In Vitro Efficacy of New Antifolates against Trimethoprim-Resistant Bacillus anthracis

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Bacillus anthracis is innately resistant to trimethoprim (TMP), a synthetic antifolate that selectively inhibits several bacterial dihydrofolate reductases (DHFRs) but not human DHFR. Previously, we were able to confirm that TMP resistance in B. anthracis (MIC > 2,048 μg/ml) is due to the lack of selectivity of TMP for the B. anthracis DHFR (E. W. Barrow, P. C. Bourne, and W. W. Barrow, Antimicrob. Agents Chemother. 48:4643–4649, 2004). In this investigation, 24 2,4-diaminopirimidine derivatives, representing a class of compounds with dihydrophthalazine side chains, were screened for their in vitro effects on B. anthracis Sterne and their selectivities for the B. anthracis DHFR. MICs were obtained by a colorimetric (Alamar blue) broth microdilution assay. Purified human recombinant DHFR (rDHFR) and B. anthracis rDHFR were used in a validated enzyme assay to determine the 50% inhibitory concentrations (IC50s) and the selectivity ratios of the derivatives. The MICs ranged from 12.8 to 128 μg/ml for all but nine compounds, for which the MICs were ≥128 μg/ml. The IC50 values for B. anthracis rDHFR ranged from 46 to 600 nM, whereas the IC50 values for human rDHFR were >16,000 nM. This is the first report on the in vitro inhibitory actions of this class of antifolates against TMP-resistant B. anthracis isolates. The selective inhibition of B. anthracis rDHFR and the in vitro activity against B. anthracis demonstrate that members of this class of compounds have the potential to be developed into clinically important therapeutic choices for the treatment of infections caused by TMP-resistant bacteria, such as B. anthracis.

The increasing incidence of antibiotic-resistant pathogens is a significant threat because of the inability to effectively treat certain infectious diseases. The development of new and efficient antibiotics is an important aspect in dealing with the challenge presented by antibiotic resistance.

Anthrax, although primarily a disease of herbivorous animals, can affect humans as a cutaneous, gastrointestinal, or respiratory disease (12). Respiratory anthrax is a rare disease but difficult to diagnose and fatal in almost all cases unless the patient is treated with antibiotics. The etiological agent of anthrax, Bacillus anthracis, is one of the category A priority pathogens on the list of the National Institute of Allergy and Infectious Diseases (NIAID) division of the National Institutes of Health (NIH). One of the objectives of NIH is to develop antimicrobics for use in the treatment of anthrax.

Several antimicrobics, including ciprofloxacin and doxycycline, are approved and available for the treatment of anthrax (14). However, resistance to several of these compounds has been documented (5, 6, 9, 18, 19, 23). In addition, penicillin-resistant B. anthracis-like bacteria have been isolated from wild nonhuman primates who died of an anthrax-like disease in Côte d’Ivoire (16).

Both dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) are proven targets for antimicrobial drugs. They play key roles in the folate biosynthetic pathway that generates the cofactors necessary for continued DNA and RNA synthesis. As reported previously, trimethoprim (TMP) and sulfonamides are not effective against B. anthracis (1, 20). The genetic basis for TMP and sulfonamide resistance is associated with the chromosomally encoded gene for each enzyme target, DHFR (2) and DHPS (24), respectively. This prompted us to pursue activities for the development of drugs that overcome the TMP resistance of B. anthracis. Various antifolates were screened for their in vitro activities against B. anthracis and the enzyme target, DHFR. Initial studies with a few of these DHFR inhibitors were reported previously (3). Because of those results, additional derivatives were examined for their effectiveness against B. anthracis and the enzyme target, DHFR.

The objectives of the current study were to (i) screen additional inhibitors of B. anthracis recombinant DHFR (rDHFR) and to determine the MICs for those that had reasonable in vitro activities (at concentrations <128 μg/ml) and (ii) assay hit compounds to determine the 50% inhibitory concentrations (IC50s) for the bacterial and the human enzymes to identify specific antibacterial DHFR inhibitors.

(Parts of this research were presented at the 45th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC.)

MATERIALS AND METHODS

Microorganisms. B. anthracis Sterne was used for initial drug screening. This strain was acquired from Rebecca Morton, Oklahoma State University Center for Veterinary Health Sciences. The B. anthracis Ames strain (NR-411) was used for confirmation of activity against a virulent strain and was obtained through the Biodefense and Emerging Infectious Research Resources Repository, NIAID, NIH. Work with the Ames strain was conducted in a CDC-registered and -approved biosafety level 3 laboratory, and appropriate biosafety and security regulations were used. The other strains used in this study included Enterococcus
faecalis ATCC 29212 and Escherichia coli ATCC 25922, which were obtained from ATCC. Upon receipt, the strains were checked for purity and stocks were grown in an appropriate medium.

**Compounds.** The compounds were provided by Basilea Pharmaceutica, Basel, Switzerland. The compounds were synthesized as described in a previous patent (13).

**MIC determinations.** The MICs of the compounds for *B. anthracis* Sterne and Ames were determined by an Alamar blue broth microdilution assay, as described previously (3, 4). In the case of the *B. anthracis*, the final inoculum was 5.0 × 10^4 CFU/ml, equivalent in turbidity to a 0.5 McFarland standard.

Sterile solutions of test and quality control (QC) drugs were prepared at the appropriate concentrations in cation-adjusted Mueller-Hinton broth (Becton Dickinson, Cockeysville, MD) (7) containing 10% Alamar blue and then aliquoted into 96-well plates at 100 µl/well. The plates were prepared and infected on the same day. Doxycycline at three concentrations (twofold dilutions, 1 to 0.25 µg/ml) was used to determine the MIC for *E. coli* ATCC 29255. For plates infected with the Sterne strain, doxycycline was used at 0.0156, 0.03125, and 0.0625 µg/ml. For plates infected with the Ames strain, the doxycycline concentrations at twofold dilutions were 0.0156 to 0.25 µg/ml. TMP-sulfamethoxazole concentrations (twofold dilutions of TMP-sulfamethoxazole from 2/38 to 0.25/4.75 µg/ml) were used for *E. faecalis* ATCC 29212. Sterility control wells and growth control wells for each agent were included in the 96-well plate.

For initial screens of the test compounds, 10-fold dilutions from 128 µg/ml to 0.128 µg/ml were used in triplicate columns. Two columns were infected, and the third column served as a color control. This also allowed observation of drug precipitation. In subsequent panels, drug concentrations were used at twofold dilutions. The MIC for each compound was evaluated four times by using the dilution range of 128 to 0.128 µg/ml. Subsequent MIC evaluations at narrower ranges were also conducted four times. Thus, the MIC for each compound was evaluated eight times in total.

After 16 h incubation, the plates were allowed to equilibrate for 30 min at room temperature before they were sealed with a sterile plate sealer (Nunc). The plate was then read visually and spectrophotometrically in an optical microtiter plate reader programmed to subtract the absorbance at 600 nm from that at 570 nm. The MICs are reported as the lowest drug concentrations yielding a differential absorbance of zero or less (i.e., the color remained blue). The MICs were compared to the acceptable limits for the QC strains (see Table 3 in reference 8) to validate drug performance and medium suitability.

For *B. anthracis* Ames, the drugs which had the best MICs for strain Sterne were prepared in 96-well plates in alternating twofold dilutions to encompass more drug concentrations. QC drugs, as well as sterility and growth control wells, were included as described above. The plates were infected, incubated, and read as described above.

**Expression and purification of Sterne rDHFR.** The strain Sterne rDHFR was expressed in *E. coli* and purified as described previously (2). Fractions containing purified enzyme were pooled and the His tag was removed by using a Novagen thrombin cleavage kit, according to the manufacturer’s instructions. Polyacrylamide gel electrophoresis analysis, performed as described in Ref. 17 with 12.5% acrylamide gels, was used to confirm the cleavage of the His tag and indicated a purity of greater than 95%.

**RESULTS**

**Initial screening.** In the initial stages, several DHFR inhibitors were tested against *B. anthracis* Sterne, the *B. anthracis* rDHFR, and the human rDHFR. Particularly good results were obtained with four derivatives from one subclass of 2,4-diaminopyrimidine analogues with a dihydrophthalazine side chain (3), referred to here as type A (Table 1).

Encouraged by this finding, we tested more compounds of type A with diverse side chains at the R1 and R2 positions. The results for these additional derivatives are presented in Table 1, along with those for the original four hit compounds (BALK7662, BALK6796, BALK6760, and BALK6763), which are marked in boldface in Table 1. Eight additional derivatives had MICs >128 µg/ml, but the results are not shown in Table 1; they are discussed in the text. We chose 128 µg/ml as the upper MIC with which to conduct the subsequent IC_{50} evaluations with the recombinant enzymes. This was primarily due to the twofold dilution testing range that we used for the initial screening. For compounds with MICs exceeding this value, no IC_{50} determinations were performed. Additionally, all of the compounds listed in Table 1 have very low levels of activity against the human rDHFR.

From the initial screening, it was determined that the best compound was BALK7662, with a MIC of ≤12.8 µg/ml and an IC_{50} for the *B. anthracis* rDHFR of 54 nM (Table 1). This compound also had reduced selectivity for the human rDHFR.
TABLE 1. Compounds of type A with MICs of <128 μg/ml for *B. anthracis* Sterne

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>Mol wt</th>
<th>MIC (μg/ml) for Sterne (Ames)</th>
<th>IC₅₀ (nM)</th>
<th>SR†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R₁</td>
<td>R₂</td>
<td></td>
<td></td>
<td>BaDHFR</td>
<td>HuDHFR</td>
</tr>
<tr>
<td>E1</td>
<td>-O-CH₃</td>
<td>No substitution</td>
<td>444.50</td>
<td>&gt;128</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C1</td>
<td>-O-CH₃</td>
<td>-CH₃</td>
<td>458.52</td>
<td>≤64 (≤64)</td>
<td>92 ± 3.3°</td>
<td>&gt;28,000°</td>
</tr>
<tr>
<td>B2</td>
<td>-O-CH₃</td>
<td>-CH₂-CH₃</td>
<td>472.55</td>
<td>≤32 (≤25.6)</td>
<td>96 ± 14°</td>
<td>&gt;27,000°</td>
</tr>
<tr>
<td>BAL17662</td>
<td>-O-CH₃</td>
<td>-CH₂-CH₂-CH₃</td>
<td>486.58</td>
<td>≤12.8 (≤16)</td>
<td>54 ± 12°</td>
<td>110,000 ± 26,000°</td>
</tr>
<tr>
<td>G1</td>
<td>-O-CH₃</td>
<td>-CH₂-CH₂-CH₂-CH₃</td>
<td>500.61</td>
<td>≤12.8 (≤12.8)</td>
<td>170 ± 26°</td>
<td>&gt;20,000°</td>
</tr>
<tr>
<td>C2</td>
<td>-O-CH₃</td>
<td>-CH₂-CH₂-CH₂-OH</td>
<td>502.58</td>
<td>≤64 (&gt;102.4)</td>
<td>120 ± 9°</td>
<td>&gt;25,000°</td>
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<tr>
<td>BAL16796</td>
<td>-O-CH₃</td>
<td></td>
<td>544.61</td>
<td>≤128</td>
<td>300 ± 4°</td>
<td>813,000 ± 31,000°</td>
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<tr>
<td>BAL16700</td>
<td>-O-CH₃</td>
<td></td>
<td>626.30</td>
<td>≤25.6 (&gt;51.2)</td>
<td>46 ± 7°</td>
<td>&gt;16,000°</td>
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<tr>
<td>C3</td>
<td>-O-CH₃</td>
<td></td>
<td>512.62</td>
<td>≤25.6 (≤12.8)</td>
<td>200 ± 6°</td>
<td>&gt;22,000°</td>
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<tr>
<td>D3</td>
<td>-O-CH₃</td>
<td></td>
<td>526.64</td>
<td>≤12.8 (≤12.8)</td>
<td>260 ± 6°</td>
<td>&gt;19,000°</td>
</tr>
<tr>
<td>A3</td>
<td>-O-CH₃</td>
<td></td>
<td>534.62</td>
<td>≤64 (≤51.2)</td>
<td>150 ± 13°</td>
<td>&gt;19,000°</td>
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<tr>
<td>A2</td>
<td>-O-CH₃</td>
<td></td>
<td>550.62</td>
<td>≤128</td>
<td>97 ± 21°</td>
<td>&gt;23,000°</td>
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<tr>
<td>B3</td>
<td>-O-CH₃</td>
<td></td>
<td>582.62</td>
<td>≤128</td>
<td>600 ± 100°</td>
<td>&gt;22,000°</td>
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<td>BAL16763</td>
<td>-O-CH₃</td>
<td></td>
<td>614.15</td>
<td>≤128</td>
<td>400 ± 13°</td>
<td>&gt;42,000°</td>
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<tr>
<td>E2</td>
<td>-OH</td>
<td></td>
<td>536.60</td>
<td>≤128</td>
<td>69 ± 21°</td>
<td>&gt;24,000°</td>
</tr>
<tr>
<td>G2</td>
<td></td>
<td></td>
<td>619.73</td>
<td>≤32 (≤25.6)</td>
<td>170 ± 7°</td>
<td>&gt;21,000°</td>
</tr>
</tbody>
</table>

* The chiral center is marked with an asterisk.

b MICS are given as the means ± standard errors of the means (n = 4).

c IC₅₀ values are given for the *B. anthracis* DHFR (BaDHFR) and the human (HuDHFR) rDHFR and are means ± standard errors of the mean. ND, not determined.

d SR, selectivity ratio, which is the IC₅₀ for human DHFR/IC₅₀ for *B. anthracis* DHFR.

e n = 3.

f n = 2.
2-phenylethanol group (compound A1) eliminates the in vitro pound B1), furan (compound F1), pyridine (compound H1), or pound A2) (Table 1). However, addition of a phenol (com-

When compounds like BAL16700 and BAL16763 were com-
pared to similar compounds containing cyclic substitutions at the R2 position (compounds C3, D3, A3, A2, and B3), various effects were observed with this series of 2,4-diaminopyrimidine analogues. All of these derivatives have MICs of less than 128 μg/ml and a lack of selectivity for the human rDHFR (compounds C3, D3, A3, A2, and B3; Table 1). The best compound in this series is compound D3, in which case the R2 substitution is cyclohexane. The next best activity is observed with a phenyl group (compound BAL16700) or a cyclopentane group (compound C3) in the R2 position (Table 1). Addition of a toluene group still maintains good activity and selectivity (compound A3), as does the addition of a phenylmethanol group (compound A2) (Table 1). However, addition of a phenol (com-

Two other derivatives, not discussed above, that contained modifications at R1 and R2 were evaluated. Compound E2, with a hydroxyl group at R1 and a phenylethanol group at R2, has a MIC less than 128 μg/ml and an IC50 of 69 μg/ml (Table 1). Compound G2, which is similar to compound BAL16700 but with a 2′-morpholinoethoxy group at R1, has a MIC of ≤32 μg/ml (Table 1).

Other derivatives that were not highly active in vitro (i.e., MICs > 128 μg/ml) included the indicated substitutions for R1 and R2, respectively, as follows: compound D1, hydroxyl and phenol; compound D2, dimethoxymethyl and phenol; compound F2, dimethoxymethyl and phenylethanol; and compound H2, methyl 2-methoxyacetyl and phenol.

**Evaluation of B. anthracis Ames.** For these evaluations, the drug concentration ranges were customized on the basis of previous results obtained with the Sterne strain. The MICs for these 10 compounds are given in parentheses after the MICs reported for the Sterne strain in Table 1. Essentially, there were no marked differences for compounds C1, B2, BAL17662, G1, C3, D3, A3, and G2 (Table 1). However, compounds C2 and BAL16700 (Table 1) showed marked increases in their MICs for the Ames strain. The reason for this was not examined further.

**Homology model.** We have generated a model of the B. anthracis DHFR structure to rationalize our experimental results and to subsequently optimize our inhibitors. As discussed previously, an X-ray structure of the TMP-resistant S. aureus F98Y DHFR mutant was selected as a template. The sequence alignments for these two proteins are shown in Fig. 1, along with the residues discussed here (highlighted in red). It should be noted that the similarity of the sequence of the strain Sterne DHFR is 100% with the sequences of the DHFRs of several virulent strains of B. anthracis, including the Ames strain (2). As the F98Y mutation causes TMP resistance in S. aureus (10), it appears likely that the corresponding tyrosine is also involved in the TMP resistance of B. anthracis (102Y) (Fig. 2). As expected by the sequence similarity, the model obtained is highly similar to the template structure, which is reflected in a root mean square deviation of 0.5 Å for the Ca atoms. Also, for the majority of the side chains in the inhibitor-binding pocket, an almost identical position is assigned.

However, several deviations which are likely to influence the binding of inhibitors can be observed (Fig. 2). In B. anthracis, a methionine is located at the position of the L6 of S. aureus. This alteration can be expected to have a negative influence on the inhibitor affinity, since this residue contributes to the shape of the subsite hosting the amino-pyrimidine moiety. The change of D28 (in S. aureus) to a glutamate is expected to result in a significant weakening of the inhibitor affinity (Fig. 2). A shift of the inhibitor of at least 0.5 Å is required to maintain the hydrogen bonding. Three mutations are observed in the proximity of the dimethoxy-phenyl moiety. The mutation of T47 to an asparagine residue (Fig. 2) not only decreases the size of this subsite but also alters the donor-acceptor pattern (Fig. 2). Although the mutations of Q20 to an asparagine and especially of the mutation of S50 to an alanine (Fig. 1, and 2) are located at the edge of the binding pocket, they can be expected to influence significantly the preference for R1 substituents, as both the shape and the hydrogen-bond donor-acceptor pattern are considerably changed.

In contrast to the change of N57G, the mutation K30Q (Fig. 1 and 2) appears to be more relevant since it renders this residue into a potential hydrogen-bond partner for substituents at the R2 position. An indirect effect might be caused by the mutation of T37 to a methionine (Fig. 1 and 2). This residue is involved in a hydrogen-bonding pattern, which fixes the position of R59 in the S. aureus DHFR. A weakening of this H-bond pattern can result in an alternative conformation of the arginine, which would significantly decrease the size of this subsite.

The preference for R1 and R2 substituents might also be influenced by the mutations of H24 and K53 to arginine residues (Fig. 1). The modeled orientations of folate and TMP in the binding pocket of the B. anthracis DHFR homology model are shown in an electrostatic surface representation in Fig. 3. The pteridine ring of the folate is buried inside the negatively charged pocket, with the glutamate moiety protruding out from the pocket, where its position is stabilized by a positively charged surface patch formed by the side chains of arginine residues R24, R53, and R58, together with lysine residues K34 and K111. The change of both the size and the donor and acceptor properties of this positively charged patch that forms the ceiling and left wall of the entrance to the folate-binding pocket, an almost identical position is assigned.
pocket could result in additional H-bond interactions. However, due to the high solvent exposure of these residues, this hypothesis requires further investigation either by structure analysis of the *B. anthracis* DHFR or by mutation experiments.

**DISCUSSION**

In 2005, we reported on a group of 2,4-diaminopyrimidine derivatives with in vitro activities against *B. anthracis* Sterne and selective activities against *B. anthracis* rDHFR but not human rDHFR (3). For five of the six derivatives, the MICs ranged from 12.5 to 128 μg/ml (3). The IC$_{50}$ values for the *B. anthracis* rDHFR ranged from 46 to 404 nM for four compounds, while the IC$_{50}$ values for human rDHFR were greater than 16,000 nM, with the best being 813,000 nM (3). To our knowledge, this was the first such report demonstrating any antifolate with activity against *B. anthracis*.

Recently, Joska and Anderson reported on several 2,4-diamino-5-deazapteridine and pyrimidine derivatives with in vitro activities against *Bacillus cereus* (15). The most potent compound was in the 2,4-diamino-5-deazapteridine group, with an MIC$_{50}$ of 1.6 μg/ml (15). Using recombinant *B. cereus* DHFR (which has 98% sequence identity with the *B. anthracis* DHFR), the investigators were able to show IC$_{50}$s ranging from 0.27 to 252 μM (i.e., 270 to 252,000 nM) (15); no comparative evaluation was reported for human DHFR. However, the authors did cite previous data obtained with some of the compounds for rat DHFR in which the IC$_{50}$ was 25 μM for the best compound (22); that compound had a MIC$_{50}$ of 2.6 μg/ml (15). The six pyrimidine derivatives were much less selective for the *B. cereus* rDHFR, with IC$_{50}$s ranging from 37 to 150 μM. Apparently, none of the antifolates in the Joska and Anderson study were evaluated against an avirulent or virulent strain of *B. anthracis* (15).

In our current investigation we have extended the findings of our previous studies with the 2,4-diaminopyrimidine group of antifolates. Additionally, the in vitro activities of several of the hit compounds have been confirmed with the virulent Ames strain. The most active compounds have small, hydrophobic side chains at positions R$_1$ and R$_2$ (e.g., compounds BAL17662, B2,
and BAL16700; Table 1). It appears that the side chain at position R2 is larger than a methyl or ethyl group for optimal activity (Table 1). A molecule without a side chain at position R2 and a methoxy group at position R1 is inactive against B. anthracis Sterne (e.g., compound E1; Table 1). A
ducts larger than cyclohexane show reduced activities, proba-
bly because of steric problems but also because of electronic properties (e.g., compounds A1, A2, B1, B3, and BAL16765 versus compound BAL16700; Table 1). A benzyl side chain is well tolerated, while a pyridine leads to a loss of activity of the molecule (e.g., compound H1 versus compound BAL16700; Table 1). Compounds with a methoxy group at position R1 have antibacterial activities similar to that of a compound with a 2’-morpholino-ethoxy side chain at the same position (e.g., compound G2 versus compound BAL16700; Table 1). This indicates a flexibility for side chains at R1, which can be further exploited.

These results are in line with those obtained with the B. anthracis DHFR homology model developed on the basis of the crystal structure of the DHFR from a TMP-resistant S. aureus strain (10). For example, the different IC50 values of compounds G1, C2, and BAL16796 reflect well the expected alteration of the preference for substituents at position R2. Of these three compounds, compound BAL16796 showed the best affinity and compound C2 showed the poorest affinity in S. aureus (data not shown). According to molecular modeling, this ranking is changed in B. anthracis due to the favorable interactions of the hydroxyl group of compound C2 and the repulsive interactions of the carbonyl group of compound BAL16796 with the Q30 (which replaced the K30 of S. aureus), thus rendering compound C2 a better inhibitor than compound BAL16796 of the B. anthracis DHFR (Table 1).

The model also helped to identify several residues which are likely to contribute to the altered ligand-binding properties of the B. anthracis DHFR. The mutations D25E and L60M appear to affect mainly the diaminopyridine scaffold of the inhibitors, whereas other mutations, in particular, S50A, K30Q, and T37M, are likely to influence the selectivity for the R1 and R2 substituents.

The data obtained in this study show that the DHFR inhibitors tested have the potential to become clinically relevant drugs. Optimization of the MIC, while keeping the excellent selectivity for the bacterial enzyme, is a next goal. A chemistry program will be guided by structural information to make the proper modifications. The development of effective antimicrobials affecting this target in B. anthracis is particularly important for the treatment of anthrax. Such a drug would generally be welcomed as a contribution to address the problem of infections caused by antibiotic-resistant bacteria.

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