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Structure-activity relationship for enantiomers of potent inhibitors of *B. anthracis* dihydrofolate reductase

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Abstract

Background—Bacterial resistance to antibiotic therapies is increasing and new treatment options are badly needed. There is an overlap between these resistant bacteria and organisms classified as likely bioterror weapons. For example, *Bacillus anthracis* is innately resistant to the anti-folate trimethoprim due to sequence changes found in the dihydrofolate reductase enzyme. Development of new inhibitors provides an opportunity to enhance the current arsenal of anti-folate antibiotics while also expanding the coverage of the anti-folate class.

Methods—We have characterized inhibitors of *Bacillus anthracis* dihydrofolate reductase by measuring the K_i and MIC values and calculating the energetics of binding. This series contains a core diaminopyrimidine ring, a central dimethoxybenzyl ring, and a dihydrophthalazine moiety. We have altered the chemical groups extended from a chiral center on the dihydropyridazine ring of the phthalazine moiety. The interactions for the most potent compounds were visualized by X-ray structure determination.

Results—We find that the potency of individual enantiomers is divergent with clear preference for the *S*-enantiomer, while maintaining a high conservation of contacts within the binding site. The preference for enantiomers seems to be predicated largely by differential interactions with protein residues Leu29, Gln30 and Arg53.

Conclusions—These studies have clarified the activity of modifications and of individual enantiomers, and highlighted the role of the less-active *R*-enantiomer in effectively diluting the more active *S*-enantiomer in racemic solutions. This directly contributes to the development of new antimicrobials, combating trimethoprim resistance, and treatment options for potential bioterrorism agents.

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Keywords

antibiotic resistance; *Bacillus anthracis*; dihydrofolate reductase; dihydrophthalazine; enantiomer; racemate

1. Introduction

Bacteria affecting human health are increasingly acquiring antibiotic resistance [1]. All strains of *Bacillus anthracis*, the causative agent of anthrax, encode for a dihydrofolate reductase (DHFR) enzyme that is not susceptible to trimethoprim, which is the only commercially available anti-DHFR therapy for bacterial infections [2–4]. Some strains of *B. anthracis* are Category A Select Agents, and they have been documented as previously engineered and weaponized by some countries [5]. This provides a unique advantage in terms of biodefense, as cellular functions not currently targeted by therapeutics are unlikely to be maliciously engineered.

DHFR inhibitors are an active and established area of development, and many recent efforts are using this target to respond to the problem of antibiotic resistance. Aside from the scaffold described herein and also previously by Basilea Pharmaceutica Ltd. [6, 7], other anti-DHFR compounds under development include Iclaprim, being pursued by Acino Pharma [8], AR-709, pursued by Evolva [9], and 7-aryl-2,4-diaminoquinazolines, pursued by Trius Therapeutics [10]. A review of recent patent literature outlined antibacterial efforts targeting DHFR specifically for bacteria relevant to human health, including *Staphylococcus aureus*, *Pneumocystis carinii* and *B. anthracis* [11].

As part of our ongoing program to develop antimicrobials capable of targeting *B. anthracis* we have extended the previously reported dihydrophthalazine-based RAB1 series [2, 12]. Completion of the X-ray crystal structure of *B. anthracis* DHFR complexed with RAB1 highlighted the long and deep hydrophobic pocket of ~ 600 Å³ normally accommodating dihydrofolate as part of the catalytic addition of protons to form tetrahydrofolate [12]. This step is essential to bacterial metabolism, and inhibition leads to depletion of precursors needed for synthesis of nucleic acids [13]. Contacts between the protein and the diaminopyrimidine ring were conserved relative to known interactions of this site with substrate or other anti-folates [14–17]. These contacts include Glu28, of which an equivalent residue is present in all known DHFR enzymes, and Phe96, which has been implicated in mediating resistance to trimethoprim [14, 18]. Overall the interactions between the protein and RAB1 were hydrophobic and included more than 20 other residues. The dihydrophthalazine moiety displayed shape complementarity to residue Leu55 and the dihydrophthalazine placement within the binding site triggered a conformational change of the side chains of Arg58 and in turn Met37. These observations provided evidence of specificity for bacterial versus human DHFR due to the terminal dihydrophthalazine moiety, as its length and volume could not be accommodated with the human DHFR binding pocket [12].

Original work on this series was carried out in conjunction with Basilea Pharmaceutica Ltd. The most promising modification was at a chiral carbon within the dihydropyridazine ring, but the chemical space that was explored was limited to linear alkyl or six-membered rings, with some extensions from these six-membered rings in only the ortho position [2]. RAB1 contains an *n*-propyl at this chiral carbon, and a preference for the *S*-enantiomer was visualized in the crystal structure [12]. This preference was mediated by the position of the terminal guanidino group of Arg58, which would sterically occlude the binding of the R-enantiomer at this position. Further, the position of Arg58 was solidified by hydrogen

bonding to the carbonyl-linker of RAB1 either directly or *via* a water molecule. In the current work, we have continued these studies by further altering the group at this chiral carbon, which is located at the protein and solvent interface, determined the effect on potency, and compared this to *in silico* calculations as well as binary co-crystal structures available for the more potent compounds (Fig. 1).

2. Material and methods

Chemical synthesis of inhibitors has been detailed in previous publications [12, 19]; separation of enantiomers has also been reported [20]. Protein expression, purification and crystallization followed that described earlier [12]. Assessment of enzymatic activity and the minimum inhibitory concentrations (MIC) were also described previously [21]. Conversion of IC₅₀ values to inhibition constant (K_i) values was achieved by using the Cheng-Prusoff equation with a determined K_m value of 16.3 μM (data not shown). X-ray data for the *R*-isobutenyl and *R,S*-isobutyl complexes were collected at the Center for Advanced Microstructures and Devices, Baton Rouge, LA, they were indexed and scaled with HKL2000 [22]. The *R,S*-isobutyl data set was extremely weak and in many frames exhibited a split pattern of diffraction; this is reflected in the value of R_{sym} (Table 1). Data for the *R,S*-trifluoropropyl complex were collected at the University of Oklahoma, Norman, OK; these were indexed with iMosflm and scaled with SCALA [23, 24]. Data for the *R,S*-phenyl and *R,S*-isopropyl complexes were collected at Oklahoma State University, Stillwater, OK, and were indexed and scaled with Saint and SADABS, as incorporated in the Proteum2 software suite [25]. Statistics for the processing and scaling of these data sets utilized Xprep [26], which reports values of R_{sym} for all data causing an apparent increase relative to other data sets (for *R,S*-phenyl the overall value is 19.2%, and for *R,S*-isopropyl the overall value is 11.6%, see Table 1). Three-dimensional structures were isomorphous with the previous structure, PDB ID 3FL8 [12].

Refinement was carried out with Phenix [27] supplemented by the program Coot [28] for model visualization and manual building. When dual occupancy of enantiomers was identified, coordinates for the enantiomeric models were split into the portion sharing the same position and the portion showing different positions. Only the later portion was used to calculate occupancy. Figures were generated with the Chimera program from UCSF [29]. Model coordinates and structure factors have been deposited with the Protein Databank and accession codes are listed in Table 1. Atomic contacts between protein and ligand molecules were calculated with the Ligand-Protein Contacts server [30], assisted by calculations from the CASPP server [31].

Calculation of energy values upon complexation (ΔE) was performed with minimization algorithms from the SYBYL software suite (SYBYL 8.1, Tripos Certara Company) and with Monte-Carlo simulations performed with in-house software (Sapient Discovery). Starting models were based on the previously published 3FL8 structure with the *S*-*n*-propyl modification [12] or with the current structure 4ELH with the *R*-isobutenyl modification. All calculations utilized a 7 Å diameter sphere within the binding site for flexibility and minimization, and incorporated terms for van der Waals and electrostatic forces as well as solvation energies. Agreement (R²) of ΔE_{binding} with measured IC₅₀ values is 0.94 when the outlier *para*-trifluoromethoxybenzyl is excluded, as no reliable IC₅₀ could be measured for this compound.

3. Results and Discussion

3.1 Potency in biological assays

Our current efforts have incorporated biochemical assessment of the inhibition constant (K_i) and microbiological assays to determine the minimum inhibitory concentration (MIC). When comparing these values it is evident that general trends in activity are shared, although the K_i values are more sensitive to individual modifications. We have used the MIC values, as global patterns are more readily discerned, in concert with the chemical profile of the modifications to classify this series into four groups (Fig. 1). The first group contains alkyl moieties comprising compounds *n*-propyl (RAB1), isopropyl, trifluoropropyl, isobutyl, isobutenyl and 1-ethylpropyl. As a class, these systems contain generally favorable MIC and K_i values, but the preference for a linear or secondary branched alkyl substituent is clear, as found with isobutyl and isobutenyl (K_i 6.9 nM and 8.2 nM, respectively) versus isopropyl and 1-ethylpropyl (K_i 13.2 nM and 12.5 nM, respectively). The second group of modifications contains a core phenyl moiety, which was among the most potent with a K_i of 5.0 nM. The addition of fluorine atoms had minimal impact on potency, with a somewhat more deleterious effect in the para (K_i 6.4 nM) versus the meta (K_i 4.8 nM) position. The MIC values for these two groups are generally favorable and range within one two-fold dilution of 1 μ g/mL, with the exception of the 1-ethylpropyl moiety that had a further elevated MIC of 4–8 μ g/mL. Larger additions to the phenyl ring, as found in the third class comprising *para*-methylphenyl, *meta,meta'*-dimethylphenyl and *ortho*-methylphenyl, were less favored, with K_i values ranging 11.2 – 30.7 nM and MIC values becoming progressively worse from 1–2 μ g/mL range up to 8 μ g/mL as the substituent increased in size. The fourth class of modifications contains larger groups built from a benzyl moiety, with K_i values from 28 nM and higher and MIC values starting at 4 μ g/mL and increasing to 16 μ g/mL. We estimate that members of both the third and fourth class would protrude out of the protein pocket and into solvent, likely accounting for their poorer inhibitory profiles (Fig. 1).

Overall, the *n*-propyl, trifluoropropyl, isobutyl, and isobutenyl components from the first class and the phenyl modifications of the second class displayed the best potency, with the top two compounds containing isobutenyl (K_i 8.2 nM, MIC 0.5 μ g/mL) or phenyl (K_i 5.0 nM, MIC 0.4–1.5 μ g/mL).

3.2 Activity of *S*- and *R*-enantiomers for most active compounds

We have also examined the enantiomeric preference within the *n*-propyl, isobutyl or isobutenyl moieties, which are among the most potent members of the current series. For each compound tested, the racemic mixture was separated into the component enantiomers and resulted in solutions with estimated 99.5% purity [20]. Among measurements of K_i and MIC parameters it is striking that little variation can be attributed to the *R*-enantiomers. In contrast, there is a definite progression of improving activity among the *S*-enantiomers that mirrors subtle improvements seen with the racemic mixture (Fig. 2). The K_i values for an *S*-enantiomer are approximately half that of its racemic mixture; for example, *S*-isobutenyl has a K_i of 4.4 nM while racemic (*R,S*)-isobutenyl has a K_i of 8.2 nM. This relationship is not as clear in the MIC values, where the racemic (*R,S*) mixture tends to display quite similar values as the *S*-enantiomer. For example, the MICs for racemic (*R,S*)-isobutenyl and for *S*-isobutenyl are 0.5–1 μ g/mL; similarly for (*R,S*)-isobutyl and for *S*-isobutyl MICs are 1–2 μ g/mL. This is likely due to the less sensitive nature of MIC experiments to small variations. Interestingly, the same correlation of racemate to *S*-enantiomer does not hold true for *n*-propyl (RAB1), which has MIC values of 1–3 μ g/mL for the racemate but 0.2–0.5 μ g/mL for *S*-*n*-propyl. These observations indicate that the *R*-enantiomer within the mixture contributes very little to the inhibition but instead serves to

dilute out the *S*-enantiomer (Figs 1, 2). It is therefore reasonable to assign an improvement in potency of racemic mixtures to more favorable interactions with the *S*-enantiomer.

3.3 Three-dimensional structure determination of binary complexes

The binary structure of *B. anthracis* DHFR co-crystallized with racemic *n*-propyl-inhibitor RAB1 has been reported [12]. As in the current work, the protein crystal was composed of eight crystallographically unique copies, which display very low structural variation (R.M.S. deviation of ~ 0.25 Å) except for the four residues at the C-terminal. These residues are an artifact of the thrombin cleavage site used to remove the 6-His tag, and they are intimately involved in crystal packing interactions. Each of these eight chains had full occupancy of only the *S*-enantiomer of RAB1.

In the current work these results have been extended to include binary co-crystal structures with inhibitor derivatives containing racemic trifluoropropyl, isopropyl, isobutyl and phenyl (Table 1). Within the crystal lattice, electron density for each chain is an average of all of the individual protein molecules corresponding to that one position from the eight crystallographically unique copies. We find that the enantiomer bound by the individual protein molecules can be mixed in some of the crystallographically unique copies. The result is a partial occupancy of each enantiomer, such that some of the individual protein molecules contain the *S*-form while others contain the *R*-form, resulting in mutually exclusive electron density for both ligand forms at that crystallographically unique copy. While the trifluoropropyl and isopropyl complexes contain only the *S*-enantiomer, isobutyl and phenyl complexes display a mixture of *S*- and *R*-enantiomers among the eight protein chains forming the crystal, though the *S*-enantiomer is still heavily favored. In addition, the co-crystal structure with 99.5% purified *R*-enantiomer of isobutenyl has been determined to confirm the binding interactions with the less active isomer (Table 1).

Overall, the positional difference observed between the five co-crystal structures from the current work is on the order of 0.7 Å R.M.S. deviation. While some side chain movements are noted around the binding site, there is also mobility in the loop containing residues 18–20. This loop bridges the substrate binding site and the NADPH site, and the structural variation is likely a result of the empty NADPH pocket as no contacts are made from this loop with the ligand. Each structure also contains electron density characteristic of an ion at the base of the helix formed by residues 45–51. Based on coordination with backbone amide atoms this ion has been assigned as a chlorine atom, and its location superposes with an oxygen atom from a phosphate moiety of NADPH, which is consistent with other structures of DHFR in the absence of NADPH (Fig 3) [32]. This interaction provides stabilization for the base of this helix in the absence of the co-factor. This site is directly adjacent to the glycine residues of Phe96-Gly97-Gly98, which is of note as Phe96 is heavily implicated in substrate binding and believed by some to mediate TMP resistance [14]. In addition, Gly97 exhibits distorted Ψ - ϕ geometry that indicates stress at this backbone position. The Cl^- is likely a fiducial marker for a system of crosstalk between the substrate and co-factor binding site within a single protein molecule that links co-factor binding to substrate binding *via* Phe96.

Another prominent and conserved structural feature in the current DHFR co-crystal structures are two (or more) ordered water molecules that fill a ~ 56 Å³ channel adjacent to the primary amine (C4 position) of the diaminopyrimidine (DAP) ring and the exterior of the protein (Fig. 3). These ordered waters have also been visualized, for example, in the DHFR from *S. aureus* [21], *P. carinii* [33], *Mycobacterium avium* [34], and *Homo sapiens* [35, 36] but not *Trypanosoma brucei* [37]. Catalysis with folic acid substrates utilizes the other primary amine (C2 position) of the DAP ring, and so it is unclear what activity would make use of this conserved feature [38].

3.4 Interactions of inhibitors with the DHFR folate binding site

For any inhibitor, regardless of which enantiomer was bound, the scaffold DAP ring and the central dimethoxybenzene ring maintain conserved inhibitor:protein contacts. Hydrogen bonds complex the nitrogen atoms of the DAP moiety with residues Met6, Val7, Leu21, Glu28, Phe96, Tyr102 and variably Ala8 and Thr115. The DAP ring occupies the same space for each derivative. However, the face of the ring rotates by $\sim 14^\circ$ around an axis composed of the nitrogen atoms in response to lateral movement of ~ 1 Å by the central dimethoxybenzene ring. This movement correlates with the derivative and enantiomer bound, and in turn pivots the side chain of Phe96, which forms a parallel-displaced face-stacked interaction with the DAP ring (Fig. 3) [39]. This lateral movement is largest when the *R*-enantiomer is bound and results in further impingement of the inhibitor into the NADPH active site. The closest contact distances to a modeled NADPH, which was not present in the current binary co-crystal structures, with the inhibitor series are 3 Å from the central ring, 2.2 Å from the linker between this ring and the DAP ring, and 2.0 Å from the DAP ring.

3.5.1 Differences arising from enantiomer-specific contacts

The remaining group on each inhibitor is the terminal dihydrophthalazine, which imparts selectivity for bacterial over human DHFR [12]. When an *S*-enantiomer is complexed, the dihydrophthalazine moiety is surrounded by residues Val32, Arg53, Leu55, Arg58 and the aliphatic portion of Lys33 (Fig. 2, blue inhibitor with orange surface). A key element to the high degree of surface complementarity is Leu55 and the deviation from planarity of the dihydropyridazine ring of the phthalazine unit.

Complexation with an *R*-enantiomer is only accommodated by placing the phthalazine moiety across the binding site and into a cleft created by residues Leu29, Gln30 and Lys33 (Fig. 2, magenta inhibitor with blue surface). In this situation, the deeper portion of the active site is vacant, and, in some instances, Leu55 rotates into a different rotamer. Residues Arg58, and, in turn Met37, have previously been visualized protruding to the inside of this pocket [12]. In the current structures, their positions show variability in concert with Leu55, but they do not move completely inward to fill this empty space. The resulting positions of the derivatized groups contact residues Leu29, Gln30 and Lys33 for *S*-enantiomers (Fig. 2, blue inhibitor with blue surface) or Ile51, Arg53 and Leu55 for the *R*-enantiomer (Fig. 2, magenta inhibitor with purple surface). A conformational change of the side chain of Arg53 is necessary to avoid steric collision, depending on the enantiomer present. In most complexes with *S*-enantiomers, either a direct or water-mediated hydrogen bond is formed between the linker carbonyl and the terminal guanidino unit of Arg53, whereas with *R*-enantiomers the shift in inhibitor position towards the NADPH site precludes this interaction.

3.5.2 Co-crystal structure with *R,S*-trifluoropropyl and *R,S*-propyl inhibitors

Among the trifluoropropyl, isopropyl, and the previously determined *n*-propyl (RAB1) systems, specific interactions are largely conserved and, despite initiating the experiments with a racemic mixture, only the *S*-enantiomer occupies the binding site for each inhibitor. The fluorine atoms of *S*-trifluoropropyl approach Gln30 (2.5 Å to the terminal oxygen) and are surrounded by the carbonyl oxygen of Pro26 (4.1 Å), Leu29 (4.5 Å), and Lys33 (5.2 Å to the terminal nitrogen) (Fig. 3).

3.5.3 Co-crystal structure with *R*-isobutenyl inhibitor

For the *R*-isobutenyl complex, the terminal guanidine moiety of Arg53 is able to stack with the planar trigonal isobutenyl moiety (Fig. 2). There is latitude in the placement of the

isobutenyl group, as the terminal planar unit displays rotational freedom of over $\sim 30^\circ$ between the eight crystallographically unique copies, and the terminal group of Arg53 can be modeled to follow this movement. When compared to the *S*-enantiomer complexed site, Arg53 extends up or out of the binding site by approximately 1.7–3 Å to avoid a steric clash with the *R*-isobutenyl inhibitor. However, this side chain appears more mobile and as such its electron density is generally less well defined than neighboring atoms.

In this crystal lattice, one chain of the eight crystallographically unique molecules displays electron density suggestive of partial occupancy of 0.45 with an *S*-isobutenyl enantiomer. Interactions of *S*-isobutenyl are as described in section 3.5.4. It is unclear how the *S*-enantiomer would have been present, as the *R*-isobutenyl sample was highly purified. However, due to the 100-fold improved affinity for the *S*-enantiomer (Fig. 2), if any trace amount remained it would be readily incorporated into the binding site.

3.5.4 Co-crystal structure with *R,S*-isobutyl inhibitor

For the isobutyl structure, all eight sites contain strong electron density for the *S*-enantiomer, with additional density in two chains corresponding to partial occupancy (0.57 and 0.41) with the *R*-enantiomer (Table 1). The interactions with the isobutyl moiety are almost identical to those found with the *R*-isobutenyl or *S*-enantiomer derivatives; the exception is the interaction of the derivatized isobutyl group with Arg53. In this case, the isobutyl is not planar, and the *R*-configuration does not form a stack with the side chain termini of Arg53, but instead displaces the side chain upward from the binding site (Fig. 4). This movement is accompanied by more diffuse electron density at residue Leu55, reinforcing the linked behavior of these residues within the binding site. There is also lateral freedom of movement at the termini of the *R*-isobutyl modification, as there was for the *R*-isobutenyl moiety, indicative of weakened interactions to dock this portion of the inhibitor.

3.5.5 Co-crystal structure with *R,S*-phenyl inhibitor

When the inhibitor contains a phenyl group at the modified position, four of the eight protein binding sites are fully occupied by the *S*-enantiomer, two contain only the *R*-enantiomer, and two show evidence of dual occupancy (*R*-enantiomer 0.59 and 0.42) with both enantiomers (Table 1). To date this is the only occurrence of 100% occupancy with only the *R*-enantiomer when a racemic mixture was utilized.

The placement of *S*-phenyl isomer allows a closer approach to Gln30 and Leu29; the face of the *S*-phenyl modification seems to rest on the side chain of Leu29 with a distance range of 3.8–4 Å (Fig 3). The approach to Gln30 is closest for *S*-phenyl of all those visualized at 3.0–3.9 Å to the most terminal portion of the side chain. The phenyl group in the *R*-enantiomer is slotted between the aliphatic portion of Arg53 (3.6–4 Å) and Leu55 (3.1–3.7 Å) with some interactions with Ile51 (3.4 Å) (Fig 3). These interactions are similar to the *R*-isobutenyl interaction, and seem predicated on the planar configuration at the derivatized site to facilitate interaction with the delocalized guanidine portion of Arg53 for *R*-enantiomers and with the face of Leu29 for *S*-enantiomers.

3.6 Correlation with calculated energy of binding

Calculation of the energetic contributions of van der Waals forces, electrostatic interactions and solvation energy to the binding of each derivative was carried out to further quantify the contribution of enantiomers within a racemic mix. These values reveal a gain of greater than 5 kcal/mol for the *S*-enantiomer versus the *R*-enantiomer (Fig. 1), with an average ΔE for the *S*-form is 40.9 (± 0.7) kcal/mol, while the average ΔE for the *R*-form is 35.3 (± 0.5) kcal/mol. The calculated energy values present a general trend that overall is highly correlated with measured K_i values. It is noted that the most potent racemic inhibitors also

have the largest difference in binding energy between the *S*- and *R*-enantiomers ($\Delta\Delta E$). For the less potent racemic inhibitors the smaller difference between the *S*- and *R*-enantiomer binding energy ($\Delta\Delta E$), such as the *para*-methoxybenzyl inhibitor, arises from less potent *S*-enantiomers (smaller ΔE) in combination with average to larger ΔE (improved potency) for the corresponding *R*-enantiomers.

These calculations further support the role of the *S*-enantiomer in determining the potency, while the *R*-enantiomer displays much weaker binding and serves in most situations to dilute out the more active component. It is reasonable that as the *S*-enantiomer becomes less potent, the presence of the *R*-enantiomer may have a greater impact. However, for the current inhibitor series the *R*-enantiomer offers no gain of potency.

4. Conclusions

It is clear that the larger values of $\Delta\Delta E$ (Fig.1) represent more potent inhibitors, and this effect on potency likely arises due to decreased competition within the racemic mixture for the binding site. However, this effect is attenuated when the derivatized modification extends beyond a phenyl or isobutyl/isobutenyl group, as longer or bulkier groups display decreased potency with the racemic mixture (Fig.1). While less favored binding of the *R*-enantiomer may play a role, it is more likely a consequence of over-growing the inhibitor with respect to the confines of the protein's binding site.

Shorter modifications display relatively constant MIC values, such as for the first group (MIC range 1–3 $\mu\text{g/mL}$). The 1-ethylpropyl derivative represents the outlier (MIC 4–8 $\mu\text{g/mL}$), likely due to the dual branched structure at the primary carbon from the phthalazine. This degree of torsional freedom is presumably difficult to accommodate within the confines of the protein site, and it appears there is an energetic penalty in the binding as a result of the constraints of the binding site. Therefore, in consideration of exposure out of the binding site and into solvent, it is not only size but also the constraint to a more planar conformation, as with phenyl and isobutenyl, which results in favorable potency.

We cannot rule out other binding modes, particularly by the less potent inhibitors. However, it is likely that protrusion into solvent accounts for poorer performance with larger modifications. The recognition of the most favorable chemical modifications and the understanding of the roles of enantiomers in potency are proving crucial for progress. The data presented here support a progression of increased affinity as the derivatized inhibitor can interact more favorably with Arg53, Leu29 and Leu55. However, potent inhibition appears to arise from an improvement of the more active *S*-enantiomer in concert with exclusion of the *R*-enantiomer. The most potent derivatives from this series contain a planar derivative: isobutenyl (K_i 8.2 nM, MIC 0.5 $\mu\text{g/mL}$) or phenyl (K_i 5.0 nM, MIC 0.4–1.5 $\mu\text{g/mL}$). Data for isobutenyl modification indicate that the presence of the *R*-enantiomer causes a relative doubling of the K_i but does not affect the MIC value.

The ability to compensate for use of chiral mixtures in a therapeutic setting, such as only considering the concentration of a single enantiomer, would be beneficial from a manufacturing standpoint as chiral separations can be costly and result in loss of material. These data are guiding further modifications, as well as assisting in the assessment of pharmacokinetics, all of which are needed for further development of this inhibitor series for therapeutic intervention for biodefense purposes against *B. anthracis*. As elements of resistance continue to emerge in bacterial populations, including the trimethoprim-resistance noted in *Enterococcus faecalis*, *Streptococcus pneumoniae* and *S. aureus*, alternative antifolates with better coverage can fill this gap in the current arsenal of therapeutics [40–43].

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Highlights

- *B. anthracis* DHFR is effectively inhibited by dihydrophthalazine inhibitors
- Derivations of these inhibitors at the chiral center markedly affect the potency
- Enzyme and whole cell inhibition and crystallography indicate best derivations
- *In silico* calculations highlight role of enantiomeric energy differences
- Potency is maximized with *S*-phenyl or *S*-isobutenyl groups on the phthalazine

Figure 1. Modifications at “R1” are designed to modulate the potency with interactions at the protein’s interface with solvent

A) K_i (Standard Error of the Mean, SEM) and MIC values were determined with racemic mixtures of inhibitors; calculation of the energy of binding for individual enantiomers is given, ΔΔE is the difference in energetics of enantiomers. **B)** Two dimensional depiction and view of the inhibitor in the binding site. The protein has a grey van der Waals surface, with the proximal region depicted as transparent dots to permit visualization of the inhibitor buried within the site. The magenta wire cage indicates the position of R1 inhibitor modifications.

a. MIC values have been published [19]

b. Values for the *n*-propyl modification IC₅₀, MIC and binary co-crystal structure have been previously published [12].

c. Binary co-crystal structures presented in current work.

Figure 2. Both enantiomers inhibit the DHFR enzyme and they have overlapping binding sites
A) K_i (Standard Error of the Mean, SEM) and MIC values were determined using purified enantiomers of inhibitors. **B)** Stereo diagram of the binding sites for each enantiomer, which overlap and maintain a high conservation of atomic contacts. Residues Leu29, Gln30 and Lys33 are included with a blue surface; for the *S*-enantiomer (cyan) this supports the derivatized position (pictured here as isobutenyl), while for the *R*-enantiomer (magenta) this surface accommodates the dihydrophthalazine moiety. The dihydrophthalazine moiety of the *S*-enantiomer is in contact with the orange surface comprising residues Val32, Arg53, Leu55, and Arg58. The isobutenyl of the *R*-enantiomer is in contact with the purple surface over Ile51, Arg53 and Leu55.

Figure 3. Features of the *B. anthracis* DHFR binding site and interactions with enantiomers for isobutyl and phenyl derivatives

A) Two conserved water molecules (red) with unknown functional significance are visualized within the protein and below the C4 nitrogen of the diaminopyrimidine ring. The inhibitor shown is *S*-isopropyl (cyan). The chloride ion (green) that mimics a phosphate of NADPH is also shown. **B)** Distances between fluorine atoms of the *S*-trifluoropropyl derivative (cyan) to side chains of Gln30 and Lys33 are shown, as well as to the carbonyl oxygen atoms of Pro26 and Leu29. **C)** The *S*-phenyl (cyan) and *R*-phenyl (magenta) derivatives are shown as a stereo picture. Residues Leu29 and Gln30 interact closely with the *S*-phenyl moiety, while Ile51, Arg53 and Leu55 interact with *R*-phenyl moiety. **D)** The

S-isobutyl (cyan) and *R*-isobutyl (magenta) derivatives are depicted, also as a stereo diagram. The “top” of the binding site (in this view) contains Arg53, which is displaced out from the binding site in response to the *R*-isobutyl enantiomer.

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Table 1

Statistics for crystallographic structure determinations

PDB ID code	<i>R</i> -isobutenyl 4ELH	<i>R</i> , <i>S</i> -isobutyl 4ELG	<i>R</i> , <i>S</i> -phenyl 4ELB	<i>R</i> , <i>S</i> -isopropyl 4ELE	<i>R</i> , <i>S</i> -trifluoropropyl 4ELF
Resolution (Å)	44 – 2.1 (2.18 – 2.10)	48 – 2.1 (2.16 – 2.10)	50 – 2.6 (2.70 – 2.60)	29 – 2.35 (2.44 – 2.35)	37 – 2.3 (2.42 – 2.30)
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell (Å)	68.4, 136.0 168.4	68.2, 135.7 168.3	67.9, 135.4 168.2	68.5, 136.4 168.6	68.3, 136.1 168.5
<i>R</i> _{sym}	5.8 (25.8)	13.2 (56.0)	19.2 (39.7)	11.6 (36.4)	4.0 (12.4)
<i>I</i> / σ <i>I</i>	24.3 (4.7)	9.7 (2.1)	14.8 (3.5)	10.2 (3.1)	10.5 (3.5)
Completeness (%)	98.8 (98.0)	95.5 (90.4)	100 (100)	97.0 (83.6)	95.8 (92.3)
Redundancy	4.4 (4.4)	4.1 (3.7)	11.0 (11.0)	5.1 (3.3)	2.5 (1.7)
<i>R</i> _{work} / <i>R</i> _{free}	19.6 / 25.2	19.4 / 26.5	24.4 / 30.9	19.7 / 25.3	22.8 / 29.0
Num residues / water	1328 / 1281	1328 / 1259	1328 / 620	1328 / 1033	1328 / 947
Protein B-factor (Å ²)	35.6	38.1	37.2	27.4	39.6
Ligand B-factor (Å ²)					
<i>R</i> -enantiomer	40.8 (8)	44.2 (2)	48.9 (4)	null (0)	null (0)
<i>S</i> -enantiomer	43.2 (1)	36.9 (8)	48.6 (6)	35.0 (8)	47.6 (8)
R.M.S. Bond lengths (Å)	0.007	0.011	0.007	0.008	0.012
R.M.S. Bond angles (°)	1.11	1.12	1.078	1.106	1.20

Values for the highest resolution shell are given in parentheses; parentheses after ligand B-factors give the number of copies of each enantiomer found within the 8 non-crystallographic protein chains of the asymmetric unit, including chains with electron density for both enantiomers.