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Structure-guided design and biosynthesis of a novel FR-900098 analogue as a potent *Plasmodium falciparum* 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr) inhibitor

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Abstract

We report here the enzymatic biosynthesis of FR-900098 analogues and establish an *in vivo* platform for the biosynthesis of *N*-propionyl derivative FR-900098P. FR-900098P is found to be a significantly more potent inhibitor of *Plasmodium falciparum* 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*Pf*Dxr) than the parent compound, and thus a more promising antimalarial drug candidate.

Malaria is one of the leading causes of global mortality, responsible for an estimated 627,000 deaths in 2012 alone.^{1, 2} Almost all cases that result in fatality are due to infections from *Plasmodium falciparum*, and the rapid emergence of pathogenic strains resistant to conventional antimalarial drugs such as chloroquine as well as current artemisinin-based therapies highlights the need for novel therapeutics.^{3–6}

Enzymes involved in the methylerythritol phosphate (MEP) pathway are essential for the biosynthesis of isoprenoids in eubacteria, and represent effective targets for therapeutic intervention due to its orthogonality to the mammalian pathway.^{7–9} The second enzyme in this pathway, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr) catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate (DXP) to 2-C-methyl-D-erythritol 4-phosphate, facilitating the production of the isoprenoid precursors isopentenyl diphosphate and dimethylallyl diphosphate.¹⁰ The phosphonate natural product fosmidomycin acts as a slow, tight-binding inhibitor of Dxr from bacteria and from plants.¹¹ Subsequently, fosmidomycin and the related natural product FR-900098 (Scheme 1) are shown to inhibit the growth of multi-drug resistant *P. falciparum* at submicromolar concentrations. The

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antimalarial activities of both compounds have been validated in mice models, albeit with a high rate of recrudescence.¹² Fosmidomycin works synergistically with lincosamides, and this combination therapy provides parasitological cures for patients with acute uncomplicated falciparum malaria.¹³

The clinical antimalarial efficacy of both of these phosphonates has prompted research efforts towards the production of derivatives with increased inhibitory activity towards *P. falciparum* and its Dxr enzyme (*PfDxr*). To improve bioavailability by masking the dianionic charge of the phosphonate moiety, ester prodrugs have been synthesized and evaluated in mouse models.^{14, 15} Derivatization at the α position has also been evaluated, where the substitution of fluorine or a partially halogenated benzene ring has led to improved inhibition behavior.^{16, 17} At the other end of the molecule, reversal of the hydroxamate moiety has also been shown to improve antimalarial properties, particularly in tandem with the aforementioned efforts.¹⁸

To generate novel *N*-acyl derivatives of FR-900098, we utilized the native enzymes FrbH, FrbG, FrbF, and FrbI from the *Streptomyces rubellomurinus* FR-900098 biosynthetic pathway,¹⁹ heterologously expressed and purified from *E. coli* BL21(DE3). FrbH catalyzed conversion of commercially available 2-amino-4-phosphonobutyrate (2APn) and cytidine 5'-triphosphate (CTP) to CMP-5'-3-aminopropyl phosphonate (CMP-5'-3APn), which was isolated by HPLC fractionation. FrbG then catalyzed hydroxylation of CMP-5'-3APn to the corresponding hydroxylamine (CMP-5'-H3APn). As this product was found to rapidly oxidize to the non-productive nitroso derivative, the FrbF acyltransferase was added in tandem to the reaction mixture, along with the acyl-coenzyme A (CoA) donor *n*-propionyl-CoA, *n*-butyryl-CoA, acetoacetyl-CoA, or malonyl-CoA. In all four cases, synthesis of the corresponding CMP-conjugated FR-900098 derivative was observed and confirmed by tandem mass spectrometry (Figure S3). Nucleotide cleavage catalyzed by the promiscuous nucleotide hydrolase FrbI was carried out only after isolation of the CMP-conjugated product by HPLC fractionation to prevent premature hydrolysis of the other CMP-conjugated intermediates.

To evaluate the antimalarial potential of FR-900098 derivatives, we generated and expressed a codon-optimized *P. falciparum* *dxr* gene, lacking the 72-residue amino-terminal signal peptide, in *E. coli* BL21(DE3). The enzymatic activity of the purified recombinant *PfDxr* was determined by monitoring NADPH consumption in the presence of substrate, yielding a k_{cat}/K_M value of $2.5 \mu\text{M}^{-1} \text{min}^{-1}$. Co-crystal structures of *PfDxr* were grown in the presence of inhibitor (fosmidomycin or FR-900098), NADP⁺, and Mn²⁺ (Table S1, Figure S1). The structures are comparable to previous *PfDxr* structures,²⁰ with root mean square deviation < 0.5 Å over 406 aligned C α atoms.

Notably, in the FR-900098 co-crystal structure, the *N*-methyl group is situated in a hydrophobic pocket flanked by Met-298 and Met-360, and engages in a favorable van der Waals contact with the indole ring of Trp-296. This observation, along with previous observation that *N*-methylated substrate-based inhibitors of *PfDxr* are more effective than their desmethyl counterparts,²¹ suggest that further extension of the inhibitor into the

hydrophobic pocket could introduce additional favorable van der Waals interactions to improve binding affinity.

Accordingly, we chose the *n*-propionyl FR-900098 derivative, here called FR-900098P, for inhibition studies against the purified *PfDxr* enzyme. Enzymatic synthesis with two steps of HPLC purification was carried out with an isolated yield of 12 %. Assays were performed at inhibitor concentrations of 0–20 nM. In our hands, inhibition constants of $K_i = 7.5 \pm 2.0$ nM for fosmidomycin and $K_i = 3.7 \pm 0.9$ nM for FR900098 were observed, comparable to previous results.¹⁸ With FR-900098P, a K_i value of 0.92 ± 0.19 nM was obtained, demonstrating it to be a significantly more potent *PfDxr* inhibitor than its parent compound. To the best of our knowledge, this is the first demonstration of an FR-900098 derivative with a modified *N*-acyl substituent as a superior Dxr inhibitor.

Given the improved potency of FR-900098P, we sought to develop an *in vivo* biosynthetic platform for its production. Although chemical synthesis can be efficient and high-yielding, biosynthesis offers certain advantages in terms of sustainability, as renewable biomass feedstocks can be used, and environmental impact, as no strong acids, organic solvents, or metal catalysts are required. We built our biosynthetic platform upon our previous *E. coli* FR-900098 production chassis.¹⁹ This engineered strain contains the entire FR-900098 pathway from *S. rubellomurinus* along with the resistance gene *dxrB*, which confers resistance to the normally toxic product. We first attempted to identify FR-900098P from the FR-900098 producing strain directly, given that *E. coli* can naturally produce propionyl-CoA and that the wild type FrbF enzyme can accept propionyl-CoA as a substrate. However, no FR-900098P was detected, likely indicating that intracellular propionyl-CoA concentrations are too low to compete with acetyl-CoA in FrbF-catalyzed acyl transfer. To overcome this problem, we investigated a mutasynthetic approach by which sodium propionate was fed to the culture at the time of induction. Once taken up by the cell, propionate can be converted to propionyl-CoA via the propionyl-CoA synthetase gene *prpE*, which can then be utilized by FrbF. This approach proved to be successful (Figure 1A), and FR-900098P was detected in the cell culture broth by tandem mass spectrometry. FR-900098, however, was still the dominant phosphonate product, as FR-900098P represented only ~7 % of the total phosphonate yield.

To further increase FR-900098P production *in vivo* using the native FR-900098 biosynthetic machinery, a metabolically engineered *E. coli* host was investigated. To create a stronger driving force for FR-900098P synthesis, propionyl-CoA concentrations in the host can be increased via two modifications. First, over-expression of *prpE* can increase the rate at which propionyl-CoA is synthesized. Second, deletion of the *prpRBCD* operon prevents propionyl-CoA from being consumed in primary metabolism via conversion to succinate and pyruvate. Both of these modifications have been reported in the literature in *E. coli* strain BAP1, which has successfully been utilized in the heterologous production of secondary metabolites.²²

To engineer the BAP1 strain for FR-900098P production, it was simultaneously transformed with the three compatible plasmids containing the FR-900098 pathway. Following selection on LB solid media with three antibiotics, a colony was isolated containing all three

plasmids. Production of FR-900098P in this host was analyzed under identical growth conditions as the BL21(DE3) host. Relative to cell culture density, the FR-900098P titer increased 3.5-fold in the BAP1 strain as compared to the BL21(DE3) strain (Figure 1B). However, it was observed that the BAP1 strain did not reach as high a density as the BL21 strain, resulting in an overall increase in endpoint FR-900098P concentration of ~50 %. This is likely due to the increased metabolic burden placed on the BAP1 strain by over-expressing *prpE* under a T7 promoter while simultaneously limiting its ability to utilize the exogenous propionate for central metabolism. As observed in the BL21(DE3) strain, the BAP1 strain also produced FR-900098 as its primary phosphonate product. However, the relative amount of FR-900098P was significantly greater in BAP1, comprising ~24 % of the total phosphonate yield.

In summary, we have identified a hydrophobic binding pocket in the *PfDxr* enzyme based on co-crystal structures with fosmidomycin and FR-900098, inspiring synthesis of the *N*-propionyl derivative FR-900098P. Through *in vitro* synthesis and evaluation with *PfDxr*, FR-900098P was found to be a more potent inhibitor than its parent compound. An *in vivo* platform was also established for FR-900098P biosynthesis utilizing both mutasynthetic and metabolic engineering strategies. Nevertheless, extensive metabolic engineering efforts would be necessary to enhance the low titer and productivity to levels competitive with established chemical synthetic platforms.²³ When coupled with further derivatization, such as esterification of the phosphonate moiety, FR-900098P could serve as a new potential antimalarial drug candidate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

a) Tandem mass spectrometry reveals no production of FR-900098P by *E. coli* BL21(DE3) (red trace) unless supplemented with exogenous sodium propionate (blue trace). b) Engineered strain BAP1 produces higher titers of FR-900098P than BL21(DE3).

DXP

R = H, fosmidomycin

R = CH₃, FR900098R = CH₂CH₃, FR900098P

Scheme 1.
Relevant chemical structures