bacillus* germination inhibitors have been tested *in vitro* but, to our knowledge, their ability to protect mammalian cells from *B. anthracis*-mediated killing has not been tested.

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In the present study, we examined the kinetics of the germination process triggered by the common *in vitro* germinants, inosine and alanine. We first determined kinetic parameters for germination using inosine and alanine as co-germinants. Michaelis-Menten analysis revealed that *B. anthracis* spore germination follows a sequential mechanism and that germinants show remarkable synergy. Because inosine is a strong germinant *in vitro*, we screened nucleoside analogs against inosine/alanine-mediated spore germination. These assays led to the identification of a series of germination inhibitors. One of these compounds, 6-thioguanosine (6-TG),⁵ was also able to inhibit spore germination in macrophages and prevent anthrax-mediated cell death. 6-TG therefore shows potential as an anti-anthrax agent.

EXPERIMENTAL PROCEDURES

Chemicals and Equipment—Unless noted, all chemicals were obtained from Sigma-Aldrich. Spore germination was monitored on a Biomate 5 spectrophotometer at 580 nm or a Lab-systems iEMS 96-well plate reader (ThermoElectron Corporation, Waltham, MA) fitted with a 540-nm cut-off filter.

Bacterial Strains and Spore Preparation—The *B. anthracis* Sterne 34F2 strain was a gift from Dr. Arturo Casadevall, Albert Einstein College of Medicine. The Sterne strain lacks the pXO2 plasmid for encapsulation but contains the pXO1 plasmid for the production of virulence factors. For spore preparation, the Sterne strain was grown on BD Biosciences nutrient agar for 5 days followed by harvesting in ice-cold water. After three washing steps, spores were separated from vegetative and partially sporulated forms by centrifugation through a 20–50% Histo-Denz gradient. The spore pellet was washed six times with water and stored at 4 °C.

Analysis of Germination—Spore germination was monitored spectrophotometrically whereby the loss in light diffraction following the addition of inosine and alanine was reflected by decreased optical density at 580 nm (for Biomate 5 spectrophotometer) or above 540 nm (for Lab-systems iEMS 96-well plate reader). 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 A_{580} of 1. The spore suspension was monitored for auto-germination at A_{580} for 1 h. Only spores that did not auto-germinate were used for subsequent assays in 96-well format.

All germination experiments were carried out in 96-well plates in a total volume of 200 μ l/well. Every experiment was done in triplicate with at least two different spore preparations. Spores were allowed to germinate upon exposure to varying concentrations of both germinants in the presence of 1 mM D-cycloserine, which inhibits alanine racemase activity in the spores. For inosine titrations, spores were exposed to varying concentrations of inosine (0.1, 0.175, 0.25, 0.5, 1, 2.5, and 5 mM) at specific constant alanine concentrations (0.075, 0.1, 0.15, and 0.25 mM). For alanine titrations, spores were exposed to varying concentrations of alanine (0.05, 0.1, 0.133, 0.2, 0.5, 1, and 5 mM)

at specific constant inosine concentrations (1, 2.5, 5, and 10 μ M). The concentration range for both co-germinants was selected to avoid data clusters in the double reciprocal plots. Spore germination was evaluated based on decrease in A_{540} at room temperature each minute for 35 min. The germination extent of each well at each time point was expressed as a fraction of the actual OD divided by the OD obtained at the beginning of germination. Relative OD values were plotted against time. All measurements show standard deviations of less than 10%.

Germination rates (v) were determined for the various combinations of germinants used. Germination rates were calculated as the slope of the linear portion immediately following the initial lag phase of relative OD values over time. All measurements were performed in triplicate. The resulting data were plotted as double reciprocal plots of $1/v$ versus $1/[\text{variable germinant concentration}]$. All plots were fitted using the linear regression analysis from the SigmaPlot v.9 software to determine apparent K_m and V_{\max} values.

Calculation of Inhibition Constants (K_i)—Purified spores were diluted in 1 ml of germination buffer. Nucleoside analogs were added to 10 mM final concentration. Spore suspensions were incubated for 15 min at room temperature while monitoring A_{580} . If no germination was detected, inosine and alanine were then added to 2.5 and 0.04 mM final concentration, respectively. Germination was monitored as above. Compounds that showed inhibitory properties were added to fresh spore aliquots at decreasing concentrations. Relative A_{580} values obtained at arbitrary time points were plotted against the logarithm of inhibitor concentrations. The data were fitted using the four-parameter logistic function of SigmaPlot v.9 software to obtain IC_{50} values (30). Inhibition constants (K_i) were calculated from a modified Chang-Prusoff equation (31).

Germination Inhibition Assays—6-TG and 6-methylmercaptapurine riboside (6-MMPR) were characterized in detail as inhibitors of germination. To determine 6-MMPR effects on inosine binding, spores were exposed to varying concentrations of inosine (0.1, 0.175, 0.25, 0.5, 1, 2.5, and 5 mM) at specific constant (0, 0.25, 0.5, and 1 mM) 6-MMPR concentrations. Alanine was kept at saturating concentrations. To study 6-MMPR effects on alanine binding, spores were exposed to varying concentrations of alanine (0.01, 0.02, 0.04, 0.06, 0.08, 0.1, and 0.2 mM) at specific constant (0, 0.25, 0.5, and 1 mM) 6-MMPR concentrations. Inosine was kept at saturating concentrations. All assays were carried out in triplicate. All plots were fitted using the linear regression analysis from the SigmaPlot v.9 software to determine apparent K_m and V_{\max} values.

Spore Germination Mediated by Murine Macrophages—Murine RAW264.7 macrophages were washed three times in phosphate-buffered saline and seeded at 8×10^5 cells/ml on 96-well fluorescent plates in RPMI medium with 10% fetal calf serum and no antibiotic (Mediatech, Herndon, VA). After the cells had adhered, the medium was replaced with RPMI supplemented with germination inhibitors. Spores were added to the cultures at an m.o.i. of 20. The plates were centrifuged at 2000 rpm for 10 min at 4 °C and then shifted to a 37 °C incubator with 5% CO_2 to synchronize infection of RAW264.7 cells. In our hands, conditioned medium alone did not elicit germination

⁵ The abbreviations used are: 6-TG, 6-thioguanosine; 6-MMPR, 6-methylmercapto-purine riboside; m.o.i., multiplicity of infection; PI, propidium iodide; v , germination rates.

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from spores. Cytopathic effects following spore challenge of RAW264.7 cells were assessed by a propidium iodide exclusion assay (32, 33). After 6 h in culture, 25 μM propidium iodide was added to each well, and the plates shaken for 10 min. Fluorescence was read in a Wallac Victor II multititer plate reader at 590 nm (PerkinElmer Life Sciences).

Fluorescence Microscopy of Spore Germination Mediated by Macrophages—RAW264.7 cells were seeded on either Lab-Tek glass chambered cover slides (Nunc, Rochester, NY) or glass bottom culture dishes (MatTek, Ashland, MA). Spores were added to the macrophages at an m.o.i. of 20, and cells were incubated at 37 °C in the presence or absence of 6-TG for 7 h. Subsequently, 5 μM SYTO 13, a cell-permeable nucleic acid staining dye (Molecular Probes, Carlsbad, CA), was used to stain both living RAW264.7 cells and the vegetative form of *B. anthracis*, whereas 0.5 μM propidium iodide was used to stain dead macrophages. Experiments were performed with an Olympus IX81 electrically motorized microscope (Center Valley, PA).

RESULTS

Spore germination is the first step in *B. anthracis* infection. Agents that impede germination, preventing the generation of vegetative bacteria, could thereby block disease induction. Efficient germination of *B. anthracis* spores can be induced by particular ribonucleosides and amino acids, specifically inosine and alanine (17, 21, 34). We determined the kinetics of the germination process triggered by inosine and alanine as well as by inosine analogs, with the hypothesis that inosine analogs could function as germination inhibitors.

Spores derived from the *B. anthracis* Sterne strain were used for germination assays. Germination of heat-activated spores was initiated by the addition of inosine and alanine and monitored at 540 nm. The optical density (A_{540}) decreases during the progression of germination. The spores were allowed to germinate upon exposure to varying concentrations of both germinants in the presence of saturating concentrations of D-cycloserine. Upon the addition of inosine and alanine to spores, we observed a rapid increase in germination, even at micromolar concentrations of both germinants.

We observed an early lag phase during the germination process, and germination rates (v) were calculated from the changes in optical density during the linear phase of the sigmoidal curves. The resulting germination rates were used for Michaelis-Menten analysis. Titrations of the germination rate with either germinant yielded hyperbolas indicating saturation of germinant binding to specific receptor sites. Rate studies were carried out using a matrix of germinant concentrations, and the data were plotted in double reciprocal format (35). V_{max} parameters were determined from the interception of the best-fitted lines with the y axis. Similarly, the interception of the fitted lines with the x axis allowed the determination of an apparent K_m .

Double reciprocal plots of $1/v$ versus $1/[\text{inosine}]$ at increasing alanine concentrations yielded a family of plots that converged in the upper left quadrant (Fig. 1A). Since every line intersects the y axis at different points, V_{max} for germination increased with alanine concentrations. Furthermore, each line intersects at different x axis values, suggesting that alanine binding

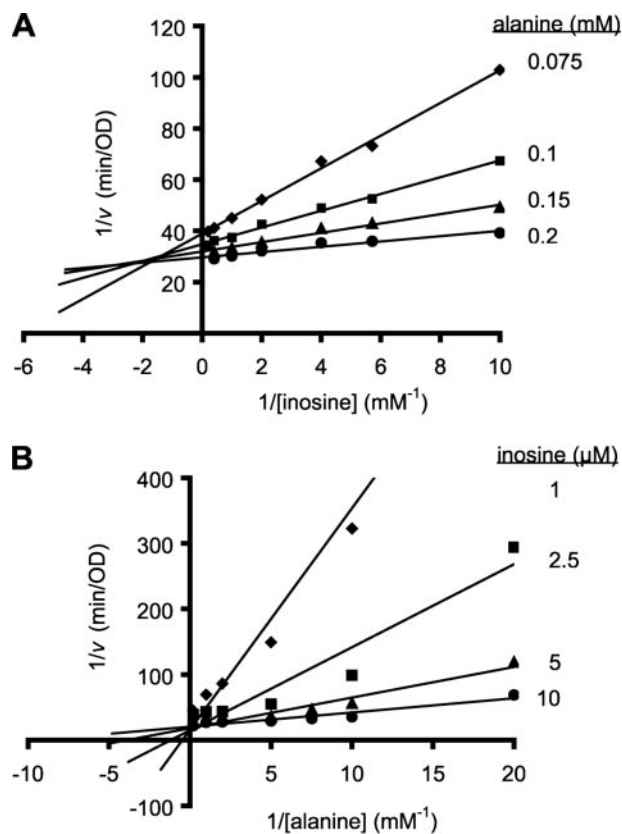


FIGURE 1. Kinetics of *B. anthracis* spore germination in the presence of inosine and alanine. Germination rates were calculated from the linear segment of optical density changes over time. A, Lineweaver-Burk plots of *B. anthracis* spore germination at variable inosine (0.1, 0.175, 0.25, 0.5, 1, 2.5, and 5 mM) concentrations and different fixed alanine (0.075, 0.1, 0.15, and 0.2 mM) concentrations. B, Lineweaver-Burk plots of *B. anthracis* spore germination at variable alanine (0.05, 0.1, 0.133, 0.2, 0.5, 1, and 5 mM) concentrations and different fixed inosine (1, 2.5, 5, and 10 μM) concentrations.

increases the affinity of spores for inosine. From these plots, apparent K_m and V_{max} values of inosine at infinite alanine concentrations were calculated to be 270 μM and 0.04 OD/min, respectively.

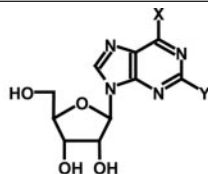
The fact that the apparent germination V_{max} increases with increasing alanine concentrations (Fig. 1A) indicates that germinants bind to separate sites. Moreover, the intersection of the curve family at a single point strongly supports the notion that both germinants are present simultaneously on their receptor(s) to achieve the synergy in both velocity and affinity. The K_m for inosine calculated at the highest and lowest alanine concentration tested were extrapolated to 35 and 164 μM , respectively. This represents an almost 5-fold increase in the affinity of the spore for inosine over a 3-fold alanine concentration range, revealing a remarkable cooperativity between the germinants.

Double reciprocal plot analysis of the effect of alanine on spore germination rate yielded a family of plots that intersect the x axis at different points, indicating that the affinity of spores for alanine goes up with increasing inosine concentrations (Fig. 1B). Thus, the affinity of either germinant increases when the complementary germinant is bound at its specific site. This is consistent with a sequential mechanism, which requires

TABLE 1

Germination inhibition constant for different nucleosides

Values were obtained in the presence of saturating concentrations of inosine and alanine. X represents substitution at the 6-position, and Y represents substitutions at the 2-position. N/A, not applicable.



Substrate	X	Y	K _i (μM)
Inosine	OH	H	N/A
6-thioinosine (6-TI)	SH	H	N/A
6-methylmercaptapurine riboside (6-MMPR)	SCH ₃	H	31
6-thioguanosine (6-TG)	SH	NH ₂	98
6-O-methylguanosine	OCH ₃	NH ₂	34
6-chloroguanosine	Cl	NH ₂	87
6-aminoguanosine	NH ₂	NH ₂	68
Xanthosine	OH	OH	777

binding of both ligands, and where binding of the first alters binding of the second ligand.

Our kinetic analysis suggested that the germination process requires inosine binding to a specific spore receptor. Therefore, we hypothesized that inosine analogs might interfere with inosine-receptor interactions and prevent germination. For inhibition assays, we selected commercially available inosine and guanosine analogs (36–38).

The inosine analogs were based on 6-thioinosine containing a sulfur substitution in place of a 6-oxo group. The 6-thioinosine analog, 6-MMPR, has a methyl group attached to the sulfur atom (Table 1). 6-TG, 6-O-methylguanosine, 6-chloroguanosine, and 6-aminoguanosine are guanosine analogs with thio, methoxy, chloro, and amino group substitutions of the 6-oxo groups. The inosine analog xanthosine has oxo groups at positions 2 and 6 (Table 1).

For inhibitor studies, *B. anthracis* spores were preincubated with nucleoside analogs for 15 min. Germination was then initiated by the addition of inosine and alanine. Germination rates were slower in the presence of nucleoside inhibitors as compared with those obtained with untreated spores. Thus, at any arbitrary time point, the optical density of inhibitor-treated spores was higher than that of untreated spores. Using this assay, we identified several potent germination inhibitors (Fig. 2).

IC₅₀ values were calculated using the inflection points of the dose-response curves (30). The IC₅₀ of the inhibitor and the apparent K_m for inosine were then used to determine the apparent K_i of the nucleoside analog using a modified Chang-Prussoff equation (31). The inosine analog 6-thioinosine did not interfere with inosine-mediated germination, even in the presence of excess alanine (data not shown). In contrast, 6-MMPR efficiently blocked inosine-triggered spore germination with a K_i of 31 μM (Table 1). Thus, the presence of a single methyl group at position 6 allowed binding to the germination receptor and interfered with the germination process. The guanosine analogs, 6-TG, 6-O-methylguanosine, 6-chloroguanosine, and

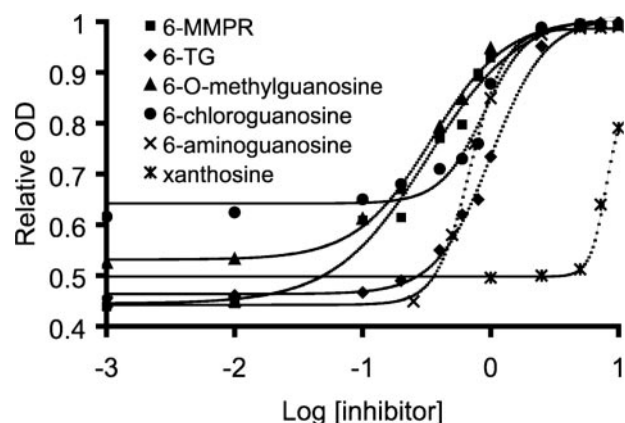


FIGURE 2. Dose-response curves of germination inhibitors. *B. anthracis* spores were incubated with increasing concentrations of nucleoside analogs. Spore germination was then triggered by adding 2.5 mM inosine and 0.04 mM alanine. The optical densities obtained at an arbitrary time point were plotted against the logarithm of inhibitor concentration and fitted to a four-parameter logistic function. Experiments were performed in the presence of 6-MMPR (closed squares), 6-TG (closed diamonds), 6-O-methylguanosine (closed triangles), 6-chloroguanosine (closed circles), 6-aminoguanosine (crosses), and xanthosine (asterisks).

6-aminoguanosine, have substitutions at positions 2 and 6 and efficiently blocked spore germination in the presence of inosine. Therefore, substitutions at positions 2 and 6 are important for receptor recognition and inhibitory properties. Xanthosine, with carbonyl groups at positions 2 and 6, on the other hand, did not interfere with spore germination. The K_i values obtained for the inosine analogs are shown in Table 1.

The nucleoside analogs 6-TG and 6-MMPR that blocked germination of *B. anthracis* are currently used in the treatment of cancer and Crohn disease, and their pharmacological properties are well established (39, 40). We used 6-TG and 6-MMPR for kinetic analysis using double reciprocal plots. To understand the competitive nature of the nucleoside analogs, we performed the inhibitor studies using alanine at saturating, inosine at subsaturating, and nucleoside analogs at variable concentrations (Fig. 3A). As expected, Lineweaver-Burk plots for the 6-MMPR inhibitor converged close to the y axis, suggesting that 6-MMPR competes with inosine. This indicates that 6-MMPR affected the affinity of the spores for inosine without greatly interfering with the maximum germination rate. Unlike inosine, 6-MMPR affected both the maximal velocity of germination and the affinity of alanine for spores (Fig. 3B). Our system yielded similar results in the presence of 6-TG, which, like 6-MMPR, competed with inosine for receptor binding.

Michaelis-Menten analysis of 6-MMPR and 6-TG inhibition of germination suggested that both nucleoside analogs inhibit germination by interfering with inosine binding to its cognate receptor. Moreover, both 6-MMPR and 6-TG were also able to induce spore germination in the presence of excess alanine and in the absence of inosine (Fig. 4). However, both the germination rate and the total decrease in optical density were significantly lower than rates observed using inosine as a co-germinant. These results indicated that 6-MMPR and 6-TG are only weak germinants. We assumed that the low germination induction potential of 6-MMPR and 6-TG is linked to their ability to inhibit inosine/alanine germination. As expected, inosine ana-

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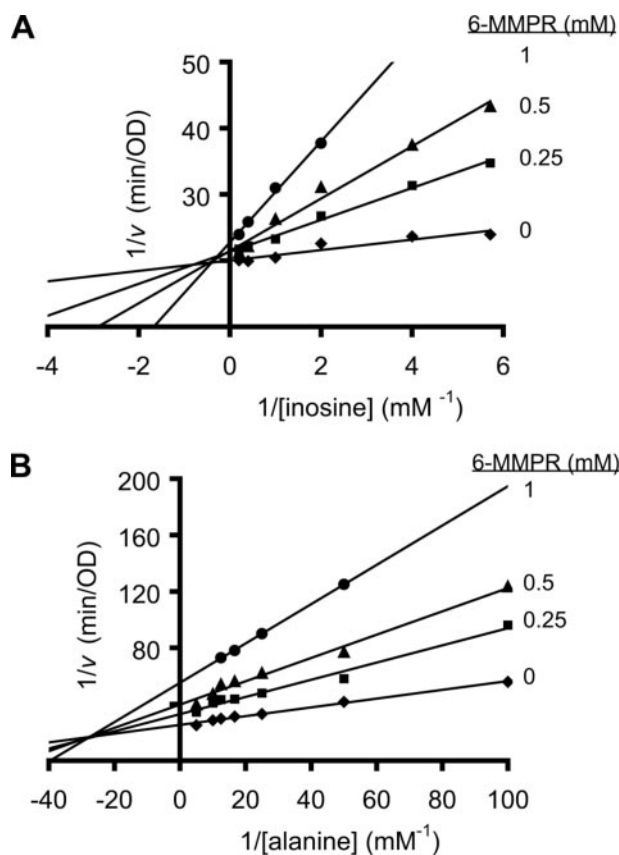


FIGURE 3. Kinetics of spore germination inhibition by 6-MMPR. *B. anthracis* spores were incubated with different concentrations of 6-MMPR, and germination was triggered with inosine and alanine. A, Lineweaver-Burk plots of *B. anthracis* spore germination at variable inosine (0.1, 0.175, 0.25, 0.5, 1, 2.5, and 5 mM) concentrations and different fixed 6-MMPR (0, 0.25, 0.5, and 1 mM) concentrations. Alanine was kept constant at 0.5 mM. B, Lineweaver-Burk plots of *B. anthracis* spore germination at variable alanine (0.01, 0.02, 0.04, 0.06, 0.08, 0.1, and 0.2 mM) concentrations and different fixed 6-MMPR (0, 0.25, 0.5, and 1 mM) concentrations. Inosine was kept constant at 1 mM.

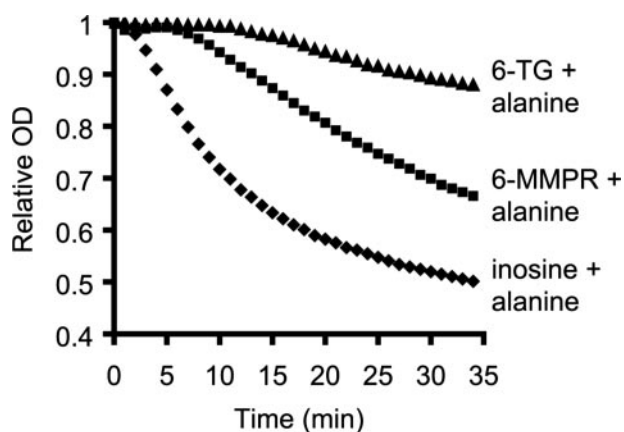


FIGURE 4. Germination properties of 6-MMPR and 6-TG. The addition of 0.25 mM alanine and 1 mM inosine causes rapid spore germination (closed diamonds). Substituting inosine for 6-MMPR (closed squares) or 6-TG (closed triangles) causes a reduction of both the germination rate and the total decrease in optical density.

logs competed with the potent germination inducer inosine for receptor binding and prevented efficient spore germination.

A productive infection and disease progression requires phagocytosis of *B. anthracis* spores and subsequent germination (41). In contrast to *in vitro* assays, intracellular factors that

promote germination of *B. anthracis* within macrophages are unknown. Multiple cellular factors have been postulated to play a role in germination within macrophages, including amino acids and nucleosides (42, 43). We tested whether the nucleoside analogs that blocked germination in *in vitro* assays also prevented germination of spores phagocytosed by macrophages. This would establish whether the tested inosine analogs represent potential therapeutics against *B. anthracis* infections. Because the vegetative form of the *B. anthracis* spore efficiently kills murine macrophages, we determined cytopathic effects following spore challenge using propidium iodide (PI) exclusion assays. We infected murine RAW264.7 macrophages with *B. anthracis* spores at an m.o.i. (spore to cell ratio) of 20 to 1. PI fluorescence intensity (and thus macrophage death) showed a marked increase within 6 h following anthrax inoculation (Figs. 5A and 6).

Strikingly, of all nucleoside analogues that efficiently blocked germination *in vitro*, only 6-TG prevented germination and cytotoxic effects in spore-infected murine macrophages. 6-TG efficiently blocked (88%) macrophage killing by *B. anthracis* spores at concentrations between 1 and 2 μM (Fig. 5B). However, a residual background of PI-positive macrophages was detected in the presence of even the highest 6-TG concentrations, possibly due to the ability of 6-TG to function as a weak germinant. No inhibition of *B. anthracis* germination was detected in the presence of the germination inhibitors 6-MMPR, 6-chloroguanosine, 6-O-methylguanosine, 2-amino-adenosine, or xanthosine, even at the highest concentrations used (Fig. 5C and data not shown).

Macrophage-spore interaction was further examined by fluorescence microscopy. The green dye SYTO 13 is cell-permeable, whereas red PI is impermeable to cellular membranes. Both dyes bind to DNA. Thus, the macrophage nucleus shows green fluorescence in healthy cells and red fluorescence in necrotic cells. Macrophages were treated with SYTO 13 and PI simultaneously. As expected, more than 90% of untreated macrophages showed green fluorescence (Fig. 6A). Exposure to *B. anthracis* spores resulted in ~ 20 and 90% PI-positive macrophages 3.5 and 7 h after infection, respectively (Fig. 6, B and C). This is consistent with the rapid increase in PI fluorescence 5–6 h following *B. anthracis* inoculation (Fig. 5A). SYTO 13 also stained vegetative bacteria, which were present in macrophages cultures 7 h after *B. anthracis* treatment (Fig. 6C, white arrow). The addition of 6-TG prevented the killing of *B. anthracis*-infected macrophages indicated by a lack of PI-positive cells, even 7 h after inoculation. Furthermore, no vegetative bacteria were detected in 6-TG-treated macrophages (Fig. 6D).

DISCUSSION

Inosine and alanine are powerful germinants of *B. anthracis* *in vitro*. Kinetic analysis of the germination process revealed that germination requires simultaneous binding of inosine and alanine and that the germinants show significant synergy. Intracellular inosine concentrations are extremely low in mammalian cells as this nucleoside is a degradation product of the purine biosynthesis pathway and is rapidly converted to uric acid (44). However, high intracellular alanine concentrations in

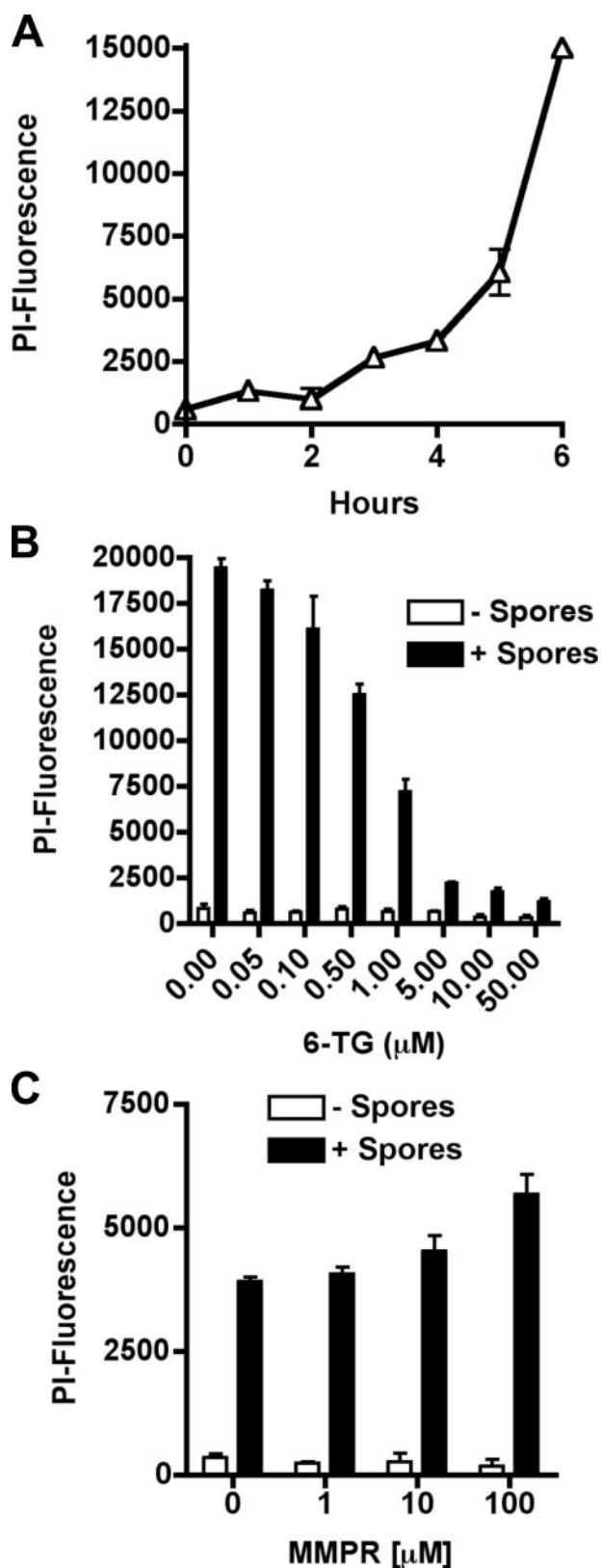


FIGURE 5. 6-TG prevents macrophage killing by *B. anthracis* spores. A, murine RAW264.7 macrophages were challenged with *B. anthracis* spores at an m.o.i. (spore to cell ratio) of 20, and cytopathic effects were determined using PI exclusion. Increasing PI fluorescence over time correlated with macrophage killing. Macrophages were incubated with increasing amounts of 6-TG (B) or 6-MMPR (C) before exposure to *B. anthracis* spores, as described in A. PI fluorescence of infected macrophages was measured 6 h after exposure to *B. anthracis* spores.

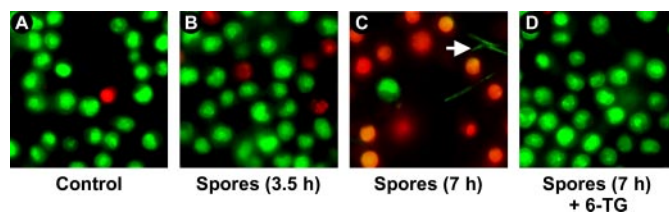


FIGURE 6. The inosine derivative 6-TG prevents macrophage killing mediated by *B. anthracis* spores. A, uninfected RAW264.7.1 macrophages stained with SYTO 13 (green, live cells) and propidium iodide (red, dead cells) show less than 5% dead cells. B, RAW264.7 cells 3 h after exposure to *B. anthracis* spores show ~20% dead cells. C, 90% of RAW264.7 cells were dead 7 h after exposure to *B. anthracis* spores. The vegetative form of *B. anthracis* is indicated by arrows. D, 6-TG-treated RAW264.7 cells 7 h after exposure to *B. anthracis* spores show that the number of dead cells is similar to the uninfected control. Furthermore, no vegetative *B. anthracis* cells are observed.

the low millimolar range might drive germination in macrophages even at suboptimal nucleoside concentrations.

Given the importance of inosine in initiating germination of *B. anthracis*, we screened inosine analogs for their ability to interfere with this process. This led to the identification of multiple powerful inhibitors that blocked germination *in vitro*. Kinetic analysis revealed that the inosine analogs 6-TG and MMPR compete with inosine for spore binding. It is not surprising that these competitive inhibitors are able to germinate *B. anthracis* in the presence of alanine, albeit with a drastically lower efficiency than inosine.

Strikingly, of all inosine analogs that blocked germination *in vitro*, only 6-TG prevented germination in macrophages. Germination in macrophages is a complex and poorly understood process. At least four germination pathways have been described in *B. anthracis* (17), including germination triggered by a combination of amino acids alone. It is conceivable that inosine is the principal germinant inside cells and that 6-TG prevents germination by competing with endogenous inosine. Low intracellular inosine concentrations might be the reason for the high efficiency of 6-TG at blocking germination of *B. anthracis* spores in macrophages. Future experiments will help to identify cellular factors that drive *B. anthracis* germination in macrophages.

It remains to be shown why 6-TG efficiently blocked germination in macrophages, whereas closely related nucleoside analogs failed when tested with macrophages. It is possible that the failure to block germination *in vivo* is due to a lack of membrane permeability for the nucleoside analogs or due to rapid intracellular degradation of these compounds. Since all inhibitors tested were able to compete with inosine *in vitro*, it is conceivable that the inosine receptors are not the main activator of germination within macrophages.

6-TG (but not any other nucleoside tested) may also compete with germinants present in macrophages other than inosine. These results would suggest that alternative receptors contribute to spore germination in macrophages. Seven different germination operons have been identified for *B. anthracis*. Two chromosomal operons, *gerS* and *gerH*, have been linked to alanine-inosine-dependent germination responses (17, 21). Studies using germination inhibitors such as 6-TG might help to identify endogenous agents that drive germination of *B. anthracis* in macrophages.

Inhibition of *B. anthracis* Spore Germination

Taken together, we established that the nucleoside analog 6-TG inhibits *B. anthracis* spore germination in macrophages and efficiently blocks cell death mediated by these spores. Future tests might identify 6-TG analogs with improved inhibition and reduced germination potential. These agents could represent powerful prophylaxes against anthrax infections. The potential of 6-TG to prevent germination and disease progression in mice infected with *B. anthracis* spores remains to be tested.

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