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Discovery, Characterization and Comparison of Inhibitors of *Bacillus anthracis* and *Staphylococcus aureus* Replicative DNA Helicases

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Abstract

Antibacterial compounds with new mechanisms of action are needed for effective therapy against drug-resistant pathogens in the clinic and in biodefense. Screens for inhibitors of the essential replicative helicases of *Bacillus anthracis* and *Staphylococcus aureus* yielded 18 confirmed hits ($IC_{50} \leq 25 \mu M$). Several (5 of 18) of the inhibitors were also shown to inhibit DNA replication in permeabilized *polA*-deficient *B. anthracis* cells. One of the most potent inhibitors also displayed antibacterial activity (MIC $\sim 5 \mu g/ml$ against a range of Gram-positive species including bacilli and staphylococci) together with good selectivity for bacterial vs. mammalian cells ($CC_{50}/MIC > 16$) suitable for further optimization. This compound shares the bicyclic ring of the clinically proven aminocoumarin scaffold, but is not a gyrase inhibitor. It exhibits a mixed mode of helicase inhibition including a component of competitive inhibition with the DNA substrate ($K_i = 8 \mu M$) and is rapidly bactericidal at $4 \times$ MIC.

Keywords

S. aureus; *B. anthracis*; helicase; high throughput screen; aminocoumarin

1. Introduction

The increasing prevalence of antibiotic-resistant bacterial strains continues to erode the efficacy of current antibiotics. The development of new antibacterials that are not subject to existing mechanisms of resistance offers a solution to this growing medical need, but successful discovery and development of new antibacterials have been difficult to achieve.¹ The bacterial DNA replication complex presents an attractive, and largely unexploited, target for development of new antibacterials. Among inhibitors which target bacterial DNA replication, only the fluoroquinolones, inhibitors of bacterial gyrase and topoisomerase, are in current clinical use.² Potent inhibitors of Gram-positive DNA polymerase have also been described but have not been tested in the clinic yet.³ The bacterial replicative DNA helicase is a promising

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target for new antibiotic discovery because it fulfills an essential role in DNA replication and exhibits no significant homology to mammalian helicases. Through the ATP-dependent unwinding of the DNA duplex, replicative helicases allow access by the rest of the replication machinery to the replication fork and thus permit duplication of the bacterial genome. These enzymes function as hexameric rings, with the DNA occupying the central channel of the hexamer.⁴ Bacterial replicative helicases have been demonstrated to be essential for bacterial growth, and inhibitors would likely be bactericidal since existing gyrase and polymerase inhibitors are known to kill susceptible bacteria. Bacterial cells contain a variety of putative or actual helicases in addition to the replicative enzyme, but they are not closely related structurally to the replicative helicases and most are involved in DNA repair or plasmid replication and are not essential for bacterial growth.

Prior screening for inhibitors of bacterial replicative helicases has yielded very few potent, selective and non-cytotoxic inhibitors. In fact, only two compounds have been described as bacterial replicative helicase inhibitors -- the natural product flavonoid myricetin inhibits *Escherichia coli* DnaB helicase⁵, and a triaminotriazine was identified in a screen for inhibitors of *Pseudomonas aeruginosa* DnaB helicase.⁶ However, these compounds also exhibit significant cytotoxicity in mammalian cell culture. Because orthologous antibacterial targets from different species contain subtle sequence differences which could make them more accessible to small molecule inhibitors,^{7, 8} we undertook the screening of compounds for inhibition of the replicative helicases from two additional species, *Staphylococcus aureus* and *Bacillus anthracis*. Surprisingly, the hit rates for the two helicases were remarkably different, and with few exceptions, hits were more potent vs. the *B. anthracis* helicase than they were vs. the *S. aureus* helicase, regardless of the screen in which they were first identified as helicase inhibitors. One of the most potent and selective inhibitors discovered (see compound **2** below) shares a portion of the aminocoumarin chemotype. While bioactive compounds with a coumarin scaffold have been known for decades, they are inhibitors of DNA gyrase and are structurally distinct from the coumarins described here.⁹ Nevertheless, favorable clinical history with this class of compounds suggests that further development of coumarin-type helicase inhibitors is feasible.

2. Results

2.1 High Throughput Screening for helicase inhibitors

Genes for the *B. anthracis* and *S. aureus* replicative helicases were cloned and expressed in *E. coli*, the enzymes were purified, and fluorescence-based assays were developed for each and optimized as high throughput screens. A total of over 186,000 small synthetic molecules were screened to identify inhibitors of the *S. aureus* or *B. anthracis* helicase-catalyzed strand unwinding reaction. Primary hits were selected and confirmed by re-assay, requiring over 50% inhibition in at least two of three replicates. The overall confirmed hit rate was about 0.08%, but when calculated separately for each helicase, it was nearly 10-fold higher for the *B. anthracis* enzyme than for the *S. aureus* enzyme (Table 1).

2.2 Characterization of confirmed hits

Confirmed inhibitors of each of the helicases were characterized further to eliminate false positives which act by mechanisms other than direct inhibition of helicase and to measure the concentration-dependence of helicase inhibition. First, hits were examined in an ethidium bromide displacement assay¹⁰ to eliminate compounds which inhibit strand unwinding by binding to the DNA duplex substrate rather than to the helicase. Second, hits were tested in a radiometric assay of helicase activity to ensure that hits block strand unwinding rather than simply quenching FAM fluorescence in the FRET assay. Several hits which resemble known intercalators or minor groove binders or were strong quenchers were eliminated by these

secondary assays. Third, hits were tested for inhibition of *Pseudomonas aeruginosa* AmpC β -lactamase in the presence of various concentrations of Triton X-100 to detect compounds acting promiscuously by a colloidal aggregate mechanism.¹¹ None of the confirmed hits exhibited inhibition of AmpC at 0.01% Triton X-100, the concentration used in the FRET helicase assays, indicating that aggregates are not likely to be responsible for the observed helicase inhibition. Finally, hits from each helicase screen were examined for inhibition of the helicase of the other species, and the concentration-dependence of inhibition (IC₅₀) was determined.

About 10% of the 160 confirmed primary hits, a total of 18 compounds were validated by these secondary assays and exhibited concentration-dependent inhibition with IC₅₀ values <25 μ M vs. at least one of the two helicases (Table 1). These fall into five chemotypes with three additional compounds as singletons (Table 2). With the exception of one chemotype (see below), confirmed hits were demonstrated by LC-MS analysis to be of correct mass and sufficient purity (>95%) for further evaluation.

2.3 Selectivity of inhibitors

In order to evaluate the selectivity of the inhibitory effects of these compounds, all confirmed hits were tested for (a) potency of inhibition of DNA replication in permeabilized *polA*-deficient *B. anthracis* cells, (b) minimal inhibitory concentration (MIC) vs. growth of *B. anthracis* and *S. aureus* cells, and counter-screened for (c) potency of inhibition of the replicative DNA polymerase Pol III_C of *B. subtilis* and (d) for effects on the viability of mammalian cells (cytotoxicity). Structures of the confirmed hits along with results of these confirmatory assays are shown in Table 2. All data are expressed in μ M to facilitate comparisons.

2.3.1 Inhibitors with a coumarin core structure—Three hits from the *B. anthracis* helicase screen and two hits from the *S. aureus* helicase screen share a coumarin chemotype (Series A, compounds 1-5, respectively, of Table 2). These coumarin-type compounds are potent screening hits, with IC₅₀ values ranging from 5-10 μ M vs. *B. anthracis* helicase. All five compounds exhibit significant inhibition of both *B. anthracis* and *S. aureus* helicases, but they are all less potent vs. *S. aureus* helicase, with IC₅₀'s ranging from about 2 to 20-fold higher than those for *B. anthracis* helicase. They are poor inhibitors of another DNA replication enzyme, *B. subtilis* replicative DNA polymerase Pol III_C (IC₅₀'s >100 μ M) confirming their selectivity for helicase. As expected for helicase inhibitors, they inhibit DNA replication in permeabilized *polA*-deficient *B. anthracis* Sterne cells, but the potency of compounds **1**, **4**, and **5** is less than that exhibited in the *in vitro* FRET-based assay. Coumarins **2** and **4** are not cytotoxic to mammalian cells when tested up to 100 μ M. Furthermore, compound **2** inhibits the growth of *B. anthracis* cells with an MIC of 6 μ M, resulting in an overall selectivity index (CC₅₀/MIC) >16.

2.3.2 Inhibitors with a benzothiazole core structure—Two hits from the *B. anthracis* helicase screen share a benzothiazole chemotype (Series B, compounds **6-7** of Table 2). They both exhibit IC₅₀ values vs. *B. anthracis* helicase of 12 μ M but are slightly less potent vs. *S. aureus* helicase. These two compounds exhibit no detectable inhibition of *B. subtilis* DNA polymerase Pol III_C, but also fail to demonstrate detectable inhibition of DNA replication in permeabilized *B. anthracis* cells. Neither of the benzothiazoles has a detectable MIC, but some cytotoxicity is observed, with CC₅₀'s of 10 and 70 μ M.

2.3.3. Inhibitors with a rhodanine core structure—Four confirmed hits from the *B. anthracis* helicase screen share a rhodanine chemotype (Series C, compounds **8-10**, Table 2). This class of hits is unique for two reasons -- they are the most potent helicase inhibitors identified, and all are approximately equipotent against both *B. anthracis* and *S. aureus*

helicase, with IC₅₀ values of 1-4 μ M. Three members of this class exhibit no detectable cytotoxicity (CC₅₀ >100 μ M), but the replacement of the 4-chloro substituent with a nitro-group (compound **10**) resulted in pronounced cytotoxicity (CC₅₀ = 11 μ M). The three non-cytotoxic rhodanines (**8**, **9** & **11**) inhibit DNA replication in permeabilized *B. anthracis* cells, but with potencies considerably poorer than those demonstrated in direct helicase assays. None exhibited a detectable MIC.

2.3.4 Inhibitors with a triazine core structure—Two confirmed hits from the *B. anthracis* helicase screen share a triazine chemotype (Series D, compounds **12** and **13**, Table 2), and they bear some resemblance to the triaminotriazine inhibitor of *P. aeruginosa* replicative helicase described previously.⁶ They are moderately potent inhibitors of *B. anthracis* helicase, with IC₅₀ values of 12 and 24 μ M, and are about half as potent vs. *S. aureus* helicase. Compound **13** fails to inhibit DNA replication in permeabilized *B. anthracis* cells and is quite cytotoxic to mammalian cells. Compound **12** inhibits DNA replication in permeabilized *B. anthracis* cells and displays about equal potency for bacterial and mammalian cell growth inhibition.

2.3.5 Inhibitors with an N-phenyl pyrrole core structure—One confirmed hit from the *B. anthracis* helicase screen and one confirmed hit from the *S. aureus* helicase screen (Series E, compounds **14** and **15**, respectively, in Table 2) share an N-phenyl pyrrole chemotype. While they appeared to be quite potent, they were the only confirmed hits that failed to exhibit the expected mass in LC-MS analysis. Further studies demonstrated that fresh DMSO solutions of re-ordered compounds exhibited no detectable helicase inhibitory activity, but solutions aged for several days in DMSO, did exhibit potent activity. This class of inhibitors was considered artifactual due to instability and not pursued further. They have been described previously as inhibitors of other targets^{12, 13}, and thus, appear to be promiscuous inhibitors as well (see Discussion).

2.3.6 Inhibitors with unique structures—Three confirmed *B. anthracis* helicase inhibitors (compounds **16**, **17** and **18**, Table 2) represent singleton chemotypes. Only compound **17** inhibited helicase *in vitro* as well as DNA replication in permeabilized cells. While not active against *S. aureus* helicase, this inhibitor exhibited an MIC against *B. anthracis* Sterne cells consistent with its IC₅₀ and displayed low cytotoxicity, yielding a selectivity index (CC₅₀/MIC) of ~2.7. Singleton **18** was about equally potent at inhibiting both *B. anthracis* and *S. aureus* helicases, but failed to produce a detectable MIC vs. either species. Computational searches of the screening library database revealed no analogs with 90% or better similarity to any of the three singletons (ChemBioOffice v 11.0, CambridgeSoft, Inc., MA).

2.4 Further characterization of the coumarin-type helicase inhibitors

Based on results from these secondary assays, three validated helicase inhibitors, compounds **2**, **12**, and **17**, exhibit sufficient potency and selectivity to warrant further optimization. However, the coumarin-type inhibitor, compound **2**, is clearly the most potent and selective (CC₅₀>100 μ M; helicase IC₅₀ ~8 μ M; MIC ~6-12 μ M, or ~3-6 μ g/ml, Table 2). Further characterization of this inhibitor, including mode of inhibition, determination of an inhibition constant (K_i), cidalty, antibacterial spectrum, and preliminary SAR studies are described below.

2.4.1 Mode of inhibition and K_i determination—Since the aminocoumarins coumermycin and novobiocin are known to inhibit the DNA gyrase function in DNA replication by competing with ATP binding¹⁴, we examined the mode of inhibition of the coumarin-type helicase inhibitor compound **2**. Assays of the ssDNA-stimulated ATPase

activity of *B. anthracis* replicative helicase in the presence of compound **2** revealed only weak inhibition (16% inhibition in the presence of 100 μ M compound **2**), indicating that this coumarin-type helicase inhibitor has little or no effect on ATP binding or hydrolysis by helicase in the absence of its duplex DNA substrate. Helicase kinetic studies with varying concentrations of ATP substrate and inhibitor suggested that compound **2** is uncompetitive with ATP at low inhibitor concentrations (Fig. 1A). However, a Dixon plot analysis ($1/V$ vs $[I]$) of the data was non-linear at inhibitor concentrations above the K_i at the two lower ATP concentrations tested, suggesting a possible second mode of inhibition at high compound **2** concentrations. Helicase kinetic studies with varying oligonucleotide substrate and inhibitor concentrations demonstrated that compound **2** exhibits a mixed competitive-uncompetitive mode of inhibition with the DNA substrate (Fig. 1B), and a Dixon plot indicates a K_i inhibition constant of ~ 8 μ M (Fig. 1C). In addition, two coumarins (compounds **1** and **2**) as well as one triazine (compound **12**) helicase inhibitor were tested and failed to inhibit gyrase at concentrations of up to 50 μ M (data not shown). This is not surprising since they lack the noviosyl sugar linkage, which is important for gyrase-inhibiting coumarins^{9, 15}. By contrast, two known gyrase-inhibiting coumarins, coumermycin and novobiocin, were potent inhibitors of gyrase in the assay (IC_{50} 's < 1 μ M) but did not inhibit *B. anthracis* helicase (data not shown). Thus, while these helicase inhibitors share a portion of the coumarin scaffold, they do not share a common biological mechanism.

2.4.2 Bactericidal—Inhibitors of DNA replication such as the fluoroquinolones and the anilinouracils are typically bactericidal^{16, 17} because successful replication and segregation of the chromosome into each cell is essential for viability. The coumarin-type helicase inhibitor compound **2** was examined and proved to be rapidly bactericidal within 5.5 hr at $4\times$ MIC (Fig. 2).

2.4.3 Initial SAR of the coumarin chemotype—To probe the structure-activity relationship for compounds **1** and **2**, nineteen additional structurally related compounds were examined against *B. anthracis* helicase (Table 3). The core bicyclic ring which is common to all five coumarin-type helicase inhibitors identified in screening was not altered, but some changes to all of the substituents were examined. The SAR results are summarized schematically in Fig 3A. Only one alteration increased the helicase inhibitory activity of **2**, replacement of the substituted phenyl ring for an unsubstituted naphthyl ring. However, this change was also associated with increased cytotoxicity (compound **1**, Table 2). Loss of the 8 position methyl group or shortening of the 3 position chain by one methylene group modestly reduced activity, but esterification of the 3 position carboxylic acid eliminated activity. These results suggest a pharmacophore representation as shown in Fig. 3B in which the acidic group and the oxygens interact with a hydrophilic region while the 4, 7, and 8 position substituents interact with a hydrophobic region.

2.4.4 Spectrum of antibacterial activity—The antibacterial spectra of the two related coumarin inhibitors, compounds **1** and **2**, as well as that of the triazine, compound **12**, were examined. The results reveal considerable breadth across the bacillus genus, with some limited activity against *S. aureus* (Table 4). Only the triazine displayed any activity against *E. coli*, and that was only in the *tolC*⁻ efflux-deficient strain. The Gram-negative outer membrane barrier or efflux may rescue *E. coli* cells from inhibition by these compounds. These results suggest that helicase inhibitors could be chemically optimized to be effective on a range of Gram-positive species.

3. Discussion

The results of this study have identified several new small molecule inhibitors of the replicative helicases of *B. anthracis* and *S. aureus*. Extensive characterization in secondary assays has

also confirmed that they act by interacting with helicase rather than with the DNA substrate and that several display antibacterial activity with minimal cytotoxicity. These compounds may provide a starting point for further optimization and the development of antibacterials with utility in biodefense or clinical settings. Four aspects of this study are of special interest. First, very few compounds inhibit *S. aureus* replicative helicase as compared to the *B. anthracis* helicase. Second, the validated hits identified in this study have not been described previously as helicase inhibitors. Third, several inhibitors share a portion of the aminocoumarin scaffold but inhibit helicase specifically, with no activity against gyrase. Fourth, compounds with an N-phenyl pyrrole chemotype are surprisingly unstable and may be a source of artifactual inhibitory activity in many screens. These points are discussed below.

3.1 Relative potency vs. two helicases

The relative difficulties in obtaining inhibitors of the *S. aureus* helicase and the differences in potency of inhibitors for the *S. aureus* vs. the *B. anthracis* helicases suggest that the *B. anthracis* helicase is more susceptible to small molecule inhibitors. The hit rate for *B. anthracis* helicase screening was nearly 10-fold higher than that for *S. aureus* helicase screening. While each screen was performed on a different library of compounds, the two libraries overlap substantially, and in addition, all hits are equipotent vs. the two helicases or more potent vs. *B. anthracis* helicase regardless of the screen from which they were identified. Structural differences in enzymatic targets from different bacterial species are known to affect the affinity for inhibitors significantly. In two recent examples, the affinities (measured as IC₅₀ values) of enoyl-ACP reductase (FabI) and peptide deformylase (Def) for inhibitors varied up to 95-fold when measured for the *E. coli* ortholog as compared to the *S. aureus* ortholog.^{7, 8} At the level of primary amino acid sequence, the overall similarity between the *B. anthracis* and *S. aureus* replicative helicases is 76%, but the similarity between the two proteins is lower in the N-terminal domain (residues 1-152) and drops to 66%. While the ATP and DNA binding sites appear to be in the C-terminal domains, recent crystallographic evidence suggests that a single-stranded DNA binding site may reside in the N-terminal domain, and it may help guide the unwound strand to primase, which binds to the helicase N-terminal domain.⁴ Interestingly, one of the three arginines (residues 116, 117, and 120) comprising this site is substituted in the *S. aureus* primary sequence with glutamine (residue 120). Possibly this difference or some other difference between the sequences is responsible for the different affinities of the two helicases for small molecule inhibitors.

3.2 Comparison with previous screening efforts and known inhibitors

The DnaB-type replicative helicases from *E. coli*, *S. aureus*, and *P. aeruginosa* have been targeted previously in anti-infective screens. Screening assay read-outs have included electrochemiluminescence¹⁸, fluorescence or FRET^{6, 19}, time-resolved FRET²⁰, scintillation proximity (SPA)²¹, and radiometric detection of ATPase inhibition²², but few hits have been described and none have progressed further in drug development. A triaminotriazine structure was shown to inhibit *P. aeruginosa* DnaB, but it displays significant cytotoxicity.⁶ A large anti-bacterial screening effort undertaken by GlaxoSmithKline, Inc. produced no hits to *S. aureus* replicative helicase.¹ While hits were obtained for another essential helicase in *S. aureus*, PcrA, which is involved in DNA repair and plasmid replication, no lead compounds could be developed from the hits.¹ Inhibitors of the *E. coli* ortholog of PcrA, helicase IV, have also been described, but no information on cytotoxicity was provided and these inhibitors do not appear to have progressed further.²² Two investigators have described inhibition of *E. coli* helicases (DnaB and RepA) by the flavone myrecitin;^{5, 23} however, this compound is quite promiscuous and cytotoxic. Similarly, intercalators and minor groove binders which interact with DNA are potent helicase inhibitors, but they lack bacterial selectivity as well.²⁴ Screening for inhibitors of *B. anthracis* replicative helicase as described in this report yielded new

inhibitors not reported previously. Possibly this is a result of screening with a new ortholog of helicase having a subtly different binding site for inhibitors.

3.3 Coumarin type inhibitors of helicase

The most potent and selective inhibitor identified in this study, compound **2**, contains a coumarin scaffold. A total of five coumarin-type helicase inhibitors were identified in the screen. They share a portion of a known bioactive chemotype, members of which inhibit gyrase by competing for ATP binding to the GyrB subunit. However, the helicase-inhibiting coumarins lack the noviosyl sugar moiety found in aminocoumarin antibacterials and consequently do not inhibit gyrase. This chemotype has a long history in antibacterial drug discovery and development. The aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A1 are produced by different *Streptomyces* strains and are potent antibacterials.²⁵ Novobiocin was marketed for several years as an antibacterial in an oral formulation (“albamycin” by Pharmacia & UpJohn), but is no longer used clinically. Its loss of favor as a clinical therapeutic probably results from its weak activity against Gram-negatives and the development of target-based resistance.²⁵⁻²⁷ There were no serious toxicity issues with the use of novobiocin for acute infections despite the fact that it competes for an ATP binding site on gyrase. In fact, it is tolerated in humans at doses as high as 3g/day.²⁸ This clinical history of the coumarin chemotype suggests that the coumarin helicase inhibitors could be well-tolerated in human therapy.

3.4 N-phenyl pyrrole type inhibitors are unstable and apparently promiscuous

The only helicase inhibitors which failed our quality control analysis by liquid chromatography and mass spectrometry were the two members of the N-phenyl pyrrole chemotype. These two compounds were not active against helicase when freshly dissolved in DMSO, but gained activity with time in DMSO solution. It is important to note that the instability of these compounds may not be generally recognized and could be responsible for their reported inhibition of HIV type 1 entry (compound NB-2¹³) and anthrax lethal factor (compound **10**¹²). In light of our results, it is important to re-examine other putative activities of this class of compounds.

4. Conclusions

This first reported screen for inhibitors of *B. anthracis* replicative helicase has yielded several validated hits which have never been reported to inhibit helicase. The strong bias towards *B. anthracis* helicase in numbers of screening hits and potency of validated hits argues that this enzyme is more susceptible to inhibition by small molecules than is the *S. aureus* helicase. The most potent and selective inhibitor, compound **2**, shares a portion of the aminocoumarin scaffold but inhibits helicase specifically.

5. Experimental

5.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study, their sources and relevant genotypes are described in Table 5.

5.2 Oligonucleotides

BHQ1 (“Hel-3’BHQ”) and 6-FAM (“Hel-5’FAM”) labeled 60-mer oligonucleotides (Table 6) were purchased from Operon, Inc. (Huntsville, AL) and Integrated DNA Technologies, Inc. (Coralville, IA), respectively, as HPLC purified. Slight variations in the mass quantitation and in RFU values required a careful calibration of each annealed batch to minimize batch-to-batch variation in the screen. The 30-mer capture strand (“Hel-Cap30”), which is complementary to

the 5'-30 nucleotides of the FAM-labeled oligo, was purchased from Operon, Inc. as desalted, unpurified. The two labeled oligonucleotide strands were annealed at a 1:2 (Hel-5'FAM:Hel-3' BHQ) ratio prior to use in the FRET quenching helicase assay. PCR primers used in this study are also shown in Table 6.

5.3 Cloning, expression, and purification of *B. anthracis* helicase

The cloning and expression of the *B. anthracis dnaB* gene and subsequent purification of the helicase has been described.²⁹ Briefly, the gene was amplified by PCR with primers HCASE45-5' and HCASE45-3' (Table 6) and *B. anthracis* genomic DNA. The amplified gene was inserted into a pET30 vector (Novagen Inc., Madison, WI) under the control of a T7 promoter and confirmed by DNA sequencing. *B. anthracis dnaB* was over-expressed in *E. coli* strain BL21 (DE3) RIL (Stratagene Inc. CA) harboring the pET30-DnaB_{BA} plasmid. Extraction of IPTG-induced cells was done as previously described (13). Protein was precipitated from the cell extract by addition of 0.25 g/ml (NH₄)₂SO₄, resuspended in buffer A (25 mM Tris-HCl, (pH 7.5), 5 mM MgCl₂, 10% glycerol, 5 mM DTT) and re-precipitated in 0.2 g/ml (NH₄)₂SO₄. The protein pellet was resuspended in buffer A and fractionated by Q-Sepharose chromatography (GE Health sciences, Piscataway, NJ), which removed any contaminating endogenous *E. coli* helicase.^{30, 31} The flow through fractions were pooled and loaded onto a 6 ml S-Sepharose column equilibrated with buffer A₁₀₀. *B. anthracis* helicase was eluted with a gradient of buffers A₁₀₀ and A₅₀₀. The peak fractions were identified by ssDNA dependent ATPase and DNA helicase activities in conjunction with SDS-PAGE. The active fractions were pooled and concentrated by ultrafiltration using a YM-30 membrane. The purified helicase was greater than 98% pure as analyzed by SDS-PAGE.^{29, 32} The enzyme was verified to be stable for several hours at room temperature, and for at least 12 weeks at -20°C in 20% glycerol storage buffer. *B. anthracis* helicase activity is dependent on the presence of ATP, with an optimum at 2.5 mM. The pH optimum is broad and centered around pH 7.8. Optimal concentrations of other assay components are as follows: 2 mM MgCl₂, 0.5 mM DTT, 0.01% Triton X-100, and 25 mM NaCl.

5.4 Cloning, expression and purification of *S. aureus* helicase

The genes for the *S. aureus* replicative DNA helicase (*dnaC*) and helicase loader (*dnaI*) were amplified by PCR from genomic DNA isolated from *S. aureus* Smith using the primers 5'Sa-dnaC and 3'Sa-dnaC for the helicase gene and primers 5'Sa-dnaI and 3'Sa-dnaI for the loader gene (Table 6). Products were sequence-confirmed and cloned in the dual expression vector pET-Duet1 (Novagen), under control of a T7 promoter/lac operator. The helicase was expressed in native form, while the loader was expressed with an N-terminal hexahistidine tag to facilitate purification. Although the helicase loader proved unnecessary either for solubility or helicase activity, the dual expression clone was used since it produced larger quantities of helicase than did a clone containing the *dnaC* gene alone in the same vector. Following overnight induction by IPTG at 14°C, cells were harvested by centrifugation and lysed in a French press. The lysate was centrifuged, and the resulting cleared supernatant was precipitated by addition of 0.2 grams of (NH₄)₂SO₄ per ml lysate. The precipitate was redissolved in buffer B (50 mM Tris pH 7.5; 1M NaCl; 10% glycerol; 2 mM 2-mercaptoethanol) containing 1 mM PMSF, and applied to an IMAC-Ni⁺⁺ column equilibrated in the same buffer. Following a wash in the same buffer, the column was eluted in a 0-200 mM imidazole gradient. The fractions containing helicase were pooled and applied to a phenyl sepharose column equilibrated in buffer B containing 1 mM EDTA; the column was eluted in gradient of decreasing NaCl (1 to 0 M) and increasing Triton X-100 (0 to 1%). The resulting helicase preparation was about 98% pure by SDS-PAGE and essentially free of nuclease activity as judged by minimal ATP-independent activity in the FRET assay. Optimal conditions for the reaction include a pH range of 7.6-8.4, a magnesium concentration of 2 mM, and an ATP concentration of 3 mM. The

enzyme was stable at room temperature for at least two hours and at -20°C in 20% glycerol storage buffer for several months.

5.5 Assays of helicase catalyzed strand unwinding

5.5.1 Fluorescent resonance energy transfer (FRET) assay—The FRET-based helicase activity assay was performed essentially as previously described^{6, 19} using labeled annealed oligodeoxynucleotides Hel-5'FAM:Hel-3'BHQ (Table 6). The assay is based on the helicase-mediated dissociation of two annealed oligonucleotides, one with a fluorescent label, the other bearing a quencher moiety. Two complementary annealed 60-mer oligonucleotides with noncomplementary 30-mer ends were labeled, respectively, with 6-carboxyfluorescein (6-FAM) and Black Hole Quencher 1 (BHQ1). When the two strands are unwound by helicase action, the 6-FAM emits an unquenched signal at 535 nm followed by excitation at 485 nm. To prevent the 6-FAM-labeled strand from reannealing with the quencher strand, an unlabeled 30-mer capture strand, Hel-Cap30, was added in 15-fold molar excess (Table 6). The FRET assay demonstrates ATP- and enzyme-dependent helicase activity and is linear in its response for at least 30 minutes at room temperature. It is tolerant of DMSO concentrations up to at least 2%.

5.5.2 Radiometric assay—Radiometric assays of helicase activity were performed as described previously^{31, 33} utilizing a radiolabeled sixty-mer:M13 partial duplex substrate (Table 6) possessing 5-nucleotide forks at both the 5' and 3' ends. A standard 20 μl reaction volume contained 25 mM Tris-HCl, (pH 7.5), 10 mM MgCl_2 , 10% glycerol, 5 mM DTT and 0.1 mM ATP, 17 fmol ($1-2 \times 10^4$ cpm/ μl) of substrate and the indicated amount of helicase. The mixtures were incubated at 30°C for 15 min and the reactions were terminated by the addition of 4 μl of 2.5% SDS, 60 mM EDTA, and 1% bromophenol blue. Displacement was monitored by polyacrylamide gel electrophoresis, followed by autoradiography.

5.6 High-throughput screening for inhibitors of helicase activity

Two small molecule libraries were screened in this study. The Microbiotix, Inc. collection (MBX) was screened for inhibitors of *B. anthracis* helicase, and the collection at the National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB) at Harvard Medical School (Boston, MA) was screened for inhibitors of *S. aureus* helicase. Both libraries were comprised of compounds purchased from commercial vendors, and while the overlap between the two libraries has not been calculated, it is likely to be significant because some of the same vendors were used. Compounds in the Microbiotix screening deck were purchased from Chembridge (San Diego, CA), Timtec (Newark, DE), and ChemDiv (San Diego, CA) while the NSRB collection contained compounds from Asinex (Moscow), Enamine (Kiev, Ukraine), Life Chemicals, Inc. (Burlington, Ontario, CA), and Maybridge (Fisher Scientific International), as well as Chembridge and ChemDiv. Compounds were selected in the molecular size range of 200 to about 500 Da and were filtered to remove unwanted and known cytotoxic fragments, including metal complexes, highly conjugated ring systems, oxime esters, nitroso and strong Michael acceptors. Compounds in the Microbiotix collection were diluted in 96-well master plates at 2.5 mM in DMSO at -20°C . Master plates were thawed at room temperature on the day of the screen, and 1 μl of compound was added to the 384-well screening plates by means of a Sciclone ALH 3000 liquid handling robot (Caliper, Inc.) and a Twister II Microplate Handler (Caliper, Inc.). Compounds in the NSRB collection were added to assay plates by pin transfer. The first two columns of wells contained the complete reaction without inhibitors (positive control), and the last two columns contained the complete reaction, but no enzyme (negative control). Screening wells contained 30 μl volume consisting of ~ 80 μM compound, 10 nM annealed oligo duplex, $15\times$ capture strand, 63 ng helicase (12 nM monomer), 30 mM Tris-HCl (pH 7.9), 0.01% Triton X-100, 0.5 mM DTT, 2 mM MgCl_2 , and 25 mM NaCl. The reaction was initiated

by the addition of ATP to 2.5 mM final concentration using a Wellmate automated microplate dispenser. Plates were incubated at room temperature for 30 min, and fluorescence was read at 530 nm in an Envision 2102 Multilabel HTS Counter (Perkin Elmer) with excitation at 490 nm. Strand unwinding catalyzed by helicase resulted in loss of quenching and a linear increase in fluorescence during the 30 minute incubation at room temperature. The capacity of the screens was 50,000 compounds in singlet per week (7,000 compounds per day). The plate Z' value³⁴ averaged 0.59, and the signal:background ratio averaged 3.6. All screening data, including the z-score, and confirmation and validation data were stored in one central database (CambridgeSoft's ChemOffice 11.0). Validated hits were re-ordered from the vendor and confirmed to be >95% pure and to be of the expected mass by LC-MS analysis. Compounds for SAR analysis were ordered from Chembridge, Inc.

5.7 Secondary assays to confirm and validate inhibitors

5.7.1 Other enzymatic assays—Hits were tested for inhibition of purified *P.*

aeruginosa AmpC β -lactamase in the presence of various concentrations of Triton X-100 to detect compounds acting promiscuously by a colloidal aggregate mechanism as described previously.¹¹ The ability of compounds to inhibit *B. subtilis* Pol IIIIC DNA polymerase was assayed by using a 96-well plate format version of the standard DNA polymerase assay as described previously.³⁵ Note that since the substrate for the Pol IIIIC assay is DNase-activated chromosomal DNA, no helicase function is required for polymerization.³⁵ Inhibition of *B. subtilis* DNA gyrase by compounds was measured by a published gel mobility assay.³⁶ Inhibition of the ssDNA-stimulated ATPase activity of *B. anthracis* helicase was measured (10 μ l volume, 100 μ M inhibitor) in the presence of 0.5 mM ATP and 200 pmole of M13mp19 single-stranded DNA as previously described.²⁹

5.7.2 Kinetic measurements—Values for K_i and IC_{50} for inhibitory compounds were determined by using the FRET-based assay under the same conditions as described for screening except that annealed oligonucleotide substrate or inhibitor concentrations were varied. All IC_{50} values were determined in duplicate using a 10-point curve consisting of two-fold dilutions of inhibitory compound from 100 μ M to 0.2 μ M. Substrate and inhibitor concentrations for kinetic experiments are noted in the figure. Rapid assessments of mode of inhibition were done by the method of Wei et al.³⁷ by determining the variation in IC_{50} values over a range of substrate and inhibitor concentrations.

5.7.3 EtBr-displacement—Hits were examined in a microplate ethidium bromide displacement assay¹⁰ to determine whether compounds bind to the DNA duplex substrate.

5.7.4 DNA replication assays in permeabilized cells—Compounds were tested for inhibition of ATP-dependent DNA replication in permeabilized Δ *polA* *B. anthracis* Sterne cells assay according to a modification of the method of Brown et al.³⁸ This assay was used to confirm whether the compounds are capable of inhibiting an intact bacterial replisome.

5.7.5 Minimum Inhibitory Concentration (MIC) and Bactericidal Assays—MICs were determined by the broth microdilution method described in the CLSI (formerly NCCLS) guidelines.³⁹ All MICs were expressed in μ M to facilitate comparisons with IC_{50} and CC_{50} values and were determined in duplicate using a 10-point curve consisting of two-fold dilutions of inhibitory compound from 100 μ M to 0.2 μ M. For bactericidal tests, inhibitors were examined in a standard method of broth culture of *B. anthracis* Sterne cells followed by plating on LB agar media and counting colony-forming units.⁴⁰

5.7.6 Determination of Mammalian Cytotoxicity—The cytotoxic concentration (CC_{50}) of compounds versus cultured mammalian cells (HeLa) was determined as the

concentration of compound that inhibits 50% of the conversion of MTS to formazan.⁴¹ Values were determined in duplicate using a 10-point curve consisting of two-fold dilutions of inhibitory compound from 100 μ M to 0.2 μ M. The “selectivity index” of a given agent is defined as the ratio of its mammalian cell cytotoxicity to its MIC value against *B. anthracis* (i.e. CC₅₀/MIC).

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Figure 1.

Kinetic analysis of coumarin-type inhibitor compound **2** vs. *B. anthracis* helicase in the FRET quenching assay. Data are displayed in the following linear transformations: **(A)** Dixon plot with 0.625 mM ($\diamond$), 1.25 mM ($\square$), 2.5 mM ($\triangle$), and 5 mM ($\circ$) ATP substrate present; **(B)** Lineweaver-Burk plot with 12.5 μ M ($\square$), 6.25 μ M ($\diamond$), 3.13 μ M ($\triangle$), and 0 μ M ($\circ$) inhibitor present; **(C)** Dixon plot with 5 nM ($\diamond$), 10 nM ($\square$), 30 nM ($\triangle$), and 100 nM ($\circ$) annealed oligonucleotide substrate present. Lines are drawn based on a linear or polynomial regression analysis of the data.

Figure 2.

Viability of *B. anthracis* Sterne cells incubated with compound **2** in broth culture at multiple concentrations. Compound **2** was added to LB cultures of *B. anthracis* Sterne cells at 0.5× MIC (○), 1× MIC (△), 4× MIC (□), or omitted from the culture ◇, and aliquots were spread on LB agar plates at various times indicated on the abscissa to determine the number of viable colony-forming units. Lines are drawn based on an exponential regression analysis of the data.

Figure 3. Summary of preliminary SAR results for coumarin type inhibitor, compound **2**. **(A)** The substructure shared by all five coumarin-type helicase inhibitors is outlined in a box, and approximate effects of specific structural alterations are noted. **(B)** A pharmacophore representation of the coumarin type helicase inhibitors.

Table 1
Summary of High Throughput Screens for Inhibitors of Two Helicases

Enzyme/Library	Compounds Screened	Confirmed Hits	Confirmed Hit Rate	Validated Hits	Validated Hit Rate
<i>B. anthracis</i> helicase/MBX ¹	78,588	1390	1.8%	150	0.19%
<i>S. aureus</i> helicase/NSRB ²	108,026	210	0.2%	30	0.03%
Total	186,614	1600	0.8%	180	0.10%

¹ MBX, Microbiotix, Inc.;

² NSRB, National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease

Table 2

Structure and Properties of Confirmed Helicase Inhibitors

No	Series	Structure	IC ₅₀ -Ba ^I	IC ₅₀ -Sa ²	MIC-Ba ³	MIC-Sa ⁴	IC ₅₀ -cell ⁵	CC ₅₀ ⁶
1	A		6	65	6	>100	107	6
2	A		12	180	10	>100	25	>100
3	A		10	42	20	24	10	10
4	A		4	15	>100	>100	100	>100
5	A		10	15	55	23	100	35
6	B		12	56	>100	>100	>100	10
7	B		12	15	>100	>100	>100	70
8	C		2	3	>100	>100	30	>100

No	Series	Structure	IC ₅₀ -Ba ¹	IC ₇₅ -Sa ²	MIC-Ba ³	MIC-Sa ⁴	IC ₅₀ -cell ⁵	CC ₅₀ ⁶
9	C		1	2	>100	>100	73	>100
10			0.5	0.7	>100	>100	n.d.	11
11	C		2	4	>100	>100	95	>100
12	D		24	47	50	75	61	68
13	D		12	21	>100	>100	>100	4
14	E		4*	4*	50*	50*	>100	>100
15	E		4*	4*	>100	>100	n.d.	>100
16	n.a		25	>100	25	>100	>100	66
17	n.a		25	>100	30	>100	27	80
18	n.a		15	12	>100	>100	>100	>100

All data are expressed in μM to facilitate comparisons.

¹IC₅₀ vs. *B. anthracis* replicative helicase;

²IC₅₀ vs. *S. aureus* replicative helicase;

³ MIC vs. *B. anthracis* Sterne;

⁴ MIC vs. *S. aureus* Smith;

⁵ IC₅₀ vs Δ *polA* *B. anthracis* Sterne permeabilized cells;

⁶ CC₅₀ vs. HeLa cells;

* results were variable due to instability of compound in DMSO (see text for details).

SAR of Coumarin Inhibitors

Table 3

R1	R2	R3	R4	Cpd. No.	IC ₅₀ (μM)
1-naphthyl	methyl	methyl	propanoic acid	1	7
2-fluoro-4-bromobenzyl	methyl	methyl	propanoic acid	2	14
2-naphthyl	methyl	methyl	acetic acid	19	25
1-naphthyl	H	methyl	propanoic acid	20	50
4-tert-butylbenzyl	methyl	methyl	acetic acid	21	57
3-methoxybenzyl	methyl	methyl	propanoic acid	22	93
4-methoxybenzyl	H	methyl	propanoic acid	23	>100
4-methoxybenzyl	H	methyl	ethyl-l-propanoate	24	>100
benzyl	methyl	methyl	acetic acid	25	>100
3-methoxybenzyl	H	methyl	propanoic acid	26	>100
trifluoromethylbenzyl	methyl	methyl	H	27	>100
3-methoxybenzyl	H	methyl	ethyl-propanoate	28	>100
benzyl	H	methyl	H	29	>100
4-methylbenzyl	methyl	ethyl	H	30	>100
2-naphthylmethyl	methyl	ethyl	H	31	>100
4-fluorobenzyl	H	methyl	H	32	>100
2-methyl-1-naphthyl	H	methyl	H	33	>100
1-naphthyl	H	methyl	ethyl-propanoate	34	>100
3-fluorobenzyl	methyl	methyl	ethyl-l-propanoate	35	>100
1-naphthyl	methyl	methyl	methyl-l-propanoate	36	>100
3-fluorobenzyl	methyl	methyl	propanoic acid	37	>100

Table 4
Average MICs ($\mu\text{g/ml}$) for Three Confirmed Hits

Bacterial Strain	Cpd 1	Cpd 2	Cpd 12
<i>B. anthracis</i> Weybridge (pXO1 ⁺ /pXO2 ⁻)	1.3	2.8	28.7
<i>B. anthracis</i> Ames 35 (pXO1 ⁺ /pXO2 ⁻)	1.3	2.8	28.7
<i>B. cereus</i> ATCC 4342	2.5	2.8	57.3
<i>B. anthracis</i> Sterne	1.3	2.8	28.7
<i>B. anthracis</i> Ames Δ ANR (pXO1 ⁺ /pXO2 ⁻)	1.3	2.8	28.7
<i>B. thuringiensis</i> ATCC 10792	2.5	2.8	57.3
<i>B. licheniformis</i> ATCC 14580	2.5	2.8	57.3
<i>B. subtilis</i> BD54	15.1	4.2	>60
<i>B. stearothermophilus</i> ATCC 12980	5.0	5.6	57.3
<i>B. megaterium</i> ATCC 12872	>40	>45	57.3
<i>S. aureus</i> ATCC 25923	2.5	11.2	43.0
<i>S. aureus</i> (MRSA) 1094	5.0	>45	57.3
<i>E. faecalis</i> ATCC 29212	>40	>45	>60
<i>E. faecalis</i> VRE ATCC 51575	>40	>45	>60
<i>E. coli</i> 700 TolC ⁺	>40	>45	>60
<i>E. coli</i> 701 TolC ⁻	>40	>45	28.7

Table 5
Bacteria strains and plasmids

Species	Strain ID	Relevant Genotype	Source (Ref.)
<i>B. anthracis</i>	Sterne 34F2	pXO2 ⁺	Colorado Serum Co.
<i>B. anthracis</i>	Sterne ΔpolA	pXO2 ⁺ ΔpolA	This study
<i>B. anthracis</i>	Weybridge	pXO1 ⁺ pXO2 ⁺	S. Leppla, NIAID
<i>B. anthracis</i>	Ames ΔANR	pXO1 ⁺ pXO2 ⁺	K. Shatalin ⁴²
<i>B. anthracis</i>	Ames 35	pXO2 ⁺	S. Leppla, NIAID ⁴³
<i>B. stearothermophilus</i>	ATCC 12980	Wild-type	ATCC
<i>B. thuringiensis</i>	ATCC 10792	Wild-type	ATCC
<i>B. licheniformis</i>	ATCC 14580	Wild-type	ATCC
<i>B. megaterium</i>	ATCC 12872	Wild-type	ATCC
<i>E. faecalis</i>	ATCC 29212	Wild-type	ATCC
<i>E. faecalis</i>	ATCC 51575	VRE	ATCC
<i>S. aureus</i>	ATCC 25923	Wild-type	ATCC
<i>S. aureus</i>	ATCC 13709	“Smith” wild-type strain	ATCC
<i>S. aureus</i>	1094	MRSA clinical isolate	R. Ellison, U. Mass Medical School
<i>B. subtilis</i>	BD54	ile-1, leu-8, metB5	N. Brown ⁴⁴
<i>E. coli</i>	700	Wild-type	G. Jacoby, Lahey Clinic, Mass.
<i>E. coli</i>	701	ΔtolC	G. Jacoby, Lahey Clinic, Mass.
<i>E. coli</i>	SG101	ΔtolC	Ghosh et al. ⁴⁵
<i>E. coli</i>	SG101-DnaCI	pET-Duet1-SaDnaC-DnaI	This study
<i>E. coli</i>	BL21(DE3)-DnaB _{BA}	pET30-DnaB _{BA}	Biswas et al. ²⁹

Table 6
Sequences of Oligonucleotide Primers and Helicase Substrates*

Hel-5'FAM	6-FAM-ATCTTTCTGAGTACGAGAGTTCTGAGCAGTTCCAATACATTTTTTTTTTTTTTTTTTTTTT
Hel-3'BHQ	TTTTTTTTTTTTTTTTTTTTTCTCTGCTTTACTGCTCAGAACTCTCGTACTCAGAAAGAT-BHQ1
Hel-Cap30	ACTGCTCAGAACTCTCGTACTCAGAAAGAT
5'Sa-dnaC	AAAAAAAGCTTCATATGGATAGAATGTA
3'Sa-dnaC	TGATTATGGTACCGAAAACTTTT
5'Sa-dnaI	AAAAAGGATCCGAAGCAATTTAAAAGTA
3'Sa-dnaI	GATTTGTATTTCATTGTGCGACCAATCATTTT
HCASE45-5'	ATCTCCATGGTCACGACGTTGTAAAACGACGGCCAGTGAATTCTGA
HCASE45-3'	TCACGACGTTGTAAAACGACGGCCAGTGAATTCTGAATCTCCATGG
Sixty-mer	GGGTCTCACGACGTTGTAAAACGACGGCCAGTGAATTCTGAGCTCGGTACCCGGGGTAGGA

* All sequences are presented in the 5' to 3' orientation