Mutations in the *Pneumocystis jirovecii* DHPS Gene Confer Cross-Resistance to Sulfur Drugs

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*Pneumocystis jirovecii* is a major opportunistic pathogen that causes *Pneumocystis* pneumonia (PCP) and results in a high degree of mortality in immunocompromised individuals. The drug of choice for PCP is typically sulfamethoxazole (SMX) or dapsone in conjunction with trimethoprim. Drug treatment failure and sulfur drug resistance have been implicated epidemiologically with point mutations in dihydropteroate synthase (DHPS) of *P. jirovecii*. *P. jirovecii* cannot be cultured in vitro; however, heterologous complementation of the *P. jirovecii* trifunctional folic acid synthetase (*PjFAS*) genes with an *E. coli* DHPS-disrupted strain was recently achieved. This enabled the evaluation of SMX resistance conferred by DHPS mutations. In this study, we sought to determine whether *DHPS* mutations conferred sulfur drug cross-resistance to 15 commonly available sulfur drugs. It was established that the presence of amino acid substitutions (T517A or P519S) in the DHPS domain of *PjFAS* led to cross-resistance against most sulfur drugs evaluated. The presence of both mutations led to increased sulfur drug resistance, suggesting cooperativity and the incremental evolution of sulfur drug resistance. Two sulfur drugs (sulfachloropyridazine [SCP] and sulfamethoxy pyridazine [SMP]) that had a higher inhibitory potential than SMX were identified. In addition, SCP, SMP, and sulfadiazine (SDZ) were found to be capable of inhibiting the clinically observed drug-resistant mutants. We propose that SCP, SMP, and SDZ should be considered for clinical evaluation against PCP or for future development of novel sulfur drug compounds.
there was cooperativity between individual mutations that led to the increased sulfu drug resistance of the double mutants.

Recently, cloning of the trifunctional PfJAS genes and their heterologous complementation in a DHPS-disrupted E. coli host strain was achieved (15). This provided an assay method that permitted a direct assessment of sulfu drug resistance conferred by mutations observed clinically (T517A and P519S) in the PfJAS genes. This work endorsed the prediction that the double mutant (T517A and P519S) had increased sulfamethoxazole resistance (threefold) relative to the WT clone. These data provided some explanation for the epidemiological evidence that identified the predominance of the double mutants clinically (18).

While S. cerevisiae is taxonomically more closely related to P. jirovecii than E. coli, the E. coli model system proved to be more robust in evaluating sulfu drug resistance because it avoided the above-mentioned complicating parameters of the S. cerevisiae model system. In this study, we utilized heterologous complementation of PfJAS in the DHPS-disrupted E. coli host strain to evaluate sulfu drug cross-resistance of PfJAS mutants (T517A and P519S) against 15 sulfu drugs. We report the sulfu drugs that were more effective than SMX and dapson (DAP), which are currently the drugs of choice to treat PCP.

**MATERIALS AND METHODS**

**Cells, growth media, and transformation.** The bacterial strain employed for molecular cloning and plasmid amplification was Escherichia coli strain MC1061 (araD139 [ΔaroABC-lev]7679 galU galK ΔλacY17 pl4 hsdR (rK-mK) mcrB). The growth medium utilized was 1× YT medium (0.5% [wt/vol] yeast extract, 0.8% [wt/vol] tryptone, 0.5% [wt/vol] NaCl).

The DHPS-disrupted E. coli strain was C600 [ΔfolP::Km] –3 595 of 0.1. Cells were then diluted 200-fold, and 5 μl of dimethyl sulfoxide was also loaded onto the center of each plate and served as the negative control.

**Construction of vectors.** (i) pGEX.PfJAS and pET28a.PfJAS. PfJAS expression constructs in pGEX 4T-2 (phagatnine S-transferase tagged) or in pET28a (six-His tagged) have been described previously (15). In this study, we employed a pET28a.PfJAS mutant construct (M596T) that permitted heterologous complementation in E. coli. The oligonucleotides used and alleles generated are summarized in Table 1. A fourth allele, which has not been reported clinically, was synthesized by using oligonucleotides 259705 and 259706 (Table 1). This synthesis yielded T517V and P519S (VRS) which was arbitrarily defined as 100% inhibition. The wild-type inhibition radius (maximum) was the highest measured radius of inhibition (for the mutant allele).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Clone name</th>
<th>Primer no.</th>
<th>Sequence (5′-3′)*</th>
</tr>
</thead>
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<tr>
<td>TRP</td>
<td>T517 R518 P519</td>
<td>259705</td>
<td>GGTGGCCAGTCTGTACCC</td>
</tr>
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<td>VRS</td>
<td>T517 R518 S519</td>
<td>259706</td>
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<td>ARS</td>
<td>T517 R518 S519</td>
<td>109216</td>
<td>GAGGAGTTGTCTCCAGG</td>
</tr>
<tr>
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<td>T517 R518 S519</td>
<td>109217</td>
<td>TCTAAGATCTATACGG</td>
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<td>T517 R518 S519</td>
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<td>CGTGAGATGTAGTAGAC</td>
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<td>T517 R518 S519</td>
<td>109219</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>CAGAACCCCTCAACGG</td>
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</tbody>
</table>

*Lowercase letters signify noncoding sequence.

**Synthesis of mutant alleles implicated with sulfa drug resistance in pGEX. PfJAS.** Three alleles having mutations at T517A and P519S (designated ARS, TRS, and ARP) are found in PCP patients who have been treated with sulfa drugs (3, 17, 18). Their synthesis was achieved by using a Quikchange XL sitel-directed mutagenesis kit (15) and has been described previously. The oligonucleotides used and alleles generated are summarized in Table 1. A fourth allele, which has not been reported clinically, was synthesized by using oligonucleotides 259705 and 259706 (Table 1). This synthesis yielded T517V and P519S for PfJAS and is designated VRS herein. The DNA sequence of mutants was confirmed by DNA sequencing analysis using a Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). Functional complementation was confirmed by transforming the clones into the E. coli C600ΔfolP::Km strain and plated onto 1× YT medium minus thymidine.

**Analysis of sulfa drug resistance by agar drug diffusion assays.** In order to evaluate the sensitivity or resistance of each DHPS allele to sulfa drugs, drug diffusion assays were performed as described previously (13). These assays were performed in 86-mm-diameter petri dishes containing 22.1 ml (±0.1 ml) aliquots of 1× YT medium solidified with 1.5% [wt/vol] agar and supplemented with the appropriate selective antibiotic. A 6.5-mm hole was made in the center of each agar plate. Sulfu drugs were prepared immediately before use and dissolved in dimethyl sulfoxide to 100 mg/ml, and 30 μl was loaded onto the center of each plate and allowed to diffuse through the agar for at least 5 h. In control plates, 30 μl of dimethyl sulfoxide was also loaded onto the center of each plate and allowed to diffuse through the agar. Each experiment was done in quadruplicate. Clones were precultured in 2× YT broth at 37°C. The E. coli cultures were harvested while in log-phase growth, washed with 1× phosphate-buffered saline (0.8% [wt/vol] NaCl, 0.2% [wt/vol] KCl, 1.44% [wt/vol] Na2HPO4, 0.24% [wt/vol] KH2PO4, pH 7.4), and normalized to an A595 of 0.100. Clones were then diluted 500-fold in 1× phosphate-buffered saline prior to inoculation. A six-spoke inoculation tool (13) was used to inoculate six individual clones radially from the center of the agar plate. Plates were then incubated at 37°C for 24 h and then cooled to 4°C until the zones of inhibition were measured. Zones of inhibition were measured to the nearest half-millimeter and then analyzed by using PRISM version 4.0 statistical analysis software (for Windows; GraphPad Software, San Diego, Calif. [http://www.graphpad.com]). Analyses performed were the determinations of means and standard deviations for all data sets. Each data set was normalized to determine resistance or sensitivity relative to the wild-type allele (TRP) according to the following equation: [mean radius (mutant) – mean radius (WT)] × 100. The mean radius (maximum) was the highest measured radius of inhibition (for the mutant VRS) which was arbitrarily defined as 100% inhibition. The wild-type inhibition was defined as the baseline or 0% inhibition.

**Analysis of the IC50 in broth cultures.** Transforms of pGEX 4T-2-PfJAS were precultured in 1× YT medium containing ampicillin (100 μg/ml) and kanamycin (30 μg/ml). Cells were harvested during mid-log-phase growth and normalized to an A595 of 0.1. Cells were then diluted 200-fold, and 5 μl was used to seed 145 μl of 1× YT medium containing 100 μg of ampicillin/ml, 30 μg of kanamycin/ml, and sulfu drugs in a 96-well plate (catalog number 167008; Numc). SCP was evaluated at 0 to 250 μg/ml in 5-μg/ml increments. SMX was evaluated at 0 to 250 μg/ml in 5- to 200-μg/ml increments. SMX and SDZ were evaluated

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**TABLE 1. Oligonucleotides used to synthesize the mutant alleles**

<table>
<thead>
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</tr>
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<td>T517 R518 S519</td>
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<td>GAGGAGTTGTCTCCAGG</td>
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</table>

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**Antimicrob. Agents Chemother.**

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FIG. 1. Inhibition of PjFAS mutants expressed from pGEX 4T-2 determined by using the agar drug diffusion assay. (a) Means and standard deviations of the zone of inhibition. (b) The mean and standard deviation was calculated for each data set which was then normalized against the wild-type allele (TRP) and expressed as percent sensitivity or percent resistance relative to the wild type. Analysis was performed by using GraphPad Prism software, version 4.00.
at 0 to 1,200 μg/ml in 50- to 100-μg/ml increments. Cultures were grown at 37°C for 24 h, the turbidity \(A_{600}\) was measured by using a Multiskan Ascent microplate reader (Thermo Labsystems), and the data were plotted with Graphpad PRISM software (version 4.00). Each experiment was performed in quadruplicate and set up by using a Rapidplate liquid-handling robot (Qiagen). The 50% inhibitory concentration (IC\(_{50}\)) was determined to be the drug concentration required to reduce the turbidity relative to the “no-drug control” by 50%.

**RESULTS**

Sulfonamide cross-resistance of mutant PjFAS alleles. In order to assess whether the mutations that conferred SMX resistance (as previously demonstrated [15]) also conferred cross-resistance to other sulfa drugs, the growth inhibition of four mutant clones (VRS, ARS, TRS, and ARP) by 15 sulfa drugs was evaluated by using an optimized agar drug diffusion assay described previously (13). The growth inhibition was analyzed as described in Materials and Methods. The absolute inhibition of each allele to each sulfa drug is presented in Fig. 1a. The WT (normalized to 0% inhibition) was compared against each mutant allele (with the maximum inhibition [for VRS] normalized to 100%) (Fig. 1b). Each sulfa drug evaluated in this study inhibited the mutants by various degrees. No consistent resistance profile was observed between the mutants across the range of sulfa drugs. The magnitude of resistance of each allele varied depending on the identity of the sulfa drug. These data were indicative of cross-resistance and suggested that the resistance conferred by an amino acid substitution (in the catalytic site of DHPS) does not establish unequivocal or comparable resistance to all sulfonamides.

While it was observed that the mutant alleles were more resistant than the WT, there were a number of exceptions where the mutant alleles were more sensitive than the WT. Specifically, these exceptions were noted: (i) VRS was more sensitive than the WT to the sulfa drugs SCP, SMP, and SDZ; (ii) TRS was more sensitive than the WT to the sulfa drugs SCP, SMP, SDZ, and SDM; and (iii) ARP was more sensitive than the WT to SCP. This was also the case in medium that was prepared by using tryptone which was free of sulfonamide antagonists (catalog number 1.02239; Merck). Evidently, the two sulfa drugs (SCP and SMP) had the highest inhibitory activity overall and appeared to be more effective against the mutant alleles relative to the WT. SDZ also showed greater inhibitory potential against the mutants VRS, ARS, and TRS than the WT, but ARP was more resistant.

The 15 sulfa drugs evaluated against PjFAS were ranked from highest to lowest inhibitory potential as follows: SCP > SMP > SMX > STZ > SAM > SDZ > SDM > SMO > SMR > SIA > SSA > DAP > SQX > SPD > SDX. The drugs SMO to SDX, which had the lowest inhibitory potential, were also the least effective against the mutant alleles. The sulfa drugs SCP to SDM (with the exception of SMX and STZ) showed some capacity to inhibit the mutant alleles relative to the inhibition of the WT. Comparison of the R groups of the “top-10-ranked” sulfa drugs revealed some common elements (Fig. 2). With the exception of SAM and SSA, the sulfa drugs that had the highest inhibitory potential have an R group that consists of an aromatic ring with a N substitution at the ortho

![FIG. 2. (a) Structure of pABA and sulfonamides. (b) Three-dimensional structural alignment of SCP and SMP.](http://aac.asm.org/Downloadedfromhttp://aac.asm.org/ on May 19, 2021 by guest)
position or adjacent to the amide group. SAM and SSA have an O substitution at the ortho position. Using this model, it was evident that DAP, which has been utilized for the treatment of PCP, had a significantly lower inhibitory potential than SMX. In fact, DAP was among the least effective inhibitors in this assay system.

The three-dimensional alignment of SCP and SMP (Fig. 2b) done by ChemSketch/3D Viewer (version 5.07; ACD/Chem software, Advanced Chemistry Development Inc., Toronto, Ontario, Canada) revealed that they maintain an identical three-dimensional spatial orientation that is unique among the sulfa drugs evaluated in this study. This result may be an indicator of a more specific fit of these two drugs into the pABA binding site of DHPS of PjFAS. Alternatively, these drugs may achieve higher intracellular concentrations either through a higher diffusion rate constant into E. coli or, conversely, a lower efflux rate.

Sulfa drug inhibition of DHPS from various species. We sought to investigate whether (i) the sulfa drug resistance pattern observed using this assay system was a function of the permeability of specific sulfa drugs through the membrane of the folP host strain, (ii) the antifolate activity of the 15 sulfa drugs was comparable between different species of FAS and DHPS, and (iii) the DHPS mutations were significant variables in sulfa drug resistance profiles. We therefore compared the resistance profile of the WT and mutant alleles expressed from pET28a of PjFAS M596T with those from previous comparable studies performed with ScFAS (13), Pf-PPPK.DHPS (PfFAS) (6), and EcDHPS (this study).

Comparison of the ratio of inhibition between the six-His tagged constructs of EcDHPS, ScFAS, PfFAS, and PjFAS M596T constructs (means and standard deviations) expressed from pET28a determined by using the agar drug diffusion assay.

![FIG. 3. Inhibition of EcDHPS, PfFAS, ScFAS, and PjFAS M596T constructs (means and standard deviations) expressed from pET28a determined by using the agar drug diffusion assay.](http://aac.asm.org/)

TABLE 2. Ranking of inhibitory potential of sulfa drugs for DHPS from various species in the E. coli C600ΔFolP strain

<table>
<thead>
<tr>
<th>Sulfa drug by rank</th>
<th>S. cerevisiae</th>
<th>P. jirovecii</th>
<th>P. falciparum</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SCP</td>
<td>SCP</td>
<td>SCP</td>
<td>SCP</td>
</tr>
<tr>
<td>2</td>
<td>STZ</td>
<td>SMP</td>
<td>SAM</td>
<td>SAM</td>
</tr>
<tr>
<td>3</td>
<td>SMX</td>
<td>SMX</td>
<td>SMO</td>
<td>SMO</td>
</tr>
<tr>
<td>4</td>
<td>SAM</td>
<td>STZ</td>
<td>STZ</td>
<td>STZ</td>
</tr>
<tr>
<td>5</td>
<td>SSA</td>
<td>SAM</td>
<td>SIA</td>
<td>SIA</td>
</tr>
<tr>
<td>6</td>
<td>SMP</td>
<td>SDZ</td>
<td>SMX</td>
<td>SAM</td>
</tr>
</tbody>
</table>

*WT only.
inhibitory sulfa drug. However, the ranking of the remaining 14 sulfa drugs differed from species to species. Furthermore, a different pattern of resistance was noted between PjFAS (T517A and P519S) and ScFAS (T597A and P599S) mutants (10). This result indicated that identical amino acid substitutions at comparable sites can have significantly different resistance outcomes, implicating subtle structural differences between the individual DHPS superstructures.

**Determination of IC$_{50}$ of PjFAS alleles to SCP, SMP, SMX, and SDZ.** In order to quantitatively determine the resistance of the four drugs found to be most inhibitory to the drug-resistant alleles, liquid growth inhibition assays were performed with 96-well microtiter plates. In agreement with the drug diffusion assays, these data indicated that SCP and SMP had significantly higher inhibitory potential than SMX (Fig. 4). The MIC (the concentration required to inhibit 100% of growth) of SCP was in the order of 60 $\mu$g/ml (0.2 mM), and the MIC of SMP was in the order of 150 $\mu$g/ml (0.5 mM), while the MIC of SMX and SDZ was closer to 700 $\mu$g/ml (2.8 mM).

A finer discrimination of resistance between each allele could be discerned by determining the IC$_{50}$ (Fig. 4 and Table 3). It was determined that the resistance pattern of each allele relative to the WT was consistent with the results of the agar drug diffusion assay (with one exception). In agreement with the SDZ drug diffusion assays, the mutants TRS, VRS, and ARS were more sensitive than the WT, while ARP was more resistant than the WT in the liquid growth inhibition experiment. Also in agreement with the SMX drug diffusion assays, it was found that all mutant alleles were more resistant than the WT in the liquid growth inhibition experiment. Furthermore, as predicted by the SMP drug diffusion assay, VRS and ARS were more sensitive than the WT, while ARP was more resistant than the WT in the liquid growth inhibition assay, which was contrary to the agar drug diffusion data. Despite this latter incongruous result, it was evident that like SDZ, SMP was capable of inhibiting the naturally occurring double mutant ARS and single mutant TRS to a greater degree than the WT.

The discordant data between the agar drug diffusion assay and the liquid growth inhibition assay for SCP remains unresolved. However, it was not surprising that sulfa drug resistance results do not always translate from agar to liquid media. A case in point is the sulfamethoxazole resistance of the PjFAS mutants determined previously (15) by using the validated Etest methodology. The MIC of 1 to 3 $\mu$g/ml determined in that study does not correlate to the MIC determined with liquid medium, which was in the order of 600 $\mu$g/ml. Clearly, the dynamics of nutrient, sulfa drug, and sulfa adduct diffusion (30) in and out of the cell are significantly different between agar and liquid.

**DISCUSSION**

A large amount of epidemiological data has linked the emergence of mutations in PjFAS to the exposure to sulfa drugs, but direct evidence demonstrating that such mutations confer resistance in *P. jirovecii* has only recently emerged (15). That previous work demonstrated that amino acid substitutions in PjFAS T$_{517}$A and P$_{519}$S (ARS) conferred a threefold-increased level of SMX resistance relative to the WT and that the individual DHPS superstructures.

**TABLE 3. IC$_{50}$ of *E. coli C600ΔfolP::Km*’ transformed with mutant or WT PjFAS**

<table>
<thead>
<tr>
<th>Drug</th>
<th>TRP</th>
<th>ARP</th>
<th>TRS</th>
<th>ARS</th>
<th>VRS</th>
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<tr>
<td>SCP</td>
<td>11.7</td>
<td>15.1</td>
<td>6.4</td>
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<td>SMP</td>
<td>28</td>
<td>37</td>
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<td>27</td>
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</tr>
<tr>
<td>SMX</td>
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<td>60</td>
</tr>
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</table>
individual amino acid substitutions functioned cooperatively, resulting in the elevated resistance of double mutant ARS.

The cross-resistance data presented herein provide a similar overall resistance profile and support the previous findings of cooperativity between individual mutations that resulted in increased resistance of the double mutant ARS. This point was not absolutely true nor was it without exception in this cross-resistance study. The single mutant ARP was more resistant than the double mutant ARS for five drugs (SCP, SMP, SDZ, and SMR). Clearly, cooperation between individual amino acid substitutions that lead to higher resistance is dependent on the drug and its fit into the pABA binding site of DHPS.

Of particular interest was the finding that the mutant ARS, which is the clinical isolate observed most frequently, was considerably more resistant than the synthetic double mutant VRS and the single mutants ARP and TRS. Liquid growth inhibition studies confirmed previous findings (15) which showed a threelfold-increased level of SMX resistance (IC_{50}) of ARS compared to that of the WT. However, the absolute drug concentration required was significantly higher in liquid growth inhibition assays than in agar drug diffusion assays. This may be explained by the significant differences in drug and nutrient diffusion in the two medium types.

This finding is in contrast with previous findings with S. cerveiae model systems (13, 14) that showed VRS to be more resistant than ARS. This finding highlights that the role of residues T517 and P519 of PfFAS (or T597A and P599S of ScFAS) is highly conserved in terms of conferring sensitivity or resistance to sulfa drugs between homologs of FAS, the tertiary context of these residues in the overall DHPS sequence and structure is critically important. That is, subtle differences in the catalytic site can have profound effects on drug resistance. Clearly, VRS is significantly more resistant than ARS in the S. cerveiae FAS structure, but ARS is significantly more resistant than VRS in the P. jirovecii FAS structure.

The data presented in this study demonstrated that the mutations T517A and P519S in PfFAS led to cross-resistance for most sulfa drugs evaluated. Clearly, mutations that lead to resistance against one drug can have broad implications for resistance against an entire class of drug. This finding would be consistent with a highly conserved drug binding site and is supported by the consistent resistance trend observed from previous model studies (13). Clearly, subtle structural differences in the pABA binding site dictate drug specificity and resistance for different species as evidenced by the different intrinsic resistance-susceptibility pattern to sulfa drugs (Table 2). Four DHPS structures have been solved, E. coli (1), Staphylococcus aureus (10), Mycobacterium tuberculosis (4), and S. cerveiae (M. C. Lawrence et al., unpublished data). It is evident that the pterin binding site is exquisitely conserved, while the pABA binding site shows significant structural variation (despite sequence conservation) and is reflected by the observed variation in sulfa susceptibility between species. SCP consistently showed the highest inhibitory potential for all DHPS enzymes, indicating that some sulfonamides may have broad-spectrum activity against DHPS from various species while some sulfonamides can act with greater species specificity. On this last point, there seemed considerable overlap in the efficacy of the top-6-ranked sulfa drugs across four different species of DHPS (Table 2). Selective diffusion through the E. coli membrane may be a significant contributor to the higher activity of SCP. If so, drug diffusion would be a key criterion to investigate for the selection of a species-specific inhibitor.

PjFAS M596T was observed to have higher susceptibility to sulfa drugs than EcDHPS and ScFAS. Based on the ranking of inhibitory activity for various sulfa drugs, it is possible that PCP therapy could be more efficacious by the choice of a sulfa drug (such as SCP or SMP) which had higher activity relative to SMX. It would seem that these data can be interpreted to suggest that antifolate compounds (sulfonamides or otherwise) can be selected through screening to have greater drug specificity to individual DHPS species. Furthermore, antifolates such as SCP that had the highest activity against the four DHPS species (including Pf-PPPK.DHPS, as described by Berglez et al. [6]) indicates that antifolates that have broad-spectrum activity can be identified.

Of the 15 drugs evaluated, 2 drugs were noted to be conspicuously superior to SMX in their inhibitory potential: SCP and SMP, which were ranked first and second. SMX, which has been the sulfa drug of choice to treat or prevent PCP infections, was ranked third. More importantly, it was observed that SDZ (ranked sixth), was superior to all other drugs due to its ability to effectively inhibit the mutant alleles (ARS, VRS, and TRS) to a greater degree than the WT. SDZ was the only drug capable of inhibiting ARS. These features make these three sulfa drugs worthy candidates for further evaluation against PCP. These data support previous findings which demonstrated that SMP was effective against a mouse model of PCP (5, 12).

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