Supplementary Information

Characterization and Application of the Fe(II) and α-Ketoglutarate Dependent Hydroxylase FrbJ

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Experimental Procedures

Materials. The compounds α-ketoglutarate (αKG), ferrous sulfate (Fe(II)SO_4), ascorbic acid, *S*-(5'-adenosyl)-*L*-methionine (SAM), and fosfomycin were purchased from Sigma-Aldrich (St. Louis, MO). Fosmidomycin was purchased from Invitrogen (Carlsbad, CA). FR-900098 was purchased from Toronto Research Chemicals (Toronto, Canada). 1-Deoxy-D-xylulose-5-phosphate (DXP) was purchased from Echelon Biosciences (Salt Lake City, UT). NADPH was purchased from Jülich Chiral Solutions GmbH (Jülich, Germany). Blank paper discs (6 mm diameter) used in bioassay were purchased from BD Diagnostics (Sparks, MD). All other reagents and materials were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Cloning, expression, and purification. The full-length *frbJ* gene was cloned as previously described[1]. Briefly, forward (5'-GCTATTAAT*CATATG*GTTGAGATTCTGAAGAAGCC CGTCA-3') and reverse (5'-CGAAATAT*AAGCTT*TCAGTGCGTCGGCTTCGCCGCCG-3') primers introduced *Nde*I and *Hind*III sites (underlined and italicized), respectively. The gene was cloned into the corresponding sites of pET28a, designed to create an N-terminal His_6-tag, and then used to transform *E. coli* BL21(DE3) competent cells. Transformed cells were cultured in LB medium supplemented with kanamycin (50 μg/ml) at 37 °C to an OD_{600} ~ 0.7 and then induced at 22 °C by addition of isopropyl-*β*-D-thiogalactoside (IPTG) to a final concentration of 0.3 mM. After 24 h, the cells were harvested by centrifugation at 10,000 x *g* and resuspended in 20 mM Tris-HCl (pH 7.65), 15% glycerol, and 0.5 M NaCl at 4 °C. The sample was freeze-thawed and then lysed further by sonication. After cellular debris was removed by centrifugation at 15,000 x *g*, the N-His_6-tagged FrbJ protein was purified using immobilized metal-affinity chromatography (IMAC) with TALON Superflow Co^{2+} resin (Clontech Laboratories; Mountain View, CA) coupled to fast-performance liquid chromatography (FPLC). The purified protein was washed three times using a Millipore Amicon Ultra-15 Centrifugal Filter Device (MWCD 10 kDa) with 50 mM HEPES-Na^+ buffer (pH 7.25) and concentrated a final time. SDS-PAGE gel electrophoresis was used to assess the presence and purity of FrbJ in the sample. Concentrated protein was stored in 20% glycerol at -80 °C.

Kinetic analysis. Experiments used to determine the kinetic parameters of FrbJ were carried out in triplicate. Each reaction mixture (100 μL final volume) contained 0.2 μM FrbJ, 200 μM Fe(II)SO_4, 800 μM ascorbic acid in 50 mM HEPES-Na^+ buffer (pH 7.25). For the determination of substrate kinetic parameters, the αKG concentration was held constant at 10 mM while the specified substrate concentration ranged from 0-2.5 mM. For the determination of K_M with αKG, the FR-900098 concentration was held constant at 1 mM while the final concentration of αKG ranged from 0-10 mM. All reactions were initiated by the addition of recombinant FrbJ and run at 25 °C. Aliquots were taken and quenched by the addition of 4% trifluoroacetic acid and were kept at 4 °C until analysis by HPLC. The kinetic parameters were obtained after analyzing the extracted ion chromatograms for product formation by LC-MS. Methods for liquid chromatography separation and mass spectroscopy were previously described[1].

Antimalarial compound library creation. Overexpression strains for DhpI and Pfs contained His_6-tags making them amenable to purification similar to FrbJ. Commercially available FR-900098 and fosmidomycin were the starting substrates for the antimalarial compound library. These two compounds (5 mM) were incubated with 25 μM purified His_6-FrbJ, 10 mM αKG, 200 μM Fe(II)SO_4, and 800 μM ascorbic acid. Reaction mixtures were kept at 25 °C for 24 h to ensure complete conversion to products.

Samples were then transferred into a Microcon® centrifugal filter device (MWCO 10,000) (Millipore; Billerica, MA) and centrifuged for 10 minutes at maximum speed to remove enzyme, yielding solutions of FR-33289 and hydroxyfosmidomycin. These hydroxylated samples, FR-900098, and fosmidomycin were then used as substrates for the second *in vitro* reaction. These four compounds (5 mM) were incubated with 25 μM purified His_6-DhpI, 25 μM purified His_6-Pfs, and 10 mM SAM at 25 °C for 24 h. DhpI, like many other methyltransferases, undergoes product inhibition by the product *S*-adenosylhomocysteine (SAH)[2]. As a result, SAH nucleosidase, which catalyzes SAH hydrolysis to adenine and *S*-ribosylhomocysteine, was added to *in vitro* reaction mixtures to ensure complete conversion to the desired methylated phosphonate. Once again, samples were transferred into a Microcon® centrifugal filter device (MWCO 10,000) and centrifuged for 10 min at maximum speed to remove enzyme, yielding solutions of *O*-methylated phosphonates. The prospective antimalarial library was then purified by HPLC separation using a method identical to that in the kinetic analysis, as mentioned earlier[1]. Fractions were collected and then checked for purity before final lyophilization to dryness.

Bioauthentication by *E. coli* bioassay. The *E. coli* bioassay strain WM6242 contains three phosphonate uptake genes *phnCDE*, expression of which creates a hypersensitive *E. coli* strain to phosphonate antibiotics[3]. The FR-900098-sensitive *E. coli* strain used in these bioassays was strain WM6242 transformed with pETDuet, pRSFDuet, and pACYCDuet to ensure ampicillin, kanamycin, and chloramphenicol resistance, respectively. Although not required in the assays described here, inclusion of these plasmids protects against any background antimicrobial activity from antibiotics in the spent medium of *E. coli* FR-900098 production strains. FR-900098-resistant *E. coli* strains were created by transforming WM6242 with pMal-c2x-*dxrB* and pMal-c2x-*ispC* (cloned under P_{tac} promoter without maltose binding protein fusion), respectively, in addition to pRSFDuet and pACYCDuet. Overnight cultures of the three strains were grown in LB media with the following antibiotic concentrations: ampicillin, 50 μg/mL; kanamycin, 25 μg/mL; and chloramphenicol, 12.5 μg/mL. Saturated overnight cultures were diluted 10-fold in LB media and 100 μL was mixed with 3 mL of 1.0% top agar. The mixture was spread on Antibiotic 2 media + 0.4 mM IPTG plates and allowed to solidify. Blank paper discs were impregnated with sample and incubated overnight at 37 °C, after which they were added to the agar plates and analyzed for zones of growth inhibition.

Bioauthentication by pfDxr inhibition. The full-length *dxr* gene from *Plasmodium falciparum* was codon-optimized for expression in *E. coli* by DNA 2.0 (Menlo Park, CA) and the sequence encoding the 72 amino acid long signal peptide was later removed to increase soluble expression[4-5]. An N-His_6-tag was introduced to the shortened protein making it amenable to purification similar to FrbJ. The inhibition of the enzymatic activity of pfDxr was determined in triplicate at 25 °C. Each reaction mixture (300 μL final volume) contained 50 nM pfDxr, 300 μM NADPH, 1 mM $MnCl_2$ in 50 mM HEPES-Na^+ buffer (pH 7.25). The reaction was monitored by NADPH oxidation at 340 nm. For the determination of IC_{50} values, substrate concentration was held at 250 μM and inhibitor concentrations of 0-500 nM were used. For the determination of K_I values, substrate concentration ranged from 0-250 μM and inhibitor concentrations of 0-100 nM were used.

1. T. W. Johannes, M. A. DeSieno, B. M. Griffin, P. M. Thomas, N. L. Kelleher, W. W. Metcalf and H. Zhao, *Chem Biol*, 2010, **17**, 57-64.
2. C. L. Hendricks, J. R. Ross, E. Pichersky, J. P. Noel and Z. S. Zhou, *Anal Biochem*, 2004, **326**, 100-105.
3. A. C. Eliot, B. M. Griffin, P. M. Thomas, T. W. Johannes, N. L. Kelleher, H. Zhao and W. W. Metcalf, *Chem Biol*, 2008, **15**, 765-770.

4. H. Jomaa, J. Wiesner, S. Sanderbrand, B. Altincicek, C. Weidemeyer, M. Hintz, I. Turbachova, M. Eberl, J. Zeidler, H. K. Lichtenthaler, D. Soldati and E. Beck, *Science*, 1999, **285**, 1573-1576.
5. B. Bae, R. E. Cobb, Z. Li, M. A. DeSieno, H. Zhao and S. K. Nair, submitted.

Supplemental Figure 1. Growth inhibition assays of (A) FR-900098-sensitive *E. coli* strain and (B) FR-900098-resistant *E. coli* strain. Discs labeled 1 were soaked with ddH_2O; discs labeled 2 were soaked with FR-900098; discs labeled 3 were soaked with FR-33289; discs labeled 4 were soaked with fosmidomycin; and discs labeled 5 were soaked with hydroxyfosmidomycin. The FR-900098-sensitive strain produced inhibition zones for all the bioactive compounds, while the FR-900098-resistant strains (containing *dxrB* or *ispC*) did not produce inhibition zones for any of the phosphonates.