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Functional genetic analysis of the *Plasmodium falciparum* deoxyxylulose 5-phosphate reductoisomerase gene

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Abstract

Novel antimalarial drugs are urgently needed to treat severe malaria caused by *Plasmodium falciparum*. Isoprenoid biosynthesis is a promising target pathway, since the biosynthetic route in *Plasmodia* is biochemically distinct from the mevalonate pathway in humans. The small molecule fosmidomycin is an inhibitor of the enzyme responsible for the first dedicated step in isoprenoid biosynthesis, deoxyxylulose 5-phosphate reductoisomerase (DXR). However, the antimalarial effects of fosmidomycin might not be specific to DXR inhibition and further validation of DXR is warranted. We present the first functional genetic validation of *Plasmodium falciparum* DXR (PF14_0641). Using a single cross-over strategy, we show that plasmid integration occurs at the DXR locus but only when DXR gene function is preserved, but not when integration disrupts gene function. These data indicate that DXR is required for intraerythrocytic development of *Plasmodium falciparum*.

Keywords

Malaria; Isoprenoid biosynthesis; drug targets

Novel antiparasitic agents are needed to treat severe malaria caused by *Plasmodium falciparum*. The isoprenoid biosynthesis pathway of the parasite is a promising drug target. Isoprenoids comprise a large and diverse group of natural products, and play important roles in electron transport, cell signaling, apoptosis, and membrane structure [1,2]. In *P. falciparum*, the fundamental isoprenoid building blocks (isopentenyl pyrophosphate and dimethylallyl pyrophosphate) are generated through eight enzymatic reactions from pyruvate and glyceraldehyde 3-phosphate [3,4]. The first dedicated enzyme of isoprenoid biosynthesis, deoxyxylulose 5-phosphate reductoisomerase (DXR), converts deoxyxylulose 5-phosphate (DXP) to methylerythritol 5-phosphate (MEP), and this metabolic route is therefore alternately referred to as the DXP or MEP pathway. *P. falciparum* produces the key intermediates of this pathway, and recombinant forms of two of the pathway enzymes have the expected catalytic activities [3,5]. The DXP pathway is also the essential and exclusive metabolic route to isoprenoids in eubacteria, including *E. coli* [6]. In contrast, the DXP pathway of the parasite biochemically differs from the human isoprenoid biosynthesis pathway, which proceeds

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through acetyl-CoA with mevalonate as a key intermediate, and this difference adds to its interest as a possible drug target.

The small molecule fosmidomycin, which inhibits recombinant *P. falciparum* DXR enzyme *in vitro*, has antimalarial activity both in cell culture and *in vivo*, and has been well-tolerated in humans [3,7–9]. Modification of the simple structure of fosmidomycin to improve its drug characteristics has been challenging [10–13].

A crucial step in drug discovery is functional validation of potential therapeutic targets. Since fosmidomycin inhibits the DXR enzyme and kills the malaria parasite, *de novo* isoprenoid biosynthesis has been presumed to be essential in *Plasmodia*. If the target of fosmidomycin is indeed the parasite DXR enzyme, one would anticipate that fosmidomycin treatment should diminish production of the DXR product, methylerythritol 4-phosphate (MEP). However, two studies have found little alteration in DXP pathway metabolites, and, most notably, levels of the DXR product (MEP) did not change in fosmidomycin-treated trophozoite stage parasites [4,14]. These studies raise the possibility that the antimalarial effects of this compound might not be exerted through DXR inhibition. To test the hypothesis that the DXR gene product is required for intraerythrocytic parasite development, we initiated studies to genetically disrupt DXR, in order to assess the role of this locus in *P. falciparum* development and survival.

To disrupt the DXR gene locus (PF14_0641), so as to assess its role in survival, we employed a control strategy for gene disruption using single cross-over integrating vectors based on pCAM-BSD, which confers blasticidin resistance (gift of David Fidock, Columbia University) (Figure 1) [15]. Integration of the knockout vector is expected to ablate function, as the recombinant gene product would lack most of the predicted active site region (aa 232–395), based on studies with the *E. coli* DXR homolog [16]; integration of a separate control vector recapitulates a functional gene with the native *Pf-DXR* 5' UTR and the vector-derived 3' UTR from the *P. berghei* *DHFR* gene (Figure 1). Vectors were generated by a PCR-based strategy, with primers designed to amplify bp 31–706 of the DXR genetic locus from 3D7 genomic DNA (A: CATGCCCGGGCTTCATCACAATAAC, B: CATGCTCGAGTTACCCACTATGTTCTGAATCAACAGGT) or bp 747–1463 of the DXR locus (C: CATGCCCGGGCAAAATGTTTACAAGACAA, D: CATGCTCGAGGTTTGTGTATATATCGGTAG). The purified PCR products were digested with *Xma*I and *Xho*I (underlined), and cloned into these sites in pCAM-BSD, to generate pCAM-BSD-DXR^{KO} and pCAM-BSD-DXR^{control}, respectively.

The DXR knockout and control plasmids (100µg each) were electroporated independently into synchronized 3D7 *P. falciparum* parasites (MR4 strain, courtesy of Pradipsinh Rathod, University of Washington) as described previously [15], and transfectants were selected by growth in the presence of 1 µg/ml blasticidin (Invitrogen). These cell lines were continually cultured in the presence of blasticidin, and screened for spontaneous integration at the genomic DXR locus. Drug-resistant parasites appeared between three to four weeks following transfection. For PCR and Southern blot analysis, genomic DNA was prepared from each strain using the Qiagen DNA mini kit per manufacturer's instructions. Plasmid-specific primers (X: TAAGAACATATTTATTAAGTGCAG, Y: GAAAAACGAACATTAAGCTGCCATA) were used in various combinations to amplify products from genomic DNA in order to screen blasticidin-resistant transfectant parasites for presence of episomal plasmids (X+Y), integration of the pCAM-BSD-DXR^{KO} plasmid (X+D), and integration of the pCAM-BSD-DXR^{control} plasmid (A+Y) (Figure 2). PCR screening of genomic DNA derived from both cell lines soon after transfection produced amplicons using plasmid-specific primers, but generated no products with integration-specific primers, indicating that the plasmids were episomal, as expected (Figure 2).

After approximately seven months of continuous culture, recombination-specific amplicons were observed with genomic DNA template from the pCAM-BSD-DXR^{control} transfectant, indicating that this population contained parasites in which the plasmid had integrated into the genomic DXR locus (Figure 2A). In contrast, primers specific to plasmid integration did not amplify template derived from the pCAM-BSD-DXR^{KO} transfectant, even after one year of continuous culture. Plasmid-specific primers X+Y readily amplified DNA from both strains, indicating continued presence of episomes and controlling for quality of the genomic DNA templates. Integrated plasmid concatamers, (which would also amplify with primers X+Y) may explain the more robust amplification seen with the control transfectants. The recombination-specific amplicon (from primers A+Y) generated from the pCAM-BSD-DXR^{control} transfectant was purified and sequenced with both genomic and plasmid-specific primers (A and Y, respectively), to confirm that this appropriately sized PCR product corresponded to the expected DXR locus sequence (data not shown).

Southern blot analyses of genomic DNA from the DXR knockout and control transfectants confirmed the PCR screening results (Figure 2B). For each strain, 2–3 µg of genomic DNA was digested to completion with *Bci*VI and *Mfe*I, separated electrophoretically on a 1.0% agarose gel, and transferred to Nytran Supercharge membrane. Blots were probed with either the same DXR amplicon (C+D) used to generate pCAM-BSD-DXR^{control}, or a *Hind*III-*Pvu*II fragment from pCAM-BSD (containing the blasticidin deaminase gene). Probes were fluorescein labeled, and hybridization and detection was performed according to the manufacturer's instructions (Amersham). Southern blotting revealed that the pCAM-BSD-DXR^{control} transfectant parasites contained a mixed population, with bands corresponding to the wild-type DXR genetic locus at 4 kb, the episomal plasmid at 3 kb, and the novel 3' integration band at 5.1 kb. Cycling of DXR^{control} transfectants off blasticidin-selection pressure for 2 weeks, followed by reapplication of blasticidin, increased the proportion of parasites within the culture that integrated. Reprobing of the blot with a control probe derived from the single copy *Isp*F locus (PFB0420w, bp 9–850) demonstrated similar loading of genomic DNA from the 3D7 parent strain and the DXR^{control} transfectant (Figure 2C).

In contrast, for the pCAM-BSD-DXR^{KO} transfectants, Southern blotting demonstrated only an episomal plasmid at 4 kb and no additional bands, which would have suggested integration at any locus (Figure 2D). No additional bands were revealed even after great overexposure of this blot (data not shown). No bands other than the native locus and episomal plasmid were identified on reprobing of this blot with the DXR amplicon (A+B) used to generate pCAM-BSD-DXR^{KO} (data not shown).

We did not detect integration of plasmid pCAM-BSD-DXR^{KO} either by Southern blot analysis or by PCR screening (which would be more sensitive for detection of site-specific integration in a subset of a mixed culture), despite prolonged intraerythrocytic culture of *P. falciparum* and multiple cycles off and on drug selective pressure. Two additional independent DXR^{KO} transfectants were each cultured for greater than 6 months without observed integration as well. Genomic DNA from the DXR^{KO} transfectant strain is readily amplified by other primer combinations (Figure 2A, X+Y primer combination), and the recombination-specific primers (X+D) readily amplify from plasmid template pCAM-BSD-DXR^{control} indicating that there is no defect with this primer combination (data not shown). In contrast, we observed site-specific integration of plasmid pCAM-BSD-DXR^{control} at the *P. falciparum* DXR locus, where integration recapitulates a functional gene. Together, these data demonstrate that the *P. falciparum* DXR locus is recombinogenic, and strongly suggests that our inability to produce a loss-of-function disruption reflects the fact that the DXR gene is essential for asexual propagation of the parasite. Alternate explanations include a possible dominant-negative effect of recombination of the pCAM-BSD-DXR^{KO} plasmid, since the expected truncated protein

product could be toxic to the cell. Additionally, we cannot formally rule out very low levels of integration of the knockout plasmid, below the level of detection by PCR screening.

With an IC_{50} against *P. falciparum* in culture between 224 – 1,268 nM, the small molecule fosmidomycin shows moderate activity against *P. falciparum* [3,9]. Although this paper demonstrates that the presumed target of fosmidomycin, DXR, is required for *Plasmodium falciparum* growth, further evidence is required to demonstrate conclusively that fosmidomycin acts through DXR inhibition. Additionally, it is not certain that a modified fosmidomycin derivative with improved drug-like qualities can be developed, since fosmidomycin contains charged phosphate, which complicates pharmacokinetic optimization of this compound.

Enzymes of the DXP pathway are attractive for development of broad spectrum antimicrobial agents, since homologues of these proteins are absent in human hosts, and their function is essential in several serious human pathogens [17–19]. We were unable to generate loss-of-function genomic disruption in the likely enzymatic target of fosmidomycin, the DXP pathway enzyme DXR, despite the ability to target recombination to this locus when gene function was preserved. Therefore *P. falciparum* DXR appears to be amenable to small molecule inhibition and essential for parasite development, supporting further drug development efforts directed against this enzyme.

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Abbreviations

DXP	deoxyxylulose 5-phosphate
DXR	deoxyxylulose phosphate reductoisomerase
MEP	2C-methyl-D-erythritol 5-phosphate
PCR	polymerase chain reaction

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Figure 1. Strategy for single cross-over disruption of *P. falciparum* DXR (PF14_0641)

Transfection plasmids contain either an insert spanning positions 31–706 of the 1.5 kb DXR coding sequence (for pCAM-BSD-DXR^{KO}), or an insert spanning positions 248–484 (for pCAM-BSD-DXR^{control}). Single cross-over homologous recombination into the wild-type locus results in a pseudo-diploid configuration. For pCAM-BSD-DXR^{KO}, both copies are truncated: one has a C-terminal truncation resulting in loss of the predicted catalytic domain (232–395), the other copy lacks a promoter and is truncated at the N-terminus. For pCAM-BSD-DXR^{control}, recombination reconstructs a functional copy of the gene as well as a presumably non-functional N-terminal truncation. Locations of PCR primers used for genetic analysis (A,B,C,D, X, and Y) are indicated by labeled arrows.

Figure 2. Genetic analysis of pCAM-BSD-DXR transfectants

A. PCR analysis. Genomic DNA isolated from blasticidin-resistant parasites transfected with pCAM-BSD-DXR^{control} (left) or pCAM-BSD-DXR^{KO} (right) was subjected to PCR using the indicated primers. For pCAM-BSD-DXR^{control}, integration events are detected by primers A + Y, while primers X + Y detect the episomal plasmid. For pCAM-BSD-DXR^{KO}, primers X + D would detect integration events, while primers X + Y detect the episomal plasmid.

B. Southern blot analysis of the DXR locus in wild-type 3D7 parental strain, pCAM-BSD-DXR^{control} plasmid, and pCAM-BSD-DXR^{control} blasticidin-resistant transfectant parasites. Genomic and plasmid DNA was digested with *Bci*VI and *Mfe*I, transferred to membrane, and probed with the DXR fragment (C/D) used as insert in the pCAM-BSD-DXR^{control} plasmid.

- C. Blot from (B) reprobed for the single copy *IspF* locus (PFB0420w, bp 9–850).
- D. Southern blot analysis of the DXR locus in pCAM-BSD-DXR^{KO} plasmid, and pCAM-BSD-DXR^{KO} blasticidin-resistant transfectant parasites. Genomic and plasmid DNA was digested with *Bci*VI and *Mfe*I, transferred to membrane, and probed with the *Hind*III-*Pvu*II fragment of pCAM-BSD that contains the blasticidin deaminase gene.