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Charged propargyl-linked antifolates reveal mechanisms of antifolate resistance and inhibit trimethoprim-resistant MRSA strains possessing clinically relevant mutations

Stephanie Reeve[‡], Eric Scocchera[‡], Jacob Ferreira, Narendran G-Dayanandan, Santosh Keshipeddy, Dennis L. Wright^{*}, and Amy C. Anderson^{*}

Department of Pharmaceutical Sciences, University of Connecticut, 69 N. Eagleville Rd., Storrs, CT 06269

Abstract

Drug-resistant enzymes must balance catalytic function with inhibitor destabilization to provide a fitness advantage. This sensitive balance, often involving very subtle structural changes, must be achieved through a selection process involving a minimal number of eligible point mutations. As part of a program to design propargyl-linked antifolates (PLAs) against trimethoprim-resistant dihydrofolate reductase (DHFR) from *Staphylococcus aureus*, we have conducted a thorough study of several clinically observed chromosomal mutations in the enzyme at the cellular, biochemical and structural levels. Through this work, we have identified a promising lead series that displays significantly greater activity against these mutant enzymes and strains than TMP. The best inhibitors have enzyme inhibition and MIC values near or below that of trimethoprim against wild-type *S. aureus*. Moreover, these studies employ a series of crystal structures of several mutant enzymes bound to the same inhibitor; analysis of the structures reveals a more detailed molecular understanding of drug resistance in this important enzyme.

Graphical Abstract

^{*}Corresponding authors: Dennis L. Wright, dennis.wright@uconn.edu, 860-486-9451; Amy C. Anderson, amy.anderson@uconn.edu, 860-486-6145.

[‡]Author Contributions

These two authors contributed equally

Accession Codes

Authors will release the atomic coordinates and structure factors have been deposited in the RCSB Protein Data Bank (PDB Codes: 5ISP, 5IST and FISQ). Authors will release the atomic coordinates and experimental data upon article publication.

Supporting Information

Supporting Information is available free of charge via the Internet at <http://pubs.acs.org>. Full mutant generation data, K_i and IC₅₀ with standard deviations, thymidine rescue MICs, crystallography diffraction and refinement statistics, OMIT map and synthetic methods.

Keywords

antifolate; MRSA; drug resistance; trimethoprim; PLAs

Introduction

Trimethoprim-sulfamethoxazole is first-line treatment for methicillin-resistant *Staphylococcus aureus* (MRSA) infections, especially those associated with community-acquired MRSA¹⁻⁴. Trimethoprim inhibits dihydrofolate reductase while sulfamethoxazole inhibits dihydropteroate synthase. Both of these essential enzymes are involved in the folate biosynthetic pathway, which is critical for the creation of one-carbon donors in metabolism⁵. While this combination has had wide success, resistant strains started to emerge in the 1990s⁶. Initial studies of clinical isolates showed that the accumulation of point mutations in the *dfrB* chromosomal gene was observed in 88 % of the tested isolates of *S. aureus*, establishing mutation as a principal mode of trimethoprim resistance⁷. Additional mechanisms of resistance include the acquisition of plasmid-encoded trimethoprim-resistant DHFRs encoded by the genes *dfrA*, also called S1 DHFR^{8,9}, *dfrG* and *dfrK*¹⁰⁻¹³; *dfrG* and *dfrK* have been observed outside the United States. Point mutations in *dfrB* confer resistance with MIC values 256 µg/mL; acquisition of S1 DHFR confers greater levels of resistance with MIC values 512 µg/mL⁷.

Analysis of several resistant clinical isolates shows that the mutation F98Y is highly prevalent, especially in combination with secondary mutations, H149R or H30N⁷. Dale, et al. showed that the IC₅₀ value for TMP increased ~400-fold with the Sa(F98Y) enzyme and reported a crystal structure of the SaDHFR enzyme with the F98Y mutation bound to NADPH, the cofactor, and dihydrofolate, the substrate⁷. Interestingly, the S1 DHFR protein natively includes a tyrosine at the 98 position as well as two other key mutations: G43A and V31I, relative to TMP-sensitive *S. epidermis*⁸. Incorporation of G43A and F98Y has been shown to confer TMP resistance⁹. Overall, studies of resistant strains confirm that new generations of antifolates targeting *S. aureus* DHFR must inhibit the mutant forms of the

enzyme, including chromosomal mutants and plasmid-encoded resistant forms, in addition to the wild-type enzyme¹².

We have been designing, synthesizing and evaluating propargyl-linked antifolates to broadly inhibit trimethoprim-resistant enzymes. Over the years, we have shown that the PLAs potently inhibit MRSA and *Streptococcus pyogenes*¹⁴, that crystal structures with the PLAs and the F98Y and F98Y/H30N mutated DHFR proteins^{15, 16} reveal the structural basis of trimethoprim resistance, and that the PLAs have low mutation frequencies and low mutation prevention concentrations¹⁷. This latter study of prospective resistance mechanisms for a lead compound (compound **1**) showed that mutations F98Y, F98I and H30N occurred with low rates of mutation (10^{-9} – 10^{-10}). Recently, we reported that specific enantiomers of the PLAs at the propargyl position steer the biaryl domain into one of two conformations, thus changing interactions with both the enzyme and the cofactor¹⁸. Some of the specific enantiomers of the PLAs were able to potently inhibit the F98Y enzyme and strain containing F98Y with K_i values of 9 nM and MIC values of 0.625 μ g/mL. In these studies, we observed in crystal structures that specific enantiomers selected the alternate α -configuration of NADPH in the cofactor binding site.

In this work, we fully characterized single- and double-step mutants of *S. aureus* DHFR that arise after exposure with an experimental antifolate, **8**. We also show the acquisition of key clinical mutations: F98Y, H30N, H149R, F98Y/H30N and F98Y/H149R, albeit at lower mutational frequencies, also confer resistance to TMP. The mutant enzymes and strains were fully characterized in order to understand fitness and the biochemical effect of the mutations. Excitingly, we show that a recently disclosed series of PLA-carboxylates (PLA-COOH) very potently inhibit both single and double mutant enzymes as well as wild-type and mutant *S. aureus* strains²⁰. Crystal structures of the single and double mutant enzymes with the PLA-COOHs reveal the mechanisms of TMP resistance as well as the basis of the potency of the new PLA-COOHs. Lessons learned from these biochemical, microbiological and structural studies of drug-resistant mutant DHFR can be applied toward the development of compounds that overcome chromosomal mutant DHFR as well as plasmid-encoded resistant DHFR.

Results and Discussion

Generation and characterization of MRSA strains resistant to compound **1**

In earlier work¹⁷, we reported an initial investigation into the potential resistance mechanisms of strain *S. aureus* 43300 to overcome inhibition by compound **1** and showed that two mutants, F98Y and F98I in the DHFR gene, were selected with low mutation frequencies (10^{-10}). To further characterize the resistance profile of the propargyl-linked antifolates, we carried out single- and double-step selection studies and characterized the resulting strains. In single-step studies, mutant selection with the ATCC quality control strain 43300 subjected to compound **1** at 6x MIC yielded three clinically observed mutations: F98Y, H30N and H149R as well as three novel mutations: F151S, F151C and D142Y. A second round of resistance selection using compound **1** and progenitor strains possessing F98Y or H149R yielded a series of both novel and clinically relevant double mutants (Table 1 and Supplemental Table 1 for comprehensive list of mutations). As strains

containing H30N/F98Y and F98Y/H149R mutants have been isolated clinically, we chose to fully characterize the fitness of these mutant enzymes and bacteria, including their single mutant counterparts (F98Y, H30N and H149R) at a biochemical, structural and cellular level.

Average overall mutation frequencies for each strain exposed to compound **1** were calculated based on the inoculum and number of colonies appearing on multiple plates with a concentration of compound **1** at 6xMIC. Sa43300 exhibits a rise in MIC to compound **1** at a very low frequency of 2.96×10^{-10} . Overall mutation frequencies are even lower to progenitor strains possessing F98Y and H149R, with rates of 6.56×10^{-11} and 3.75×10^{-11} . Specific mutational frequencies were then calculated based on the number of sequenced colonies with a particular mutation. If the generation of double mutants occurs in a step-wise fashion, the combined frequency of resistance could be as low as 10^{-21} . Evaluation of bacterial fitness, measured by doubling time, shows that the majority of the mutant strains exhibit only minor losses in growth time (1.08–1.2 x doubling time of wild-type), with the exception of Sa(F98Y/H149R), which preserved or slightly improved doubling time (Table 1). Overall, these studies show that the mutant strains are relatively fit when compared to wild-type.

The recombinant mutant DHFR enzymes were created by site-directed mutagenesis of the wild-type enzyme and purified using affinity chromatography. Michaelis-Menten kinetics were measured for each enzyme using previously published assay conditions using 12.5–100 μ M NADPH and 12.5–100 μ M DHF (Table 2)¹⁷. Overall, all enzymes, other than Sa(H149R), have k_{cat}/K_M values within approximately 2-fold of the wild-type value. Sa(H149R) has a significantly reduced k_{cat}/K_M value (6-fold reduction), which is a consequence of higher K_M values for both DHF and NADPH. Interestingly, the double mutant Sa(F98Y/H149R) compensates for the low efficiency of the single Sa(H149R) mutant as the Sa(F98Y/H149R) enzyme restores the K_M value for DHF and NADPH to nearly wild-type values. This compensatory relationship is also observed with the Sa(F98Y/H30N) double mutant. The single H30N mutation suffers a significant decrease in NADPH K_M (31.21 to 79.89 μ M); this K_M value is restored to a value near wild-type in the double Sa(H30N/F98Y) mutant.

Design of PLAs to Overcome Resistance in *S. aureus*

The goal of our program is to design and develop PLAs that efficiently inhibit the growth of wild-type MRSA and major strains of trimethoprim-resistant MRSA while maintaining a low rate of resistance. Knowledge of the prospective resistance to an earlier generation PLA (compound **1**) has been critical in guiding the development of newer generation PLAs that overcome the resistance conferred by the point mutations. Specifically, studies described above show that the mutations F98Y, H30N and H149R and their double mutant combinations, are viable enzymes and confer fitness to the bacteria. Overcoming these mutant strains will be critical to developing an antifolate that is not only effective against TMP-resistant clinical strains, but also for ensuring a PLA that will be effective for a more durable lifetime.

We analyzed three critical sources of information to design new PLAs to overcome mutation-based resistance. Firstly, we examined crystal structures of PLAs bound to wild-type and F98Y mutant enzymes to elucidate the structural basis of potency. From this analysis, it was apparent that the pocket in the enzyme that binds the glutamate moiety of the dihydrofolate substrate¹⁵ was not occupied and a properly placed substituent may lead to greater potency. We therefore incorporated a carboxylate moiety into some of the PLAs (compounds **8–12**, Table 3) in order to garner ionic interactions with basic residues near the surface of the active site (Figure 2d, specifically note Arg 57). Secondly, we examined previously identified structure-activity relationships^{15, 17, 18} and tested several compounds likely to inhibit the single mutant enzymes (Tables 3 and 4). Finally, we also incorporated the knowledge that when the propargyl position is substituted with a small alkyl group, specific enantiomers steer the biaryl domain of the PLAs and often have different affinities for the enzyme and for inhibiting the growth of the bacteria¹⁸. Therefore, we evaluated several single enantiomers (**5–7, 9, 11–12**, Table 3) in this study in addition to racemic mixtures and compounds with unsubstituted propargyl positions.

All of the compounds exhibit good potency (K_i values less than 16 nM; IC_{50} values are shown in Supplemental Table 3) against the single mutant enzymes Sa (F98Y) and Sa(H30N) with only minor losses relative to wild-type (Table 4). Compound **2** lost the greatest affinity for the Sa(H30N) enzyme with a 12.6-fold loss. Activity against the single mutant Sa(H149R), however, was more compromised. TMP loses activity against the Sa(H149R) mutant by 69-fold. The PLAs possess a range of affinity for this enzyme, ranging from compound **12** with a K_i value of 153 nM to compound **2** with a K_i value of 1362 nM. Interestingly, compound **3** with unconstrained 3' and 4' methoxy groups maintained reasonable affinity (119 nM) when compared to compound **2**, showing that flexibility may be critical for affinity to the mutant enzymes. Against the double mutant, Sa (F98Y, H30N), TMP loses 130-fold in affinity. In general, the dioxalane compounds (**2** and **7**) as well as compounds **4–6** lose significant affinity for the Sa (F98Y, H30N) double mutant enzyme (180 to 300-fold loss).

The design of compounds **8–12** is predicated on a possible interaction with a conserved arginine to provide compensatory interactions in these mutant enzymes. Pleasingly, compounds **9–12** show much greater affinity for the double mutant Sa (F98Y, H30N) enzyme with K_i values ranging from 19–45 nM. Maintaining activity against the double mutant Sa (F98Y, H149R) enzyme is clearly more challenging as compounds **1–8** and TMP show K_i values of 107–2059 nM. However, compounds **11** and **12** show significant inhibition for this enzyme, with K_i values of 69 and 55 nM, respectively. Again, it appears that the presence of the ionized carboxylates may provide critical additional interactions to compensate for reduced contacts elsewhere in the complex.

The compounds were then tested for inhibition of the growth of wild-type and mutant strains of *S. aureus* (Sa(F98Y), Sa(H30N), Sa(H149R), Sa(H30N, F98Y) and Sa(F98Y/H149R) (Table 5). The antibacterial activity of TMP was clearly crippled by even the single mutations and reached a 50–100 $\mu\text{g/mL}$ MIC value against the double mutant strains. PLAs **1–9** were more potent against the wild-type strain than TMP and many were more potent against the single mutants (MIC values between 0.078 and 5 $\mu\text{g/mL}$). However, PLAs **1–9**

also suffered significant losses against the strains with double mutations in DHFR. Excitingly, PLAs **10–12** have superior activity against the wild-type strain as well as strains with both single and double mutants. Notably, the MIC value for compound **12** against either of the double mutant strains is only 4-fold higher than the MIC value of TMP for wild-type *S. aureus*. On-target activity for the compounds was verified by supplementing the medium with thymidine and measuring MIC values. These MIC values rise by 64x – 4081x, validating that the compounds inhibit DHFR.

Structural Studies

A series of crystal structures with the wild-type and mutant enzymes elucidates the structural changes caused by the selected mutations. Mutants Sa (F98Y), Sa (H30N) and Sa (H30N, F98Y) DHFR were co-crystallized with NADPH and compound **8**; the structure of the wild type Sa DHFR complex with NADPH and **8** has been previously described (PDB: 5HF0; Figure 1a)²⁰. The structures of the wild-type and Sa (H30N) enzymes feature mixed occupancy of α - and β -NADPH in the cofactor binding site (Figure 2), similar to previous observations^{15, 18}.

The structural basis of antifolate resistance in these F98Y mutant enzymes appears to involve the loss of a critical hydrogen bond between the carbonyl backbone of Phe 92 and the conserved 4-amino group of the inhibitor. Comparisons between structures of wild-type SaDHFR and those with the F98Y mutant enzymes show that the F98Y mutation induces a rotation of the Phe 92 backbone carbonyl by 1.1 Å, which disrupts the hydrogen bond between the carbonyl oxygen and the N₄-amino group of the diaminopyrimidine ring (Figure 3). The hydrogen bond between the Phe 92 carbonyl and the 4-amino group in the wild-type enzyme is 2.9 Å; in the mutated structure it is 3.9 Å. There is a new hydrogen bond between Tyr 98 and the 4-amino group, but with reduced strength (3.4 Å). Loss of this conserved hydrogen bond to Phe 92 would represent a significant loss in affinity.

The presence of the Asn 30 mutation results in the re-organization of a water network between the active site residues and the inhibitor, effectively eliminating a hydrogen bond between the pyrimidine ring and protein. In the structure with wild-type SaDHFR, a critical water molecule bound to the N₂-amino group of the diaminopyrimidine (2.9 Å; cyan in Figure 1c) is involved in a water network with the backbone carbonyl of Tyr 109 (2.8 Å) and the imidazole side chain of His 30, effectively tying the diaminopyrimidine to two protein residues (Figure 4). In fact, the distance of this water molecule bound to His 30 is quite conserved, having an average value of 2.9 Å over several SaDHFR:PLA crystal structures. In the structures with the Asn 30 mutation, the water molecule (magenta in Figure 4) more closely coordinates with the mutated Asn residue instead of the inhibitor, reducing the effective hydrogen bonding interactions of the inhibitor. Measurements of the distance of the water molecule to the 2-amino group show that the bonds are 3.4 Å and 3.5 Å in the SaH30N and Sa(F98Y/H30N) structures, respectively; these distances are too far to create effective hydrogen bonds. This disruption of the water network linking the inhibitor to the active site residues is similar to that reported in our earlier structure¹⁶.

The double mutant enzyme, F98Y/H30N shows both the shifted carbonyl for Phe 92 as well as the altered water network at Asn 30, greatly decreasing the hydrogen bonding interactions of the 2- and 4-amino groups of the pyrimidine ring. There is a striking functional synergy between these two mutations. Not only does the second mutation further weaken inhibitor binding by disrupting contacts at the pyrimidine ring, but also restores the catalytic activity of the enzyme to near wild-type values (biochemical data in Table 2). The two cooperative effects provide a powerful mechanism for the organism to become drug resistant.

In general, the PLAs show significantly increased potency for the mutant enzymes relative to TMP. One reason for this increased potency is the coordination of a water molecule, observed in both the F98Y and F98Y/H30N structures, between the carboxylate group on **8** and Arg 57 (2.5 Å) (Figure 5). The extra hydrogen bonds created by the coordinated water molecule and Arg 57 compensate for interactions lost by the pyrimidine ring.

In addition, the presence of α -NADPH plays a role in the increased potency of the PLAs for the mutant enzymes. The displaced nicotinamide of α -NADPH coordinates a water molecule (2.4 Å) that stabilizes the carbonyl of Phe 92 (3 Å), preserving the hydrogen bonding interactions between Phe 92 and the inhibitor (Figure 3). β -NADPH does not coordinate this water molecule, does not stabilize the Phe 92 carbonyl and effectively allows the loss of the hydrogen bond. Diffraction data and refinement statistics are reported in Supplemental Table 5 and electron density (2Fo-Fc) for structures shown in Supplemental Figure 1.

Conclusion

Trimethoprim is a first-line therapeutic for treating infections caused by *S. aureus*. Over time, TMP resistance in the form of mutations in the chromosomal gene as well as plasmid-encoded insensitive DHFRs has threatened the use of this agent. Here, we select mutant *S. aureus* strains that confer resistance to both TMP and the PLAs, focusing on those strains that have been observed to confer TMP resistance clinically. In general, the mutation frequency for the PLAs is very low (10^{-10} – 10^{-11}). A series of fitness experiments show that the mutant enzymes and strains, and especially double mutants, are as fit as wild-type. Evaluation of TMP and a series of twelve PLAs shows that the PLAs are generally much more potent than TMP against the mutant enzymes and strains. Specifically, the PLAs possessing a carboxylate moiety (compounds **9–12**) exhibited good potency against the difficult double mutant enzymes and strains. These carboxylate PLAs also show low mammalian cell toxicity and good metabolic stability, making them promising drug leads²⁰.

Crystal structures of one of the PLA-carboxylate compounds (compound **8**) bound to NADPH and SaDHFR (wt), SaDHFR (F98Y), SaDHFR (H30N) and SaDHFR (F98Y/H30N) show that the mutations specifically diminish the binding of the 2- and 4-amino groups on the pyrimidine ring. The carboxylate moiety forms water-mediated hydrogen bonds with an active site arginine, compensating for some of the lost pyrimidine interactions and restoring affinity to the mutant enzymes. Overall, the PLA-COOH compounds appear to be excellent antifolate leads that maintain potency for wild-type enzymes and strains while overcoming resistance features in mutant enzymes and strains.

Experimental

Inhibitors

Trimethoprim is commercially available (Sigma Aldrich). The PLAs are previously published as follows: Compound **1** was published¹⁴, compounds **2**, **4–7** were published¹⁸ and compounds **8–12** are described²⁰. Compound **3** was not previously published. Its synthesis followed published routes¹⁴ and characterization is shown in Supporting Information. All inhibitors 95% pure as determined by HPLC.

The ¹H and ¹³C NMR spectra were recorded on Bruker instruments at 500 MHz. Chemical shifts are reported in ppm and are referenced to residual CHCl₃ solvent; 7.24 and 77.23 ppm for ¹H and ¹³C respectively. The high-resolution mass spectrometry was provided by the University of Connecticut Mass Spectrometry Laboratory using AccuTOF mass spectrometer with a DART source. TLC analyses were performed on Sorbent Technologies silica gel HL TLC plates. All glassware was oven-dried and allowed to cool under an argon atmosphere. Anhydrous dichloromethane, ether, and tetrahydrofuran were used directly from Baker Cycle-Tainers. Anhydrous dimethylformamide was purchased from Acros and degassed by purging with argon. Anhydrous triethylamine was purchased from Aldrich and degassed by purging with argon. All reagents were used directly from commercial sources unless otherwise stated. A pre-mixed heterogeneous mixture of CuI (10%/w) in Pd(PPh₃)₂Cl₂ – (Pd/Cu) was used for the Sonogashira coupling. Purity analyses were performed with reversed phase high performance liquid chromatography (RP-HPLC) performed on a Shimadzu Prominence 20 instrument fitted with a Luna 5 μm C18(2) 100 Å column (5 μm, 4.6 mm × 250 mm, Phenomenex) and detected using a UV diode array at 254 nm. Two separate determinations, method A with isocratic 50% (v/v) MeCN in 50% mM potassium phosphate monobasic at pH 7.0 and method B with isocratic 80% (v/v) MeOH in 20% mM potassium phosphate monobasic at pH 7.0, were employed to determine compound purity. Compounds were diluted in HPLC grade methanol and filtered prior to analysis. Sample concentrations were 1 mg/mL, and injection volumes were 1 μL–3 μL. The purity of the final compounds were found to be 95%.

Procedure for the synthesis of 5-(3-(3,4-dimethoxy-5-(pyridin-4-yl)phenyl)prop-1-ynyl)-6-ethylpyrimidine-2,4-diamine (3)—To a 20 mL screw cap vial with stir bar was added (0.23 mmol, 0.065 g, 1 eq) ethyl-iododiaminopyrimidine, (0.02 mmol, 0.013 g, 0.08 eq) Pd/Cu and (2.3 mmol, 0.23 g, 10 eq) KOAc. Argon purged anhydrous DMF (0.05 M, 4.6 mL) was added followed by alkyne (0.30 mmol, 0.075 g, 1.3 eq). The reaction mixture was stirred under argon for 15 min and degassed once using freeze/pump/thaw method. The vial was sealed under argon, heated at 60 °C and reaction monitored by TLC. At the end of the reaction, the dark reddish brown solution was concentrated and product purified by flash column chromatography (for pre-absorption of crude mixture SiO₂ in 10%/w of cysteine - 1.5 g, NH₂ capped SiO₂ – 1.5 g), 13 g SiO₂ for column, gradient elution from 2% – 8% MeOH/CH₂Cl₂) to afford the coupled pyrimidine as white solid. (0.045 g, 50% yield). TLC R_f = 0.2 (5% MeOH/CH₂Cl₂); ¹H NMR (500 MHz, Chloroform-d) δ 8.62 (d, J = 5.9 Hz, 2H), 7.41 (d, J = 5.9 Hz, 2H), 7.02 (s, 1H), 6.95 (s, 1H), 5.10 (s, 2H), 4.81 (s, 2H), 3.91 (s, 3H), 3.89 (s, 2H), 3.60 (s, 3H), 2.68 (q, J = 7.6 Hz,

2H), 1.20 (t, J = 7.6 Hz, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 173.8, 164.6, 161.0, 153.5, 150.0, 146.1, 145.7, 133.4, 133.2, 124.2, 121.2, 112.6, 96.2, 90.6, 76.2, 61.1, 56.2, 30.0, 26.3, 12.8; HRMS (DART, M+ + H) m/z 390.1955 (calculated for C₂₂H₂₄N₅O₂, 390.1930).

Biological

Mutant Generation and Identification—Resistant strains were selected by plating 100 µL of overnight culture (approx. 10¹² CFU/mL) of progenitor strain on Isosensitest (Oxoid) agar plates containing 6x MIC of **1** and incubated at 37 °C for 18 hours. Single colonies were isolated and the *dhfrB* gene was identified by directly sequencing the colony PCR product. For colony PCR, cells were lysed using 1 mg/mL lysostaphin and 20 µg/mL proteinase K in 0.1 M Tris, pH 7.5. The gene was amplified using sense primer (5'-ATGACTTTATCCATTCTAGTTGC-3'), anti-sense primer (5'-TTATTTTTTACGAATTAAATGTAG-3') and rTaq Polymerase (Takara) following standard PCR conditions. PCR products were purified using Promega Wizard SV Gel and PCR Clean Up system and sequenced using the sense primer.

The mutational frequency of **1** was determined by the number of resulting colonies divided by the total inoculum (1x10¹¹ CFU/mL) for each progenitor strain. The frequency of the specific mutations was determined by multiplying the mutational frequency for the inhibitor-strain pair by the frequency of sequenced colonies containing the specific mutation.

Minimum Inhibitory Concentrations (MICs)—Minimum inhibitory concentrations were determined according to Clinical and Laboratory Standards Institute's guideline for Standard Micro-dilution broth²¹ assay using a final inoculum of 5 x 10⁵ CFU/mL in Isosensitest Broth (Oxoid). The MIC was defined as the lowest concentration of inhibitor to visually inhibit growth. Growth was monitored at A₆₀₀ after 18 h of incubation at 37 °C. MICs were confirmed, calorimetrically, using Presto Blue (Life Technologies). Isosensitest broth supplemented with 10 µg/mL thymidine was used to determine any off-target antibacterial activity²².

Growth Curves—LB media (50 mL) was inoculated with 1 mL of overnight culture. Growth was monitored at A₆₀₀ every 30 minutes. The doubling time was determined from the linear portion of the growth curve by the following equation:

$$\text{Doubling Time} = \frac{\Delta \text{Time} * \log 2}{\log(\text{Final Conc.}) - \log(\text{Initial Conc.})}$$

Generation, expression and purification of Sa(F98Y, H149R) and Sa(H149R)

DHFR Enzymes—The generation, expression and purification of Sa(F98Y), Sa(H30N) and Sa(F98Y, H30N) DHFR enzymes have been previously reported^{15, 16}. Sa(WT) and Sa(F98Y) in pET-41a(+) constructs were used for the generation of Sa(H149R) and Sa(F98Y, H149R) DHFR plasmids via QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) using sense primer 5'-CTAGATGAGAAAAATACAATTCCACGTAC-3' and anti-sense primer 5'-CGAATTAAATGTAGAAAGGTACGTGGAAT-3' following the manufacturer's instructions. Mutagenesis was confirmed via sequencing. The recombinant

Sa (H149R) and Sa (F98Y, H149R) enzymes was over-expressed in *E. coli* BL21 (DE3) (Invitrogen) cells with 1 mM IPTG following previously reported procedures. Pellets were lysed using 1x BugBuster (Novagen) and DNase A (ThermoFisher Scientific) and purified using nickel affinity chromatography (5Prime). Protein was desalted using a PD-10 column (GE Healthcare) into buffer containing 20 mM Tris pH 7.0, 20% glycerol, 0.1 mM EDTA, 2 mM DTT and stored in aliquots at -80°C .

Enzyme Kinetics—Enzyme kinetics were determined by Lineweaver-Burke plots generated by enzyme activity assays using 12.5–100 μM DHF with 20 μM NADPH for DHF K_M and V_{max} or 12.5–100 μM NADPH with 50 μM DHF for NADPH K_M . K_M values were determined by non-linear regression analysis using GraphPad.

Enzyme Inhibition—Enzyme inhibition assays were performed by monitoring the rate of NADPH oxidation by DHFR via absorbance at 340 nm at room temperature in assay buffer containing 20 mM TES, pH 7.0, 50 mM KCl, 0.5 mM EDTA, 10 mM beta-mercaptoethanol, and 1 mg/mL BSA using 0.1 mM NADPH and 2 $\mu\text{g/mL}$ enzyme. Inhibitor, in DMSO, was added to enzyme:NADPH mixture and allowed to incubate for 5 minutes before the addition of 0.1 mM DHF in 50 mM TES, pH 7.0. The inhibitor concentration and volume are based on the conditions that result in a 50% reduction in enzyme activity.

Crystallization of Sa(F98Y) DHFR:NADPH:8—Sa(F98Y) DHFR at 14.25 mg/mL was incubated with 1 mM **8** and 2 mM NADPH for three hours. It was crystallized in a solution containing 0.1 M MES pH 6.0, 0.2 M Sodium acetate, 13% PEG 10,000 and 20% gamma-butyrolactone. Crystals were frozen with well solution containing 25% glycerol.

Sa(H30N) DHFR:NADPH:8—Sa(F98Y) DHFR at 14.25 mg/mL was incubated with 1 mM **8** and 2 mM NADPH for three hours. It was crystallized in solution containing 0.1 M MES pH 5.75, 0.3M Sodium acetate, 17% PEG 10,000 and 20% gamma-butyrolactone. Crystals were frozen with well solution containing 25% glycerol.

Sa(H30N, F98Y) DHFR:NADPH:8—Sa(F98Y/H30N) DHFR at 21 mg/mL was incubated with 1 mM **8** and 2 mM NADPH for three hours. It was crystallized in a solution containing 0.1 M MES, pH 6.0, 0.2 M Sodium acetate and 13% PEG 10,000 and 20% gamma-butyrolactone was added at 20%. Crystals were frozen using 25% glycerol. All data were collected at SSRL on beamline 7-1. Data were indexed, refined and scaled with HKL2000. Phenix was used for molecular replacement using 3F0Q²³ as a model and for structure refinement^{24–26}. All diffraction data and refinement statistics are reported in Supplemental Table 5 and electron density (2Fo-Fc) for structures shown in Supplemental Figure 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

PLA	propargyl-linked antifolate
DHFR	Dihydrofolate Reductase
TMP	trimethoprim
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
PLA-COOH	carboxylate propargyl-linked antifolates
WT	wild-type

References

1. Frei C, Miller M, Lewis J, Lawson K, Hunter J, Oramasionwu C, Talbert R. Trimethoprim-sulfamethoxazole or clindamycin for community-associated MRSA (CA-MRSA) skin infections. *J Am Board Fam Med*. 2010; 23:714–719. [PubMed: 21057066]
2. Gorwitz R, Jernigan D, Powers J, Jernigan J. Participants in the CDC-convened experts' meeting on management of MRSA in the community. Strategies for clinical management of MRSA in the community: Summary of an experts' meeting convened by the Centers for Disease Control and Prevention. 2006 community.
3. Nathwani D, Morgan M, Masterton R, Dryden M, Cookson B, French G, Lewis D, Infections BSfACWPoC-oM. Guidelines for UK practice for the diagnosis and management of methicillin-resistant *Staphylococcus aureus* (MRSA) infections presenting in the community. *J Antimicrob Chemother*. 2008; 61:976–994. [PubMed: 18339633]
4. Liu C, Bayer A, Cosgrove S, Daum R, Fridkin S, Gorwitz R, Kaplan S, Karchmer A, Levine D, Murray B, Rybak M, Talan D, Chambers H. America, Infectious Diseases Society of America, Clinical practice guidelines by the infectious diseases society of America for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children. *Clin Infect Dis*. 2011; 52:e18–55. [PubMed: 21208910]
5. Zhou W, Scocchera E, Wright D, Anderson A. Antifolates as effective antimicrobial agents: new generations of trimethoprim analogs. *Med Chem Comm*. 2013; 4:908–915.
6. Houvinen P, Sundstrom L, Swedberg G, Skold O. Trimethoprim and sulfonamide resistance. *Antimicrob Agents Chemother*. 1995; 39:279–289. [PubMed: 7726483]
7. Dale G, Broger C, D'Arcy A, Hartman P, DeHoogt R, Jolidon S, Kompis I, Labhardt A, Langen H, Locher H, Page M, Stuber D, Then R, Wipf B, Oefner C. A single amino acid substitution in *Staphylococcus aureus* dihydrofolate reductase determines trimethoprim resistance. *J Mol Biol*. 1997; 266:23–30. [PubMed: 9054967]
8. Dale G, Broger C, Hartman P, Langen H, Page M, Then R, Stuber D. Characterization of the gene for the chromosomal dihydrofolate reductase (DHFR) of *Staphylococcus epidermis* ATCC 14990: the origin of the trimethoprim-resistant S1 DHFR from *Staphylococcus aureus*? *J Bacteriol*. 1995; 177:2965–2970. [PubMed: 7768789]
9. Heaslet H, Harris M, Fahnoe K, Sarver R, Putz H, Chang J, Subramanyam C, Barreiro G, Miller JR. Structural comparison of chromosomal and exogenous dihydrofolate reductase from *Staphylococcus*

- aureus* in complex with the potent inhibitor trimethoprim. *Proteins*. 2009; 76:706–717. [PubMed: 19280600]
10. Kadlec K, Schwarz S. Identification of a novel trimethoprim resistance gene, *dfiK*, in methicillin-resistant *Staphylococcus aureus* ST398 strain and its physical linkage to the tetracycline resistance gene *tet(L)*. *Antimicrob Agents Chemother*. 2009; 53:776–778. [PubMed: 19015335]
 11. Nurjadi D, Friedrich-Janicke B, Schafer J, Van Genderen P, Goorhuis A, Perignon A, Neumayr A, Mueller A, Kantele A, Schunk M, Gascon J, Stich A, Hatz C, Caumes E, Grobusch M, Fleck R, Mockenhaupt F, Zanger P. Skin and soft tissue infections in intercontinental travellers and the import of multi-resistant *Staphylococcus aureus* to Europe. *Clin Microbiol Infect*. 2015; 21:567, e1–10. [PubMed: 25753191]
 12. Nurjadi D, Olalekan A, Layer F, Shittu A, Alabi A, Ghebremedhin B, Schaumber F, Hofmann-Eifler J, Genderen P, Caumes E, Fleck R, Mockenhaupt F, Herrmann M, Kern W, Abdulla S, Grobusch M, Kremsner P, Wolz C, Zanger P. Emergence of trimethoprim resistance gene *dfiG* in *Staphylococcus aureus* causing human infection and colonization in sub-Saharan Africa and its import to Europe. *J Antimicrob Chemother*. 2014; 69:2361–2368. [PubMed: 24855123]
 13. Sekiguchi J, Tharavichitkul P, Miyoshi-Akiyama T, Chupia V, Fujino T, Araake M, Irie A, Morita K, Kuratsuji T, Kirikae T. Cloning and characterization of a novel trimethoprim-resistant dihydrofolate reductase from a nosocomial isolate of *Staphylococcus aureus* CM.S2 (IMCJ1454). *Antimicrob Agents Chemother*. 2005; 49:3948–3951. [PubMed: 16127079]
 14. Viswanathan K, Frey K, Scocchera E, Martin B, Swain P, Alverson J, Priestley N, Anderson A, Wright D. Toward new therapeutics for skin and soft tissue infections: propargyl-linked antifolates are potent inhibitors of MRSA and *Streptococcus pyogenes*. *PLoS ONE*. 2012; 7(2):e29434. [PubMed: 22347365]
 15. Frey K, Liu J, Lombardo M, Bolstad D, Wright D, Anderson A. Crystal structures of wild-type and mutant methicillin-resistant *Staphylococcus aureus* dihydrofolate reductase reveal an alternative conformation of NADPH that may be linked to trimethoprim resistance. *J Mol Biol*. 2009; 387:1298–1308. [PubMed: 19249312]
 16. Frey K, Lombardo M, Wright D, Anderson A. Towards the understanding of resistance mechanisms in clinically isolated trimethoprim-resistant, methicillin-resistant *Staphylococcus aureus* dihydrofolate reductase. *J Struct Biol*. 2010; 170:93–97.
 17. Frey K, Viswanathan K, Wright D, Anderson A. Prospectively screening novel antibacterial inhibitors of dihydrofolate reductase for mutational resistance. *Antimicrob Agents and Chemother*. 2012; 56:3556–3562. [PubMed: 22491688]
 18. Keshipeddy S, Reeve S, Anderson A, Wright D. Nonracemic antifolates stereoselectively recruit alternate cofactors and overcome resistance in *S. aureus*. *J Am Chem Soc*. 2015; 137:8983–8990. [PubMed: 26098608]
 19. Oefner C, Parisi S, Schulz H, Lociuoro S, Dale G. Inhibitory properties and X-ray crystallographic study of the binding of AR-101, AR-102 and iclaprim in ternary complexes with NADPH and dihydrofolate reductase from *Staphylococcus aureus*. *Acta Cryst*. 2009; D65:751–757.
 20. Scocchera E, Reeve S, Keshipeddy S, Lombardo M, Hajian B, Sochia A, Alverson J, Priestley N, Anderson A, Wright D. Charged non-classical antifolates with activity against gram-positive and gram-negative pathogens. *ACS Med Chem Lett*. 2016 in press.
 21. CLSI. Performance standards for antimicrobial susceptibility testing; sixteenth informational supplement. Vol. M100-S16CLSI. Wayne, PA: 2012.
 22. Molina F, Jimenez-Sanchez A, Guzman E. Determining the optimal thymidine concentrations for growing Thy- *Escherichia coli* strains. *J Bacteriol*. 1988; 180:2992–2995.
 23. Frey K, Georgiev I, Donald B, Anderson A. Predicting resistance mutations using protein design algorithms. *Proc Natl Acad Sci*. 2010; 107:13707–13712. [PubMed: 20643959]
 24. Adams P, Afonine P, Bunkóczi G, Chen V, Davis I, Echols N, Headd J, Hung LW, Kapral G, Grosse-Kunstleve R, McCoy A, Moriarty N, Oeffner R, Read R, Richardson D, Richardson J, Terwilliger T, Zwart P. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Cryst*. 2010; D66:213–221.
 25. Emsley P, Cowtan K. Coot: Model-building tools for molecular graphics. *Acta Cryst*. 2004; D60:2126–2132.

26. McCoy A. Solving structures of protein complexes by molecular replacement with Phaser. *Acta Cryst.* 2007; D63:32–41.

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Figure 1.
Structure of Trimethoprim and PLAs

Figure 2.
Stereo view of wild-type SaDHFR (purple) bound to β -NADPH (salmon), α -NADPH (yellow) and compound **8** (green).

Figure 3. The shift in the position of the carbonyl of Phe 92 in the F98Y mutant (cyan) eliminates a hydrogen bond to the 4-amino group of the inhibitor. The black bar represents the 1.1 Å shift. The water molecule bound to the nicotinamide ring of α -NADPH and Phe 92 is shown.

Figure 4.

Two water molecules (blue and red) tie the pyrimidine ring to His 30 (wt) and Tyr 109. The H30N mutation (magenta) pulls one of the water molecules (magenta) 1 Å away from the 2-amino group, eliminating the hydrogen bond.

Figure 5.
A water molecule forms hydrogen bonds between the carboxylate and Arg 57.

Table 1

Mutant Strain Selection using Compound **1**

DHFR Mutation	Progenitor Strain	Single Nucleotide Polymorphism	Colonies Sequenced (%)	Mutational Frequency ^a	Doubling Time (min)
WT	---	---	---	---	32.04
F98Y	Sa43300	TTT to TAT	2/19 (10.5)	3.11 x 10 ⁻¹¹	34.53
H30N	Sa43300	CAT to AAT	3/19 (15.8)	4.67 x 10 ⁻¹¹	35.62
H149R	Sa43300	CAT to CGT	7/19 (36)	1.65 x 10 ⁻¹⁰	38.34
F98Y/H30N	Sa(F98Y)	CAT to AAT	2/16 (12.5)	8.2 x 10 ⁻¹²	35.06
F98Y/H149R	Sa(H149R)	TTT to TAT	1/1 (100)	1.23x10 ⁻¹¹	30.89

^a Overall resistance frequency for Sa43300 with compound **1** at 6x MIC = 2.96 (±1.58) x 10⁻¹⁰

Overall resistance frequency for Sa43300(H149R) with compound **1** at 6x MIC = 3.75x 10⁻¹¹

Overall resistance frequency for Sa43300(F98Y) with compound **1** at 6x MIC = 6.56 (± 1.57) x 10⁻¹¹

Table 2

Mutant Enzyme Characterization

DHFR	K _M (DHF) (μM)	V _{max} (DHF)	K _M (NADPH) (μM)	k _{cat} DHF (s ⁻¹)	k _{cat} /K _M DHF
WT	17.5	62.93	31.21	41.13	2.4
F98Y	8.38	68.38	57.08	44.76	5.3
H30N	24.49	45.76	79.89	29.91	1.2
H30N, F98Y	11.24	39.98	51.17	26.1	2.3
H149R	63.54	42.44	303.4	27.74	0.4
F98Y, H149R	5.24	44.98	45.08	29.40	5.6

Table 3

Structure of Lead PLA Inhibitors

Cmpd	R _p	R ₁	R ₂	R ₃	Ar
1	CH ₃	H	OCH ₃	H	Pyridine
2	CH ₃	H	OCH ₂	OCH ₂	Pyridine
3	H	H	OCH ₃	OCH ₃	Pyridine
4	CH ₃	OCH ₃	H	H	Pyridine
5	R-CH ₃	H	OCH ₃	H	Pyridine
6	R-CH ₃	OCH ₃	H	H	Pyridine
7	R-CH ₃	H	OCH ₂	OCH ₂	Pyridine
8	H	OCH ₃	H	H	Phenyl- <i>p</i> -COOH
9	S-CH ₃	OCH ₃	H	H	Phenyl <i>p</i> -COOH
10	H	H	OCH ₃	H	Phenyl <i>p</i> -COOH
11	R-CH ₃	H	OCH ₃	H	Phenyl <i>p</i> -COOH
12	S-CH ₃	H	OCH ₃	H	Phenyl <i>p</i> -COOH

Table 4K_i (nM)^a of PLAs against WT and mutant enzymes

Cmpd	Sa(WT)	Sa(F98Y)	Sa(H30N)	Sa(H149R)	Sa(H30N, F98Y)	Sa(F98Y, H149R)
TMP	3.43	14.68	6.89	240.8	595.1	1729
1	2.83	13.14	12.98	681.7	191.7	2059
2	2.53	3.01	33.25	1363	485.9	779.3
3	2.09	12.13	4.54	119.3	449.3	107.1
4	4.51	19.71	5.90	1563	820.3	878.5
5	2.68	8.57	16.72	1154	801.2	894.5
6	1.64	16.69	16.53	269.6	417.9	422.3
7	5.21	13.60	8.85	174.0	345.7	289.1
8	4.76	11.75	3.89	130.9	158.3	142.2
9	5.51	11.83	3.74	862.4	35.87	295.3
10	1.64	11.36	3.36	323.9	45.17	184.4
11	1.33	7.88	3.54	111.5	19.10	69.41
12	2.09	5.57	3.34	153.1	16.57	55.77

^aK_i errors reported in Supplemental Table 2

Table 5

Minimum Inhibitory Concentrations (µg/mL)

Cmpd	Sa43300	Sa(F98Y)	Sa(H30N)	Sa(HI49R)	Sa(H30N, F98Y)	Sa(F98Y, HI49R)
TMP	0.3125	10	2.5	2.5	50	100
1	0.0781	2.5	2.5	2.5	20	80
2	0.0781	1.25	5	5	40	40
3	0.0195	0.625	0.625	0.625	20	40
4	0.625	2.5	2.5	2.5	20	40
5	0.0391	0.625	0.625	0.625	40	20
6	0.0195	1.25	0.625	1.25	10	20
7	0.0195	1.25	2.5	2.5	20	20
8	0.0195	0.1563	0.1563	0.1564	6.25	>50
9	0.0391	0.1563	0.1563	0.0781	>40	>40
10	0.0195	0.1563	0.1563	0.1563	5	10
11	0.0098	0.0781	0.0781	0.0391	1.25	2.5
12	0.0098	0.0781	0.0781	0.0781	1.25	1.25