New Antifolate Inhibitors for *Mycobacterium avium*

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Abstract: The present study extends our previous work regarding new antifolates for *Mycobacterium avium* (MAC) dihydrofolate reductase (DHFR). The objectives of this study were to synthesize and test new derivatives in the general class of 2,4-diamino-5-methyl-5-deazapteridines in an effort to improve solubility and selectivity for the MAC DHFR, while maintaining lack of selectivity for the human DHFR. New 6-[2', 5'-dialkoxyphenyl) methyl]-substituted DMDP analogs were synthesized as previously described. Three clinical isolates of MAC (NJ211, NJ3404, and NJ168) and *M. tuberculosis* H37Ra (MTB) were used to evaluate the new derivatives. A previously described colorimetric (alamarBlue®) microdilution broth assay was used to determine minimal inhibitory concentrations (MIC). Purified recombinant human (rDHFR), MAC rDHFR, and MTB rDHFR were used in a validated enzyme assay to obtain IC_{50} values and to determine selectivity ratios (SR) for the derivatives. For the MAC strains, the MICs ranged from ≤ 0.25 to > 16 μg/mL. The most active derivative against MAC was SRI-20920 which had MICs of 0.25, 0.25, and 8 μg/mL for the three strains, respectively. The most selective derivative was SRI-20730 with IC_{50s} of 29 and 67,781 nM for MAC rDHFR and hDHFR, respectively, and a SR of 2,337. MICs for MTB ranged from 4 to >64 μg/mL and the SR, in general, ranged from 0.32 to 2.5. These results further substantiate the utility of this group of DMDP derivatives for selective activity against MAC.

Key Words: Antifolates, DFHR, deazapteridines, *M. avium*, *M. tuberculosis*, *in vitro*, MIC, IC_{50}.

INTRODUCTION

Through the use of the NIH sponsored Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF), we were previously able to identify the antimycobacterial activity of a class of antifolate compounds, derivatives of 2,4-diamino-5-methyl-5-deazapteridine (DMDP). Initial screening by TAACF indicated that many of the DMDPs have activity against *Mycobacterium tuberculosis* (MTB). Using this information, we proceeded to re-synthesize similar compounds for use in a study designed to evaluate their effectiveness against *M. avium* (MAC). As a result, a specific group of DMDPs with excellent *in vitro* efficacy against MAC were identified (≤ 0.13 μg/mL). More importantly, these compounds had a higher selective activity for MAC dihydrofolate reductase (DHFR) than for human DHFR [1, 2]. This is significant because MAC is intrinsically resistant to trimethoprim (TMP), a commonly used drug for prokaryotic DHFRs [2]. DHFR is a key enzyme in the folate biosynthetic pathway. The enzyme catalyses the reduction of dihydrofolate to tetrahydrofolate, derivatives of which function in single carbon transfers at various oxidation states for the synthesis of purines, methionine, glycine, pantothenate, thymidylate, and N-formylmethionyl-tRNA [3, 4]. Inhibition of DHFR leads to a depletion of the pool of tetrahydrofolate derivatives and results ultimately in inhibition of DNA, RNA and protein synthesis. DHFR is therefore an important target for medicinal chemistry, including antibacterial agents [5, 6]. The interest in developing antifolates for mycobacterial agents such as MAC is very apparent, particularly in recent years [1, 2, 7-17].

In an effort to expand this program, and develop more effective DMDPs in this class, we have synthesized several new derivatives with dipropoxy substitutions on the basic structure at both the R2 and R5 positions (Fig. **1**). In this study, we have evaluated these new derivatives for their *in vitro* activity against three strains of MAC as well as their selectivity against MAC rDHFR and human rDHFR. The derivatives were also evaluated for *in vitro* activity against MTB H37Ra and selectivity against MTB rDHFR. Another purpose in this continued investigation was to improve solubility properties of the previous inhibitors. This was addressed by preparing salt forms of existing compounds and modifying substitutions on the R2 and R5 positions for new derivatives.

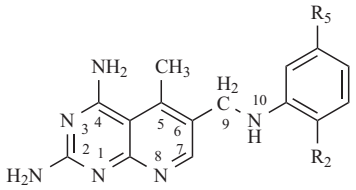

Fig. (1). Basic structure of DMDP derivatives that are shown in Table **1**. Modifications at R2 and R5 are denoted in Table 1.

RESULTS

Three strains of MAC were used to evaluate the *in vitro* activity of the new DMDP derivatives listed in Table **2**, NJ168, NJ211, and NJ3404. These are the same strains used previously [2]. In the present evaluation, MAC NJ168

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seemed to have a higher level of resistance to the new DMDP derivatives than the other two MAC strains. This was in general the case in our previous study [2]. With this strain, four derivatives had an MIC ≥ 16 μg/mL (SRI-20858, -20903, -20923, and -20924) (Table **2**). MAC strain NJ3404 appeared to be the most susceptible strain with two MICs equal to 4 μg/mL (SRI-20730, and 20858) and all the rest ≤ 1.0 μg/mL (Table **2**). This was similar to the results we reported previously for the initial group of DMDP derivatives [2]. MAC strain NJ211 was also somewhat susceptible to most of the derivatives, with only one MIC >16 μg/mL (SRI-20858), two at 4 μg/ml (SRI-20835 and SRI-20923) and all the rest falling below 2.0 μg/mL (Table **2**).

For *in vitro* activity against MTB, we used the H37Ra strain to compare the results obtained with the MAC strains. Although this is an avirulent strain, it has proved to be adequate in the past to evaluate *in vitro* activity for the DMDP derivatives [1]. Overall, the new DMDP derivatives demonstrated weaker activity against this organism than they did with the MAC strains (Table **3**). All of the MICs fell above 4 μg/mL, and most were ≥ 8 μg/mL (Table **3**). This is the general pattern that we have observed for other DMDP derivatives [1] and can be explained by the reduced selectivity of the DMDP derivatives for the MTB DHFR, as described previously and in this manuscript [7, 18].

Enzyme inhibition assays were performed with rMAC DHFR and human rDHFR to compare activity and to obtain SR from the IC_{50} values. Both of these enzymes have been used previously in our studies [2, 19]. The MAC DHFR used in this study was tested with TMP and the IC_{50} was determined to be 5,570 ± 770 nM [19], consistent with previous studies [2]. The MAC DHFR was also tested with methotrexate, a non-specific antifolate, and the IC_{50} was 5.1 ± 0.083 nM [19]. Likewise, the human rDHFR used in this study was tested with TMP and methotrexate and the IC_{50} values were 1.62 ± 0.0058 x 10^6 and 24.3 ± 7 nM, respectively [19].

In general, the IC_{50} data indicates that all the new DMDP derivatives are more selective for the MAC DHFR than the human DHFR. This is consistent with our findings for other derivatives in this group [2]. With the exception of two, all the IC_{50} values for the MAC rDHFR were below 29 nM, with the lowest value being 7.6 nM for SRI-20864.

These data can best be evaluated by dividing the derivatives into three groups. The first group would contain derivatives numbers 1 and 2 (SRI-8858 and -20730). The second group would contain derivatives numbers 3-11 and the third group would contain derivatives numbers 12-14. Derivatives in the first group are salt forms of the two lead compounds that resulted from our first round of drug selection [2]. Those two lead compounds were SRI-8686 and -20094, respectively, both of which had very good *in vitro* activity and selectivity for the MAC DHFR [2]. A problem associated with the non-salt forms was insolubility at higher concen-trations. As shown in Table **2**, both salt forms retain very good *in vitro* activity against all three strains of MAC, ranging from 0.25 to 4.0 μg/mL. In addition, selectivity for the MAC DHFR is maintained while selectivity for the human DHFR is reduced, particularly for SRI-20730 (Table **2**). Both derivatives have fairly good *in vitro* activity for MTB (≈ 8 μg/mL) (Table **3**), but selectivity against the MTB rDHFR is much less than for the MAC DHFR (Table **3**).

The second group of derivatives (numbers 3-11) are compounds that were synthesized around the basic structure of the two lead compounds from our previous studies, SRI-8686 and -20094, by incorporating various dipropoxy substitutions at the R2 and R5 positions (Fig. **1**, Table **1**). All of these derivatives maintained their selectivity for the MAC rDHFR and reduced selectivity for the human rDHFR (Table **2**). However, none of these showed an improved SR over SRI-20730.

There were some interesting results with regard to certain matched pairs of derivatives. For example, modification at the R5 position by addition of two methylene groups (SRI-20835 and -20858), as apposed to the same modification at the R2 position, decreases the *in vitro* effectiveness and reduces the selectivity for the human rDHFR by some four-fold. When this modification takes place with an extended group, as with SRI-20923 and -20924, only slight changes are observed with the *in vitro* effectiveness, and essentially no change occurs with the selectivity for the human or MAC rDHFR (Table **2**).

With regard to the third group of derivatives (numbers 12-14), the calculated Log(p) values were reduced by almost half and improved solubility was noted during the preparation and testing of solutions. Consequently, it was possible to obtain more consistent IC_{50} values for the human rDHFR (Table **2**). All three of these derivatives showed excellent *in vitro* activity against MAC NJ211, with MIC values ranging from 0.5 to 1.0 μg/mL (Table **2**). Good selectivity was observed with the MAC rDHFR and reduced selectivity was apparent with the human rDHFR (Table **2**).

Selective activity for the MTB rDHFR was considerably reduced for all the derivatives, with IC_{50s} ranging from 669 to 10,735 nM (Table **3**). This resulted in much lower SR values than observed with the MAC rDHFR, with values ranging from 41 to 0.32 nM (Table **3**). These results are similar to previously observed IC_{50} values for other DMDP derivatives against MTB rDHFR and are possibly associated with variation in properties [18] and binding characteristics of the two enzymes.

MATERIALS AND METHODS

Synthesis of DMDP Derivatives

Synthesis of the class of compounds used in this study was accomplished according to the method of Piper *et al.* which has been described previously [1, 2, 20]. In effect, the appropriate 5-substituted 5-deaza-6-bromomethylpteridine was reacted with the appropriate commercial aniline to obtain the N-linked analogs, respectively. The 10-CH_2-linked analogs were prepared by Wittig coupling of the reaction product of triphenylphosphine and 5-methyl-5-deaza-6-bromomethylpteridine. This was done with the appropriately substituted and commercially available benzaldehyde derivative followed by catalytic hydrogenation [20]. The hydrochloride salt forms, SRI-8858 and -20730 were prepared using 2N HCl in methanol. All compound structures were verified by mass and ^{1}H—labeled nuclear magnetic reso-

Table 1. Chemical and Structural Properties of DMDP Derivatives Whose Base Structure is Depicted in Figure 1

#	SRI #	Molecular Wt.	Log (p) ¶	R2	R5
1	8858 (HCl form)	368.43	3.15	-O-CH_2-CH_3	-O-CH_2-CH_3
2	20730 (HCl form)	396.49	4.12	-O-CH_2-CH_2-CH_3	-O-CH_2-CH_2-CH_3
3	20815	382.46	3.64	-O-CH_2-CH_3	-O-CH_2-CH_2-CH_3
4	20835	396.49	4.05	-O-CH_2-CH_3	-O-CH_2-CH_2-CH_2-CH_3
5	20858	396.49	4.05	-O-CH_2-CH_2-CH_2-CH_3	-O-CH_2-CH_3
6	20864	354.41	2.81	-O-CH_3	-O-CH_2-CH_3
7	20903	382.46	3.72	-O-CH_3	-O-CH_2-CH_2-CH_2-CH_3
8	20905	368.43	3.30	-O-CH_3	-O-CH_2-CH_2-CH_3
9	20920	382.46	3.64	-O-CH_2-CH_2-CH_3	-O-CH_2-CH_3
10	20923	410.51	4.54	-O-CH_2-CH_2-CH_3	-O-CH_2-CH_2-CH_2-CH_3
11	20924	410.51	4.54	-O-CH_2-CH_2-CH_2-CH_3	-O-CH_2-CH_2-CH_3
12	20959	428.49	2.16	-O-CH_2-CH_2-O-CH_3	O-CH_2-CH_2-O-CH_3
13	20972	472.58	2.84	-O-CH_2-CH_2-O-CH_2CH_3	O-CH_2-CH_2-O-CH_2CH_3
14	20973	400.43	2.72	-O-CH_2-O-CH_3	O-CH_2-O-CH_3

¶ Calculated Log(p) values were obtained using ChemDraw Ultra® (CambridgeSoft.Com, Cambridge, MA) structural analysis package. These values were obtained by Crippen's fragmentation [21].

Table 2. Minimal Inhibitory Concentrations (MIC) and IC_{50} Data for DMDP Derivatives. DMDP Derivatives were Evaluated Against MAC NJ168, NJ211, and NJ3404 for MIC. IC_{50} Values were Determined for MAC and Human rDHFR and Selectivity Ratios (SR) Calculated by the Following Formula: IC_{50} Human DHFR / IC_{50} MAC rDHFR. IC_{50} Values Represent the Average from Two or More Determinations. * Precipitation at Higher Concentrations Prevented Precise Measurements

#	SRI #	MAC NJ168	MAC NJ211	MAC NJ3404	Hu-DHFR	MAC-DHFR	MAC
		MIC (µg/mL)	MIC (µg/mL)	MIC (µg/mL)	IC_{50} (nM)	IC_{50} (nM)	Selectivity Ratio
1	8858	2	0.25	1	2,737	22	124
2	20730	2	0.5	4	67,781	29	2,337
3	20815	8	0.5	0.25	2,441	28	87
4	20835	8	4	1	4,054	22	184
5	20858	>16	>16	4	17,946	25	718
6	20864	8	0.5	≤0.25	1,372	7.6	181
7	20903	16	2	0.5	1,277	13	98
8	20905	8	1	0.25	978	24	41
9	20920	8	0.25	0.25	5,507	18	306
10	20923	>16	4	1	>2,494*	22	>113
11	20924	>16	1	0.5	>2,494*	19	>131
12	20959	ND	1	ND	26,826	42	639
13	20972	ND	1	ND	35,236	71	496
14	20973	ND	0.5	ND	3,375	16	211

Table 3. Minimal Inhibitory Concentrations (MIC) and IC_{50} Data for DMDP Derivatives. DMDP Derivatives were Evaluated Against MTB H37Ra. IC_{50} Values were Determined for MTB rDHFR and Human rDHFR and Selectivity Ratios (SR) Calculated by the Following Formula: IC_{50} Human DHFR / IC_{50} MTB rDHFR. IC_{50} Values Represent the Average from Two or More Determinations

#	SRI #	MTB H37Ra	Hu-DHFR	MTB-DHFR	MTB
		MIC (μg/mL)	IC_{50} (nM)	IC_{50} (nM)	Selectivity Ratio
1	8858	4-8	2,737	5,085	0.54
2	20730	8	67,781	1,661	41
3	20815	64	2,441	2,471	0.99
4	20835	>64	4,054	ND	--
5	20858	>64	17,946	>3,973	>4.5
6	20864	4	1,372	3,924	0.35
7	20903	32	1,277	ND	--
8	20905	16	978	3,027	0.32
9	20920	ND	5,507	2,037	2.7
10	20923	ND	>2,494*	669	>3.7
11	20924	ND	>2,494*	876	>2.8
12	20972	32	35,236	ND	ND
13	20959	32	26,826	10,735	2.5
14	20973	8	3,375	ND	ND

nance (NMR) spectroscopy. Sample purity was assessed by thin-layer chromatography (TLC) and elemental analysis. All compounds gave single spots by TLC and were within acceptable combustion parameters (0.4%).

Properties of DMDP Derivatives

Calculated Log(p) values were obtained using ChemDraw Ultra® (CambridgeSoft.Com, Cambridge, MA) structural analysis package. According to the software, these values were obtained by Crippen's fragmentation [21].

Minimal Inhibitory Concentration (MIC)

The MICs were determined for *Mycobacterium avium* strains NJ168, NJ211 and NJ3404, and *M. tuberculosis* H37Ra (ATCC 25177) using a colorimetric microdilution broth assay done in 96-well (U-bottom) plates as reported previously [1, 2]. A frozen culture in assay broth (Middlebrook 7H9 broth supplemented with albumin-dextrose-catalase enrichment and 0.2% glycerol) was thawed and diluted in broth to 1-2 x 10^5 CFU/mL, and used as the inoculum. Working stocks of the test compounds were prepared in DMSO at a concentration of 10.24 mg/mL. Drug concentrations for assay were prepared by serial two-fold dilutions in assay broth and 0.05 mL was added to appropriate wells in duplicate. The final amount of DMSO was 1.3 % and did not affect the growth of the test strains. The assay included inoculated solvent and viability controls and uninoculated drug dilution and medium controls. Plates were inoculated with 0.05 mL of standardized culture and incubated at 36-37 C for six days. The REDOX indicator alamarBlue® (Trek Diagnostic Systems) was then added to each well as a mixture with Tween 80, and the plates incubated for an additional 18-20 hr. The plates were read in an optical microtiter plate reader programmed to subtract the absorbance at 600 nm from that at 570 nm to blank out turbidity and absorbance due to oxidized dye. The MIC was reported as the lowest concentration of drug yielding a differential absorbance of zero or less. This approximated the color change of blue to pink that was observed visually after metabolic reduction of dye and represented the concentration at which no visible growth occurred. Ethambutol was used as a positive control.

MAC, MTB and Human rDHFR

Cloning, expression, and purification of MAC rDHFR has been described previously [22]. It was cloned into the vector pET15b at the *Nde*I, *Bam*HI restriction sites and expressed in *E. coli* strain BL21 Star (DE3)pLysS as a fusion protein with a His tag [2]. Purification of soluble expressed rDHFR was accomplished by binding the His tag portion of the fusion protein to a His bind (Novagen, Inc. Madison, Wis.) resin column. The His tag fusion protein was then removed by cleavage with 0.5 U of thrombin per mg of recombinant protein for 1 h on ice [2]. MTB rDHFR was cloned into a pCRT7/CT-TOPO vector (Invitrogen Life Technologies) and expressed as a 6X-His tagged fusion protein and purified in the same manner. Purified human rDHFR was kindly provided by Anatrace (Maumee, Ohio) and has been described by us previously [2].

DHFR Assay and Enzyme Inhibition

The DHFR activity for both human and mycobacterial enzymes was measured at 30ºC as the decrease in absorbance at 340 nm [2]. The reaction mixture (1 mL) contained 10 mM 2-mercaptoethanol, 0.1 mM NADPH, 0.1 mM dihydrofolate, 1 mM EDTA, 50 mM potassium phosphate (pH 7), and enzyme. The reaction was initiated by the addition of dihydrofolate after preincubation of the other components for 3 min. For inhibition assays, various concentrations of inhibitor were added to the mixture before the 3–min preincubation period. The 50% inhibitory concentration (IC_{50}) was determined from a plot of the $\log_{10}$ of the drug concentration versus percent inhibition as the amount of inhibitor required to inhibit the reaction by 50% (KC Junior software, Bio-Tek). The amount of enzyme in each inhibition assay was about 0.0024 U/mL, which yielded a mean rate of –0.029 ± 0.0010/min for MAC rDHFR (standard error of the mean; n = 7) and –0.031 ± 0.0027 for human rDHFR (standard error of the mean; n = 6). Linearity was maintained for >7 min. One unit of enzyme is defined as the amount which reduces 1 μmol of dihydrofolate/min using a molar extinction coefficient of 12,300 M^{-1} [2]. The SR was determined as the ratio of the human rDHFR IC_{50} to the MAC rDHFR IC_{50}. The same assay was used for the MTB rDHFR.

DISCUSSION

In previous studies we have demonstrated the effectiveness of DMDP derivatives against various MAC strains. Our original hypothesis was that this class of antifolates would have selective activity against MAC, an organism with inherent resistance to TMP. Trimethoprim is a commonly used antifolate with selective activity against several prokaryotic DHFRs. This reasoning was based upon the following chemical principles of DHFR inhibition.

The basic pharmacophore for DHFR inhibition can be represented as a 2,4-diamino-1,3-diazine either fused or not to another aromatic system. This group is the basis for the potent binding and inhibition of these molecules for every DFHR enzyme. DHFR functions to reduce the 7,8-double bond of folate by protonation of the 8-nitrogen, and subsequent attack of a hydride anion (from the NADPH cofactor) at the 7-carbon to balance the charge and complete the reduction. The change from a 2-amino-4-oxo system in folate to a 2,4-diamino system in the typical DHFR inhibitor increases the basicity of the nitrogen of the 1,3-diazine. This modification results in aberrant protonation at the 1-position rather than the 8-position and as a result, this intermediate cannot accept a hydride ion and collapse to product. Consequently, the end product is an intermediate bound through a salt bridge to the enzyme; this is the basis for the majority of the binding energy of these analogs to DHFR. Selectivity and some added potency, in relation to the non-classical lipophilic antifolates, is engineered through substitution of the molecule at the end distal to the salt bridge with sterically demanding hydrophobic groups. These substitutions can either bind more strongly or weakly with prokaryotic versus eukaryotic enzymes resulting in the selectivity that is seen for TMP. Basically, most non-classical lipophilic antifolates can be represented by the general structure shown in Fig. **2**.

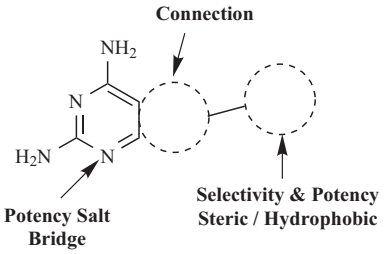

Fig. (2). Basic structure of non-classical lipophilic antifolates.

Most alterations in the "new" lipophilic antifolates are variations in the connection, not where the primary potency is derived (Fig. **2**). Simplistically, the connector region links the critical 2,4-diamino-1,3-diazine pharmacophore to the hydrophobic region that controls selectivity for different DHFR enzymes (Fig. **2**). These variations in the connector are simple modifications of an "old" paradigm realizing the critical nature of the 2,4-diamino-1,3-diazine pharmacophore [23].

Previous studies by our group have identified a series of DMDP derivatives that have exceptional *in vitro* activity against MAC that appears to be based upon their lipophilic properties and high selectivity for the MAC DHFR [1, 2]. The basic structure of this series is shown in Fig. **1**. Various derivatives in this series demonstrated good *in vitro* efficacy against three strains of MAC [2]. Additionally, it was generally apparent that particular substitutions at the R2 and R5 positions resulted in improved selectivity for the MAC DHFR but decreased selectivity for the human DHFR [2]. This resulted in enhanced SR values. These modifications did not affect the overall *in vitro* efficacy for MAC [2]. From those studies, a lead compound was developed that had exceptional *in vitro* activity against MAC and high selectivity for the MAC rDHFR but low selectivity against human rDHFR [2]. That compound was SRI-20094 [2]. With this lead information, other derivatives were synthesized using this information and the base structure depicted in Fig. **1**. These new derivatives were the basis for this study.

It is apparent from this study that the new DMDP derivatives carry similar properties of *in vitro* activity against MAC and selectivity for the MAC DHFR. Although no major improvement was observed over SRI-20094, the salt form of that derivative (SRI-20730) did maintain its *in vitro* activity for MAC and selectivity for the MAC DHFR. The salt form has improved solubility which enhances its capability to be used in experimental conditions. This should be useful for future efficacy testing in animals. Although the 2, 5-di-butyl derivative in this series was synthesized {SRI-20095, m.w. 424.54, calculated Log(p) = 4.96}, it proved to be too insoluble to manage effectively in solution. Further extended substitutions in this series were not considered because the molecular weights and calculated Log(p) values began to exceed those recommended in Lipinski's Rule of Five [24, 25].

Improved solubility was also obtained by use of *n*-O-CH_2-CH_2-O-CH_3, -O-CH_2-CH_2-O-CH_2CH_3, and -O-CH_2-O-CH_3 substitutions at the R2 and R5 positions. The calculated Log(p) values for these three derivatives (SRI-20959, -

20972, and -20973) were reduced by about a half and molecular weights remained at < 500, still keeping their characteristics in line with Lipinski's Rule of Five [24, 25]. Although these more soluble derivatives had diminished selectivity for MAC rDHFR, they did show reduced selectivity for the human rDHFR and maintained the low *in vitro* activity against MAC that has been observed with other derivatives in this group. This information should be useful for developing more selective soluble derivatives that can be properly evaluated in an animal model.

None of the new derivatives were as effective against MTB *in vitro* and did not show better selective activity against the MTB DHFR than the MAC DHFR. This finding is consistent with previous evaluations and does, however, demonstrate the selective nature of the DMDP derivatives against MAC. These results further demonstrate the apparent differences between the DHFRs of these related mycobacterial species, as suggested previously [18]. Hopefully, future studies involving the molecular structure of these two enzymes will help to better understand the specific reasons for this diversity.

As with other studies involving *in vitro* activities of similar compounds against *M. avium*, a diversity of MICs was noted for the three different strains used in this study [2]. In fact, variability in the susceptibility to antimycobacterial drugs is one of the three major differences between the *M. avium* complex and *M. tuberculosis* in regard to *in vitro* activity [26]. Thus, our results are not surprising. There are two possible explanations for this but sufficient information is not available to conclude the actual reason. One suggestion might be the strain variation in the *M. avium* cell envelops, which might contribute to the differences in drug penetration amongst members of the *M. avium* complex. Another possibility might be a variation in the binding sites of the assorted *M. avium* DHFRs. If binding site variation is the explanation, a complete understanding will have to wait until a crystal structure of the *M. avium* DHFR becomes available.

These results further support our original hypothesis that DMDP derivatives can be developed with selective activity against MAC. As indicated in our previous investigation, however, further improvement might be necessary to establish a SR that is adequate for optimum *in vivo* efficacy. Selective ratios in the neighborhood of 32,500 to 74,000, as reported for TMP and *E. coli* DHFR [27], might be more appropriate. With the enhanced solubility properties of these new DMDP derivatives, and the high-quality *in vitro* activity and selectivity properties obtained, it should be possible to continue refinement of these new compounds. Current studies are in progress to evaluate some of these lead compounds in an animal model to test *in vivo* efficacy.

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