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Resistance to the antimicrobial agent fosmidomycin and an FR900098 prodrug through mutations in the deoxyxylulose phosphate reductoisomerase gene (dxr)

Christopher M. Armstrong  
*Washington University School of Medicine in St. Louis*

David J. Meyers  
*Johns Hopkins University*

Leah S. Imlay  
*Washington University School of Medicine in St. Louis*

Caren Freel Meyers  
*Johns Hopkins University*

Audrey R. Odom  
*Washington University School of Medicine in St. Louis*

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There is a pressing need for new antimicrobial therapies to combat globally important drug-resistant human pathogens, including *Plasmodium falciparum* malarial parasites, *Mycobacterium tuberculosis*, and Gram-negative bacteria, including *Escherichia coli*. These organisms all possess the essential methylerythritol phosphate (MEP) pathway of isoprenoid biosynthesis, which is not found in humans. The first dedicated enzyme of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr), is inhibited by the phosphonic acid antibiotic fosmidomycin and its analogs, including the N-acetyl analog FR900098 and the phosphoryl analog fosfoxacin. To identify mutations in *dxr* that confer resistance to these drugs, a library of *E. coli dxr* mutants was screened at lethal fosmidomycin doses. The most resistant allele (with the S222T mutation) alters the fosmidomycin-binding site of Dxr. The expression of this resistant allele increases bacterial resistance to fosmidomycin and other fosmidomycin analogs by 10-fold. These observations confirm that the primary cellular target of fosmidomycin is Dxr. Furthermore, cell lines expressing Dxr-S222T will be a powerful tool to confirm the mechanisms of action of future fosmidomycin analogs.

Isoprenoids represent the most diverse class of natural products and are essential to all living cells (1, 2). Notable isoprenoids include such critical molecules as chlorophyll, ubiquinone, and cholesterol. All isoprenoids are derived from a common set of precursors, the 5-carbon molecules isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) (3). Both IPP and DMAPP are synthesized *de novo* by all free-living organisms, and a failure to produce these compounds is incompatible with life. Most eukaryotes, including humans, utilize the six-step mevalonate (MVA) pathway, which produces IPP and DMAPP from acetyl-coenzyme A (CoA) (4). In contrast, an alternative independently evolved route, the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, is utilized by Gram-negative bacteria, a subset of Gram-positive bacteria, plastid-containing eukaryotes, including parasitic protozoa in the *Apicomplexa* phylum, and plants (which additionally use the MVA pathway in the cytoplasm) (4, 5). Unlike the MVA pathway, the MEP pathway utilizes pyruvate and glyceraldehyde 3-phosphate in a seven-step enzymatic cascade to produce IPP and DMAPP (6, 7) (Fig. 1A). The MEP pathway is essential in key human pathogens, including *Plasmodium falciparum* malarial parasites and the bacterium *Mycobacterium tuberculosis*, and in many Gram-negative bacterial pathogens (8–11). Because this pathway is absent in humans, MEP pathway enzymes represent an attractive target for antimicrobial development (12, 13).

To date, the most successful antimicrobial agents targeting the MEP pathway are the phosphonic acid fosmidomycin (FSM) and its N-acetyl and phosphoryl analogs, FR900098 and fosfoxacin (Fig. 1B). FSM and its analogs inhibit the growth of a wide range of organisms that utilize the MEP pathway, including *P. falciparum*, bacteria, and plants (8, 14–17). Of these, FSM has been investigated most extensively. Enzymatic analysis has shown that FSM potently inhibits the first dedicated step of the MEP pathway, the reduction/isomerization of 1-deoxy-D-xylulose 5-phosphate (DOXP) to MEP by the enzyme DOXP reductoisomerase (Dxr) (EC 1.1.1.267) (18). FSM appears to act as a slow tight-binding inhibitor that binds within the active site in competition with the DOXP substrate (19). However, *in vivo* analysis has shown that the subsequent step in the pathway, the cytidylation of MEP by the enzyme MEP cytidyltransferase (IspD), may be a secondary target of FSM (20).

Initial clinical studies have demonstrated that fosmidomycin is safe and moderately effective in the treatment of clinical malaria (21, 22); the drug is currently in phase IIb studies in combination with piperaquine (23). FSM has several limitations as a clinical therapeutic. Its highly charged nature results in poor pharmacokinetics and a plasma half-life of 3.5 h (24, 25). In addition, there is an unacceptably high rate of recrudescence malaria in children treated with the FSM combinations tested so far, likely related to this rapid plasma clearance (25, 26). Finally, FSM is excluded from most cells, with the exception of organisms, such as *P. falciparum* and *Escherichia coli*, which actively transport this compound (27, 28). Because of this impermeability, FSM is ineffective against many potential pathogens, such as *Toxoplasma gondii* (29) and *M. tuberculosis* (10). Thus, substantial efforts have been made to develop more cell-
We established the utility of a FSM-resistant enzymologic effects of FSM-resistant resistance to FSM in vivo. Our results demonstrate that FSM-resistant dating the mechanism of antimicrobial activity of a novel modified protocol for error-prone PCR (41). The reaction buffer included have been collected in the study of FSM and its Dxr target (19, 24, 36–38), yet no mutation in dxr has been reported to confer resistance to FSM. Targeted mutagenesis studies have identified residues in Dxr that affect enzymatic activity (39, 40), yet some of these variant Dxr enzymes bind FSM even when enzymatic activity has been abolished. In the current study, we took a targeted mutagenic approach to identify FSM-interacting residues. We used error-prone PCR to screen for possible sequence changes in dxr and ispD that confer resistance in E. coli to FSM. Using this approach, we demonstrated that mutations in dxr can confer resistance to FSM in vivo and in vitro, and we characterized the enzymologic effects of FSM-resistant dxr mutant proteins. Finally, we established the utility of a FSM-resistant E. coli strain for validating the mechanism of antimicrobial activity of a novel FR900098 prodrug. Our results demonstrate that FSM-resistant E. coli will be a valuable tool in screening efforts to identify novel inhibitors targeting the Dxr active site and for confirming the mechanisms of action of rationally designed fosmidomycin and FR900098 analogs.

**MATERIALS AND METHODS**

**Generation of mutant libraries of E. coli dxr and ispD.** A library containing a collection of either dxr mutants or ispD mutants was created using a modified protocol for error-prone PCR (41). The reaction buffer included Tris (pH 8.3), KCl, MgCl₂, dGTP, dCTP, dTTP, dATP, forward primer, reverse primer, Taq DNA polymerase (Sigma-Aldrich) in a final reaction volume of 50 µ. The forward primer used for dxr amplification was 5′-GGGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGAAGCAACTCACCATTCTGGG-3′, and the reverse primer 5′-GGGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGGCAACCACTCATTTGGATG-3′. The reverse primer used for dxr amplification was 5′-GGGGGACACTTTTGACAAAAAGGCTGGTTATGTATTTCTCCCTGTAGGATGGTTCG-3′. PCR products were then cloned into the vector pDONR221 using the Gateway BP reaction (Invitrogen), which was used to transform chemically competent E. coli strain TOP10 cells (Invitrogen), and colonies containing library DNA were selected for on LB agar plates containing 50 µg/ml kanamycin (Sigma-Aldrich). Each transformation yielded approximately 10,000 individual colonies, which were scraped off the plates and pooled in LB liquid medium just prior to DNA purification. The plasmid libraries were then used to insert either the ispD or dxr gene into the bacterial expression vector pDX-cdbB using the Gateway LR reaction (Invitrogen) to yield many pDX-ispD and pDX-dxr variants. Construction of the pDX-cdbB vector proceeded as follows: the genomic dxr sequence from E. coli strain K-12 substrain MG1655, along with flanking regions assumed to comprise the transcriptional promoter and terminator, were previously TOPO cloned into the proprietary vector pCR2.1 (Life Technologies). In order to create a Gateway destination plasmid, with expression under the control of the dxr promoter, a 1,531-bp sequence flanked by the BamHI and EagI sites, and including the entire E. coli-derived sequence (1,455 bp), was cut out of the pCR2.1-derived vector and inserted into pBR322 (New England BioLabs) between the BamHI and EagI restriction sites. Ninety-eight percent of the dxr coding sequence, located between the BanII and BbvCI restriction sites, was removed by restriction enzyme digestion and replaced, via blunt-end ligation cloning, with sequence amplified from the proprietary Gateway destination vector pDEST17 (Life Technologies). This sequence had been amplified using the forward primer 5′-CAGGATATCACAAGTTTGTACAAAAAAGCTGGGTTTATGTATTCTCCTGATGGATGGTTCG-3′ and the reverse primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCATGGCAACCACTCATTTGGATG-3′. Screening of the dxr and ispD libraries. For each library, a total of 6×10⁶ CFU of TOP10 cells (transformed with library pDX-ispD or pDX-dxr) were plated across six LB/amp plates, three containing 100 µM FSM (Invitrogen) and three containing 50 µM FSM, and grown at 37°C for 18 h. The resulting colonies were restreaked on plates with LB/amp plus 50 µM FSM. The plates were left at ambient temperature, and additional colonies permeable analogs of FSM or its highly related analog, FR900098 (30–35).

A wealth of enzymatic, crystallographic, and clinical data have been collected in the study of FSM and its Dxr target (19, 24, 36–38), yet no mutation in dxr has been reported to confer resistance to FSM. Targeted mutagenesis studies have identified residues in Dxr that affect enzymatic activity (39, 40), yet some of these variant Dxr enzymes bind FSM even when enzymatic activity has been abolished. In the current study, we took a targeted mutagenic approach to identify FSM-interacting residues. We used error-prone PCR to screen for possible sequence changes in dxr and ispD that confer resistance in E. coli to FSM. Using this approach, we demonstrated that mutations in dxr can confer resistance to FSM in vivo and in vitro, and we characterized the enzymologic effects of FSM-resistant dxr mutant proteins. Finally, we established the utility of a FSM-resistant E. coli strain for validating the mechanism of antimicrobial activity of a novel FR900098 prodrug. Our results demonstrate that FSM-resistant E. coli will be a valuable tool in screening efforts to identify novel inhibitors targeting the Dxr active site and for confirming the mechanisms of action of rationally designed fosmidomycin and FR900098 analogs.

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**FIG 1** Mutations in MEP pathway genes confer resistance to fosmidomycin (FSM). (A) FSM inhibits catalysis by Dxr of the conversion of DOXP to MEP, with concomitant oxidation of NADPH. (B) Structures of inhibitors used in this study. (C) Schematic of selection strategy for ispD or dxr variants that confer resistance to FSM.
that formed after another 48 h were also restreaked. After final growth at 37°C for 24 h, a single colony from each plate was grown in liquid LB/amp medium, and the plasmid was isolated using the QIAprep spin miniprep kit (Qiagen). Calcium chloride competent MG1655 E. coli cells (a kind gift of David Hunstad, Washington University) were transformed with the isolated plasmid, and transformants were streaked onto plates with LB/amp plus 50 μM FSM. Strains that survived to grow after retransformation were further analyzed.

**Measurement of growth inhibition of E. coli.** Growth inhibition of E. coli was evaluated as previously described (20). In brief, overnight cultures of E. coli were diluted 1:100 into fresh LB medium supplemented with either the appropriate antibiotic or no antibiotic if no plasmid was maintained and grown to an optical density at 600 nm (OD600) of 0.5. Cultures were diluted to 105 CFU/ml in 200 μl of a well of a 96-well plate, with the indicated amounts of the inhibitory compound. Bacteria were grown in a FLUOstar Omega microplate reader (BMG Labtech) at 37°C and 700 rpm, with serial OD600 measurements. The GraphPad Prism software was used to calculate inhibitory constants (50% effective concentrations [IC50]) during logarithmic growth (8 to 10 h). All IC50 values in the text reflect the means from three or more independently performed experiments.

**Site-directed mutagenesis of dxr.** The vector pCR2.1-dxr was created by using PCR to amplify the 2,817-bp sequence between nucleotides 192528 and 193544 on the E. coli chromosome, which was then cloned into the vector pCR2.1 using the TOPO-TA cloning kit (Invitrogen). The vector pACYC-dxr was created by cloning the 2,911-bp insert between the HindIII and NotI sites in pCR2.1-dxr and ligating it into the vector pACYC184 with the 910-bp HindIII/EagI fragment removed. The vector pACYC-dxr was created using the QuikChange site-directed mutagenesis kit (Stratagen) and primers with the sequences 5′-CCAGACC TTTGTGTATCATGTTAGCGGTGTCAGGAAATTATTTGCCCCCA TCG-3′ and 5′-CGATGCGGCCTAATAATTTCGCTGACAGCGTAC CATGTATGACAAACGGCTGG-3′, which alter nucleotides at positions 663 and 664 in the open reading frame (ORF) from TT to CA. This mutation changes the codon for serine 222 to a threonine and creates a novel AccI restriction cleavage site at positions 658 to 663. Site-directed mutagenesis was confirmed by successful digestion with AccI and Sanger sequencing.

**Purification of recombinant Dxr.** Either wild-type dxr or dxr-S222T was cloned into the vector BG1861 using ligation-independent cloning (42), and recombinant protein was purified as previously described, with some minor modifications (20). Cells were lysed in lysis buffer (25 mM Tris [pH 7.5], 250 mM NaCl, 1 mM MgCl2, 0.1% Triton X-100, 5 mM dithiothreitol [DTT], 20 mM imidazole, 1 mg/ml lysozyme, 200 μM phenylmethylsulfonyl fluoride [PMSF], EDTA-free Complete Protease Inhibitor Tablets [Roche]) and then sonicated to complete lysis and shear DNA. Protein was purified over nickel agarose beads (Gold Biotechnologies) and then further purified over a HiLoad 16/60 Superdex 200 (GE Life Sciences) size exclusion column, and fractions were collected using an AKTAexplorer 100 air fast protein liquid chromatography (FPLC) (GE Life Sciences). Fractions containing the recombinant protein were subsequently combined and concentrated using Amicon Ultra-15 10,000-nominal molecular weight limit (NMWL) centrifugal filters (Millipore). Final protein concentrations were obtained using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific Pierce).

**Dxr enzyme activity.** Recombinant Dxr enzyme activity was measured by monitoring the oxidation of NADPH to NADP+ by tracking the absorbance at 340 nm in a POLARstar Omega microplate reader (BMG). Standard reactions were performed in a 50-μl volume in reaction buffer (25 mM Tris [pH 7.5], 100 mM NaCl, 7.5 mM MgCl2, 0.1 mg/ml bovine serum albumin [BSA]) with 200 μM DOP (Echelon), 400 μM NADPH (Sigma-Aldrich), 50 ng of Dxr, and the appropriate concentration of inhibitory compound. The Km [DOPX] was determined at fixed NADPH concentrations and DOPX concentrations from 0 to 5 mM. The reaction mixtures were incubated for 15 min at 37°C in the presence of all components except for DOPX. The reactions were initiated by adding DOPX, and NADPH conversion was continuously measured for up to 30 min. Kinetic parameters were determined by nonlinear regression (GraphPad Prism). All 50% inhibitory concentration (IC50), Km, Kmax, and Vmax values in the text reflect the means of the results from three or more independently performed experiments.

**Allelic replacement of the genomic dxr locus.** Allelic replacement was performed with a modified version of the pkO vector system (43). The vector pKO-dxr-S222T was created by cutting a 2,875-bp BamHI/PstI fragment from the vector pACYC-dxr-S222T and ligating it into pKO, after removal of the 2,036-bp BamHI/PstI fragment. The pKOV-dxr-S222T vector was used to transform either E. coli MG1655 or E. coli BW25113 ΔbamBΔtolC (a kind gift of Gerard Wright, McMaster University, Ontario, Canada) (44). Colonies were grown on LB with either 30 μg/ml (for MG1655) or 6 μg/ml (for BW25113 ΔbamBΔtolC) of chloramphenicol at 30°C for 48 to 72 h. Colonies were picked and grown overnight at 42°C in LB at the appropriate concentration of chloramphenicol. Cells were diluted and grown to an OD600 of approximately 0.5, at which point they were diluted 200- and 2,000-fold. Two hundred microliters of each diluted culture was plated on LB medium supplemented with 5% sucrose and grown at 37°C overnight. A total of eight colonies was isolated and screened for allelic replacement. This evaluation involved PCR amplification of the dxr locus from genomic DNA (gDNA), followed by digestion of the PCR product with the restriction enzyme AccI. An AccI cut site is present in dxr-S222T but absent from the wild-type allele. Allelic replacement was validated by Sanger sequencing.

**RESULTS**

**Identification of FSM resistance alleles of dxr but not ispD.** In vitro, FSM inhibits the first dedicated enzyme of the MEP pathway, deoxyxylulose phosphate reductoisomerase (Dxr), also known as IspC. Surprisingly, previous metabolic profiling of FSM-treated cells indicated that the downstream MEP pathway enzyme IspD (methylerythritol phosphate cytidylyltransferase, EC 2.7.7.60) was an unexpected second intracellular target of FSM (20). Since FSM was not found to inhibit recombinant IspD in vitro at physiologically relevant concentrations, our previous studies suggested that the inhibition of IspD was likely indirect. We evaluated whether amino acid changes in Dxr and/or IspD might confer FSM resistance in order to confirm which enzyme is the primary intracellular target and to establish a system for validating the antimicrobial mechanisms of action of future FSM analogs (Fig. 1C).

To investigate mutations in dxr and ispD that might confer FSM resistance, we used error-prone PCR to generate expression libraries of dxr and ispD mutants in the model Gram-negative bacterium E. coli (see Fig. 1B for experimental scheme). Transformed cells were selected on plates containing lethal concentrations of FSM (50 or 100 μM). Based on similar error-prone PCR protocols (41), the estimated complexity of the dxr library was ~105 individual clones. For every 105 CFU plated, an average of 350 colonies were observed, suggesting that 35 separate FSM-resistant dxr mutants were present (0.35% of the library). A total of 48 resistant colonies were restreaked onto FSM-containing medium (100 μM), and the seven colonies with the most robust growth were analyzed further. A comparable number of cells were transformed with the ispD library. ispD transfectants were not observed on FSM-containing medium until 2 days of growth, at which time eight total colonies from the ispD library were evident; all eight were restreaked onto LB/amp plates containing 100 μM FSM. This suggests a much lower rate of resistant clones (0.0013%) for the ispD library than that with the dxr library.

To confirm that FSM resistance was plasmid dependent, plas-
mids were isolated from seven dxr library (pDX-dxr) clones and all eight ispD library (pDX-ispD) clones. Restriction digest evaluation demonstrated that two of the eight ispD plasmids did not contain an ispD insert. All plasmids with the proper inserts, along with both dxr and ispD wild-type controls, were used to transform the wild-type fosmidomycin-sensitive K-12 derivative E. coli strain MG1655. These transformants were evaluated for FSM resistance by determination of the half-maximal effective concentration (EC_{50}) at 8 h of growth (mid-logarithmic growth phase), compared to that of the controls. None of the ispD library transformants demonstrated FSM resistance (see Fig. S1 in the supplemental material). In contrast, strains expressing pDX-dxr plasmid-borne FSM-resistant dxr alleles demonstrated a 2- to 10-fold decrease in sensitivity to FSM compared to that with strains expressing plasmid-borne wild-type dxr (Table 1; see also Fig. S2 in the supplemental material).

Two mutants in particular, dxr1 and dxr3, showed significantly increased EC_{50}s of 53 μM (95% confidence interval [CI], 40 to 71 μM; P < 0.0001) and 45 μM (95% CI, 37 to 55 μM; P < 0.0001), respectively, compared to a control EC_{50} of 4.4 μM (95% CI, 3.7 to 5.3 μM), and were therefore chosen for additional analysis. The sequencing of dxr from each vector revealed that dxr1 and dxr3 contained the exact same set of mutations. Five amino acid changes were encoded by dxr1 and dxr3: N38Y, M68L, T202A, and S222T, which confer FSM resistance. The S222T mutation was used for additional analysis.

Enzymatic analysis of DxR-S222T protein. The structure of the E. coli Dxr-FSM complex has been solved and demonstrates that Ser222 coordinates the phosphonate moiety of the inhibitor (36–38) (Fig. 2C). Structural modeling suggested that dxr-S222T confers resistance to FSM by directly reducing the susceptibility of the target enzyme, Dxr, to inhibition by FSM. We therefore compared the FSM susceptibility of purified recombinant wild-type Dxr enzyme to that of the Dxr-S222T variant. As expected, the S222T variant was 30-fold more resistant to FSM than the wild-type enzyme (IC_{50}, 1,100 nM [95% CI, 598 to 1,840 nM] compared to 34 nM [95% CI, 27 to 41 nM] [t test, P < 0.0001] [Table 2 and Fig. 3A]). During these assays, we observed that in the absence of FSM treatment, mutant enzyme activity was substantially reduced compared to that of wild-type Dxr. Since FSM is expected to bind in a manner similar to that of the natural Dxr substrate DOXP, we therefore evaluated the effect of the S222T variant on Dxr enzyme kinetics (Fig. 3B). In our hands, the mean ± standard error of the mean (SEM) K_{m} (DOXP) of wild-type Dxr was 150 ± 22 μM, similar to previously reported values (19). In contrast, the K_{m} (DOXP) of Dxr-S222T was increased 7-fold (mean ± SEM, 1,000 ± 140 μM; t test, P < 0.001; Table 2). The maximum reaction velocity (V_{max}) of Dxr-S222T was not significantly different from that of the wild-type variant (mean ± SEM, 12 ± 0.3 versus 11 ± 0.5 μmol/min/mg; t test, P = 0.31; see Table 2). While K_{m} as an enzymatic constant is not an exact measurement of substrate binding, it is typically correlated with substrate-enzyme affinity. Our data therefore suggest that the S222T mutation causes a decreased affinity for DOXP without a change in the reaction rate.

FSM is a slow tightly binding inhibitor that is competitive with the structurally similar DOXP substrate (19). Since Dxr-S222T is less sensitive to FSM and appears to have a reduced affinity for DOXP, it is likely that FSM resistance in Dxr-S222T reflects a reduced affinity for FSM. The K_{m} parameter is an approximation of the binding affinity between an enzyme and its inhibitor. A comparison of the K_{m} values of wild-type Dxr (Fig. 4A) and Dxr-S222T (Fig. 4B) suggests a 70-fold decrease in affinity for FSM compared to that of the wild-type Dxr (mean ± SEM, 11 ± 0.6 nM versus 730 ± 14 nM, P < 0.0001; Table 3). The S222T mutation therefore appears to exert a profound effect on the ability of FSM to interact with Dxr, and under FSM selection, the benefit conferred by FSM resistance appears to outweigh any potential defects caused by a decrease in DOXP affinity.

Allelic exchange of S222T in E. coli strains. The MEP pathway is essential for growth in bacteria, and Dxr catalyzes a rate-limiting step in this pathway (45–47). During initial screening and identification of the FSM-resistant DXR-S222T allele, mutant dxr vari-

### Table 1: EC_{50} in E. coli upon episomal (pDX-dxr) expression of wild-type (dxr-wt) or FSM-resistant dxr alleles (dxr1 to -7) 

<table>
<thead>
<tr>
<th>dxr-allele</th>
<th>EC_{50} (μM) (mean [95% CI])</th>
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<tbody>
<tr>
<td>dxr-wt</td>
<td>4.4 (3.7–5.3)</td>
</tr>
<tr>
<td>dxr1</td>
<td>53 (40–71)</td>
</tr>
<tr>
<td>dxr2</td>
<td>17 (12–23)</td>
</tr>
<tr>
<td>dxr3</td>
<td>45 (37–55)</td>
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<td>20 (13–30)</td>
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<tr>
<td>dxr5</td>
<td>12 (9.0–15)</td>
</tr>
<tr>
<td>dxr6</td>
<td>9.8 (6.7–14)</td>
</tr>
<tr>
<td>dxr7</td>
<td>6.8 (5.0–9.1)</td>
</tr>
</tbody>
</table>

* Data are from at least three independent experiments.*

### References


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ants were expressed episomally, and the native dxr locus was still present in the genome. We therefore evaluated whether dxr-S222T alone was sufficient to support the growth of E. coli when the native dxr gene was disrupted. The pKOV plasmid system (43) was used to exchange the wild-type allele of dxr with the dxr-S222T allele (see Fig. S3 in the supplemental material). Compared to the expression of wild-type dxr, expression of dxr-S222T did not cause a significant change in growth rate in E. coli (Fig. 5A) (doubling time under these conditions, 56 ± 2 min for the wild-type strain versus 58 ± 3 min for the FSM-resistant strain ([t test, P = 0.74]). These results demonstrate that strains expressing the dxr-S222T allele grow at a rate comparable to that of the wild-type strains, despite the 7-fold increase in $K_m$ for the Dxr substrate DOXP. As expected, the dxr-S222T-expressing strain was resistant to FSM, with an EC$_{50}$ >4-fold higher (4.1 μM; 95% CI, 1.6 to 10 μM) than that of the strain expressing the wild-type allele (0.93 μM; 95% CI, 0.70 to 1.2 μM; P < 0.001; Fig. 5B and Table 4).

Effects of S222T allele on FR900098 prodrug. While FSM has shown some promise as a potential antimarial therapeutic, its hydrophilicity contributes to poor cellular permeability and short plasma half-life (35). One strategy to improve the efficacy and pharmacokinetics of FSM and its analogs is to generate neutral prodrugs in which the negatively charged phosphonyl group is masked. However, because intracellular activation is required, these prodrugs cannot be tested directly in vitro for inhibition of Dxr activity. Since the FSM-resistant strain carrying the dxr-S222T allele differs from wild-type E. coli by a single amino acid, we hypothesized that strains with the dxr-S222T allele could be used in a simple definitive cellular assay to verify the mechanisms of action of novel FSM analogs, including prodrugs requiring intracellular activation to release a Dxr inhibitor. To demonstrate the utility of this approach, we evaluated the MG1655-dxr-S222T strain for resistance to a phosphonamidate prodrug designed to undergo intracellular activation to release FR900098, a close structural analog of fosmidomycin (D. J. Meyers, E. Enotas, J. M. Smith, C. M. Armstrong, A. R. Odom, T. A. Shapiro, and C. L. Freel Meyers, unpublished data) (Fig. 1B). FR900098 released from the prodrug is predicted to bind to Dxr in a manner similar to that of FSM. We find that the S222T mutation confers approximately 35-fold enzymatic resistance to FR900098, which closely mimics the effect of this mutation on FSM inhibition (IC$_{50}$: 24 nM [95% CI, 21 to 29 nM] and 890 nM [95% CI, 560 to 1,400 nM] against wild-type and S222T mutant enzymes, respectively) (Table 4; see also Fig. S4 in the supplemental material). Initial experiments indicated that the prodrug exerts minimal antibacterial activity against E. coli, making it difficult to interpret its effect on the mutant strain (Table 4; see also Fig. S5 in the supplemental material).

Because many compounds lack efficacy against E. coli as a result of active cellular export, E. coli strains with selective disruption of efflux pumps have been developed that demonstrate enhanced susceptibility to many compounds (48, 49). Therefore, we replaced the native dxr locus with dxr-S222T in the efflux-defective E. coli strain BW25113 ΔbamBΔtolC (44). In this strain, as in MG1655, expression of the dxr-S222T allele confers substantial resistance to FSM (EC$_{50}$: 0.37 μM [95% CI, 0.22 to 0.64 μM] versus 5.0 μM [95% CI, 2.4 to 11 μM] for BW25113 ΔbamBΔtolC and BW25113 ΔbamBΔtolC-dxr-S222T, respectively; [t test, P < 0.0005]) (Fig. 6A). Resistance to FR900098 was also observed (EC$_{50}$: 100 μM [95% CI, 66 to 150 μM] versus 570 μM [95% CI, 270 to 1,200 μM] for BW25113 ΔbamBΔtolC and BW25113 ΔbamBΔtolC-dxr-S222T, respectively; P < 0.001) (Fig. 6B and Table 4). For the FR900098 prodrug, the strain with the dxr-S222T allele was 7-fold more resistant than the isogenic parent (EC$_{50}$, 67

### Table 2 IC$_{50}$ and kinetic constants for recombinant wild-type dxr and dxr-S222T

<table>
<thead>
<tr>
<th>Dxr allele</th>
<th>IC$_{50}$ (mean [95% CI]) (nM)</th>
<th>$V_{max}$ (mean [SEM]) (μmol/min/mg of enzyme)</th>
<th>$K_m$ (mean [SEM]) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dxr wt</td>
<td>34 (27–41)</td>
<td>12 ± 0.4</td>
<td>150 ± 23</td>
</tr>
<tr>
<td>dxr-S222T</td>
<td>1,100 (600–1,800)</td>
<td>11 ± 0.5</td>
<td>1,000 ± 270</td>
</tr>
</tbody>
</table>

* Data are from at least three independent experiments. Representative curves shown in Fig. 3A.
TABLE 3 Inhibitory kinetics of recombinant wild-type and S222T Dxr

<table>
<thead>
<tr>
<th>Dxr variant</th>
<th>( K_{m} ) (( \mu M ))</th>
<th>( V_{max} ) (( \mu mol/min/mg ) of enzyme)</th>
<th>( K_{i} ) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dxr-wt</td>
<td>180 ± 29</td>
<td>13 ± 2.2</td>
<td>11 ± 0.9</td>
</tr>
<tr>
<td>Dxr-S222T</td>
<td>900 ± 210</td>
<td>11 ± 0.6</td>
<td>730 ± 14</td>
</tr>
</tbody>
</table>

*Data are from at least three independent experiments. Representative curves shown in Fig. 4.*

DISCUSSION

The non-mevalonate MEP pathway for isoprenoid biosynthesis is a promising antimicrobial target, since this pathway is well validated and essential in a number of human pathogens but absent in humans. In this work, we identified and characterized a novel mutation (S222T) in the *E. coli* deoxyxylulose phosphate reductoisomerase (Dxr), which confers resistance to the antimalarial and antibacterial agent fosmidomycin (FSM). FSM, a phosphonic acid antimicrobial, is the canonical MEP pathway inhibitor and antibacterial agent fosmidomycin (FSM). As a tool to confirm this intracellular target, we have developed and validated a novel system for testing the mechanisms of action of FSM analogs, such as FR900098, and prodrugs requiring intracellular activation to release inhibitors of Dxr.

The structural basis of FSM binding to Dxr has been well described in multiple studies (36–38). The phosphonate moiety of FSM, and presumably the phosphate moiety of the DOXP substrate, are anchored by hydrogen bonding to four amino acids in the Dxr active site: Ser186, Ser222, Asn227, and Lys228 (Fig. 2C). These interactions are anchored by hydrogen bonding to four amino acids in the substrate/FSM binding pocket and disrupt at least one, if not multiple, hydrogen-bonding interactions. Consistent with this prediction, the Dxr-S222T variant confers FSM resistance in *in vitro* and *in vivo*, and, in a similar manner, also requires higher DOXP concentrations for effective catalysis. Surprisingly, *E. coli* strains that solely express the Dxr-S222T variant are viable and do not exhibit a substantial growth defect under laboratory growth conditions, even though Dxr is essential for bacterial growth and catalyzes a rate-limiting step in the MEP pathway. Since a single point mutation confers resistance, and resistance does not come with an apparent fitness disadvantage, the genetic barrier to FSM resistance may be quite low. The development of FSM resistance will require careful monitoring during clinical treatment with FSM or related agents. The S222T variant therefore represents a potential genetic biomarker of resistance to active-site inhibitors of Dxr.

FSM is small and not easily modified without reducing potency. These limitations have led to substantial efforts to develop more cell-permeable analogs of FSM or its highly related analog FR900098 (30–35). Because many FSM derivatives are prodrugs that require intracellular activation, it is often impossible to test the efficacy of these compounds directly on the Dxr target enzyme *in vitro*. As a tool to confirm this intracellular target, we have validated the S222T mutant strain using a cell-permeable prodrug of the FSM analog FR900098 (D. J. Meyers et al., unpublished data). As seen with fosmidomycin, the S222T mutation confers 35-fold resistance to FR900098 *in vitro* and 5.5-fold resistance *in vivo* (Tables 2 and 4; see also Fig. S4 and S6 in the supplemental material). We observed a very similar increase in the EC50 of a novel FR900098 prodrug. Our results confirm that Dxr is the intracellular target for this compound following FR900098 release from the prodrug. In addition, these studies support the use of this prodrug approach in antibacterial strategies to address (i) the poor pharmacokinetics of FSM and analogs and/or (ii) the development of resistance to this compound class through mutation of
active uptake via GlpT. Finally, our results underscore the value of the S222T mutant strain for use in future drug discovery efforts as we seek to improve prodrg scaffolds for optimal cellular uptake and retention in bacteria or in intracellular pathogens, which demonstrate natural resistance to FSM as a consequence of poor uptake.

New antimalarial agents are in great demand due to widespread resistance to former first-line agents and emerging resistance to the newer artemisinin-based therapies. Within countries that are endemic for malaria, the disease affects very high-risk patient groups, specifically young infants and pregnant women. In addition, many individuals require multiple courses of treatment per year. For these reasons, new antimalarial therapies must be exceptionally safe. Development efforts targeting the MEP pathway aim to generate nontoxic compounds through the inhibition of a target that is not present in humans. Since prodrugs are not expected to exert inhibitory activity directly against the target and are therefore not easily tested biochemically, compound development may veer off target during development, thus eliminating the parasite selectivity and safety advantages. Our FSM-resistant E. coli strains are isogenic with wild-type bacteria, with the exception of a single amino acid change in a single protein, and bacterial growth inhibition is straightforward to assay. We expect that these strains will be of great utility in confirming the mechanism of action in future efforts to develop inhibitors targeting the substrate-binding pocket of Dxr for antibacterial, antitubercular, and antimalarial therapies.

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REFERENCES


