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Expression, characterization and inhibition of *Toxoplasma gondii* 1-deoxy-D-xylulose-5-phosphate reductoisomerase

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ABSTRACT

The apicomplexan parasite *Toxoplasma gondii*, the causative agent of toxoplasmosis, is an important human pathogen. 1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) in the non-mevalonate isoprene biosynthesis pathway is essential to the organism and therefore a target for developing anti-toxoplasmosis drugs. In order to find potent inhibitors, we expressed and purified recombinant *T. gondii* DXR (*Tg*DXR). Biochemical properties of this enzyme were characterized and an enzyme activity/inhibition assay was developed. A collection of 11 compounds with a broad structural diversity were tested against *Tg*DXR and several potent inhibitors were identified with K_i values as low as 48 nM. Analysis of the results as well as those of *Escherichia coli* and *Plasmodium falciparum* DXR enzymes revealed a different structure–activity relationship profile for the inhibition of *Tg*DXR.

The unicellular protozoan parasite *Toxoplasma gondii*, the causative agent of toxoplasmosis, is an important human pathogen.[1] In healthy adults, toxoplasmosis typically only produces mild, flu-like symptoms and the parasite becomes dormant. However, three factors make *T. gondii* a threat to public health. First, the parasite is highly promiscuous, infecting almost all warm-blooded animals including humans, with cats being the definitive host. Humans are infected by contacting cat feces contaminated with the mature oocyst form or by consumption of undercooked meat carrying tissue cysts. It is estimated that ~30% of world population is chronically infected with *T. gondii.* A recent CDC (Centers for Disease Control and Prevention) report disclosed that the prevalence of this infection in the US is ~11%.[1,2] Second, approximately one third of women infected for the first time with *T. gondii* during pregnancy will pass the parasite to the fetus where it can cause serious neurological damage to the fetus. Infection in particular the first trimester can lead to stillbirth. Third, the parasite poses a significant threat to immunocompromised persons, such as HIV-AIDS, cancer or organ transplant patients. Under these conditions latent infection can reactivate to fulminant Toxoplasma encephalitis, a life-threatening condition. Immunocompromised patients therefore may require recurrent treatment as current treatments are unable to clear the chronic infection. This is also true for immunocompetent patients suffering from recurring ocular toxoplasmosis. Current therapy is largely limited to anti-folate therapy. Long-term use of sulfonamides in particular has significant side effects including hypersensitivity. New therapeutic agents are therefore needed to treat toxoplasmosis.

1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) in the MEP (2-*C*-methyl-D-erythritol-4-phosphate) isoprene biosynthesis pathway is a novel target for developing anti-infective drugs.[3–5] As shown in Figure 1, unlike humans and animals that use the mevalonate pathway, most bacteria and apicomplexan parasites, including *T. gondii* and *Plasmodium* spp. (malaria parasites), use exclusively the MEP pathway to synthesize isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), essential intermediates for the synthesis of isoprenoid compounds. DXR is the 2nd enzyme of the pathway, catalyzing the reduction and isomerization of 1-deoxy-D-xylulose-5-phosphate (DXP) to 2-*C*-methyl-D-erythritol-4-phosphate (MEP) using Mg^{2+} and NADPH as enzyme cofactors (Fig. 1). Fosmidomycin (Fig. 2), a naturally occurring antibiotic, has been found to be a potent inhibitor of DXR[6] and possess antibacterial and antimalarial activities in preclinical studies and clinical trials.[7–10] This has further validated DXR as a promising drug target. Due to the poor pharmacokinetics of fosmidomycin (very short half-life in plasma and low oral availability), there is considerable interest in finding more potent and stable DXR inhibitors.[11–21] Medicinal chemistry studies by us and other groups have resulted in the synthesis of structurally diverse DXR inhibitors and representative examples

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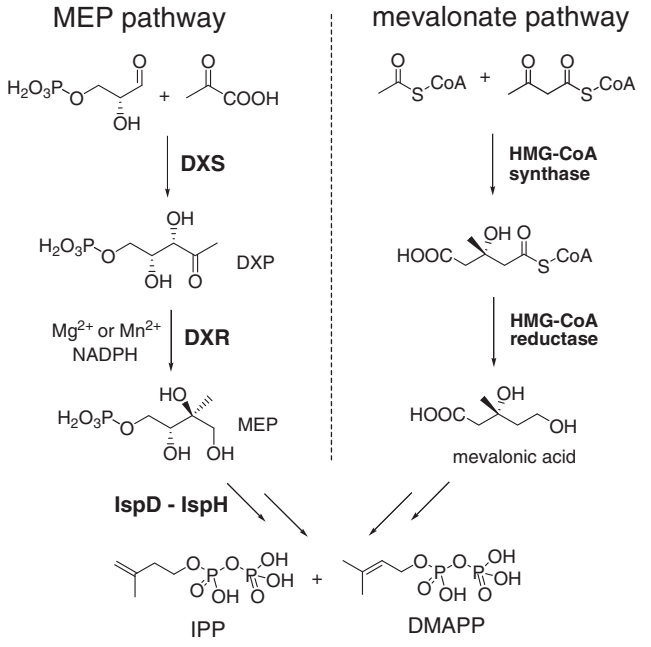

Figure 1. Non-mevalonate and mevalonate isoprene biosynthesis pathways.

are shown in Figure 2. None of these DXR inhibitors showed activity against *T. gondii* growth. This was surprising considering our finding that *T. gondii* DXR (*Tg*DXR) is essential to the growth of this organism.[22] *T. gondii* resistance to fosmidomycin is due to limited drug uptake, as previously found for certain bacteria.[23,24] The parasite cell membrane represents a permeability barrier for the compound. This is supported by the observation that fosmidomycin can effectively kill a strain of *T. gondii* engineered to express the bacterial GlpT, a known transporter of fosmidomycin, thus validating *Tg*DXR as a target for developing novel anti-toxoplasmosis drugs.[22] Here, we report the expression, purification and biochemical characterization of recombinant *T. gondii* DXR (*Tg*DXR). The inhibitory activity as well as structure–activity relationships of *Tg*DXR inhibitors are also presented.

First, we performed multiple protein alignments of the putative *Tg*DXR (NCBI Reference Sequence: XP_002370806.1) with *Escherichia coli* (*Ec*) and *Plasmodium falciparum* (*Pf*) DXRs, and the result is shown in Figure 3. Similar to *Pf*DXR, *Tg*DXR was found to carry an additional 67 amino acid residue extension at the N-terminal, when compared with the *E. coli* enzyme. This sequence likely represents the bipartite apicoplast targeting peptide,[8] since both proteins localize to the apicoplast of the parasites. In addition, *Tg*DXR possesses a very long linking sequence (224–285) with 62 residues between the NADPH binding domain (68–223) and the metal/substrate binding domain (286–513). However, in *Ec*DXR and *Pf*DXR, no more than 13 amino acid residues, which are mostly located in an α-helix that is away from the enzyme's active site, link the two domains. Nevertheless, the low homology among these three linker peptides (Fig. 3) as well as the structural information from *Ec*DXR and *Pf*DXR suggest the segment 224–285 of *Tg*DXR may not be important for enzyme activity. Except for these differences, these three enzymes share an overall high degree of similarity.

We next cloned the catalytic domain (68–513) of *Tg*DXR and inserted it into the expression plasmid pET24b. The plasmid was transformed into *E. coli* BL21-CodonPlus strain and cultured in LB medium containing kanamycin and chloramphenicol.[25] His6-tagged recombinant *Tg*DXR was expressed and purified using a standard Ni-affinity column chromatography to ~90% purity, showing an apparent molecular mass of ~45 kDa (Supplementary data on-line Fig. S1).

The recombinant enzyme was biochemically characterized and found to be able to catalyze the conversion of DXP to MEP in the presence of Mg^{2+} and NADPH.[26] The reaction rate was monitored at 340 nm, where NADPH UV absorbance is maximal. First, the activity was tested in a HEPES buffer (50 mM, pH 7.6) containing *Tg*DXR (100 nM), DXP (100 μM), NADPH (100 μM), 50 μg/mL BSA (bovine serum albumin) and varying concentrations of $MgCl_2$. As shown in Figure 4a, the activity of *Tg*DXR is dependent on Mg^{2+}, the enzyme is completely inactive in the absence Mg^{2+} and activity increases with higher [Mg^{2+}] until reaching a maximum at 4 mM Mg^{2+}. We noted that activity of the enzyme can also be supported by Mn^{2+} and Co^{2+}, two additional commonly used divalent metal ions, as illustrated in Figure 4b. In the presence of Mn^{2+} (2 mM) *Tg*DXR exhibits essentially the same activity as with Mg^{2+}, and shows approximately half of the activity with Co^{2+} (2 mM). In addition, we measured the pH-dependence of *Tg*DXR and the results demonstrated a pH optimum of 7.5–8.0 for this enzyme (Fig. 4c), although significant activity can be observed for a range from pH 6.5 to 8.5. We next determined the K_m value for the substrate DXP, which is necessary for the calculation of K_i values (inhibition constant) of *Tg*DXR inhibitors. Enzyme activities were measured in the presence of increasing concentrations of DXP (from 10 to 450 μM) and, as shown in Figure 4d, the K_m value of *Tg*DXR for DXP was determined to be 25.5 ± 3.7 μM when fitted into Michaelis–Menten equation. This is comparable to K_m values of *Ec*DXR (99 μM),[13] *Pf*DXR (106 μM)[15] and *Mycobacterium tuberculosis* DXR (47 μM).[23]

Upon optimization of the *Tg*DXR enzyme assay conditions, the inhibitory activity of compounds **1**–**11** (Fig. 2) was determined in order to explore their structure–activity relationships (SAR) for this enzyme.[26] These selected compounds represent a broad structural diversity and are particularly suited for the initial SAR study. Fosm-

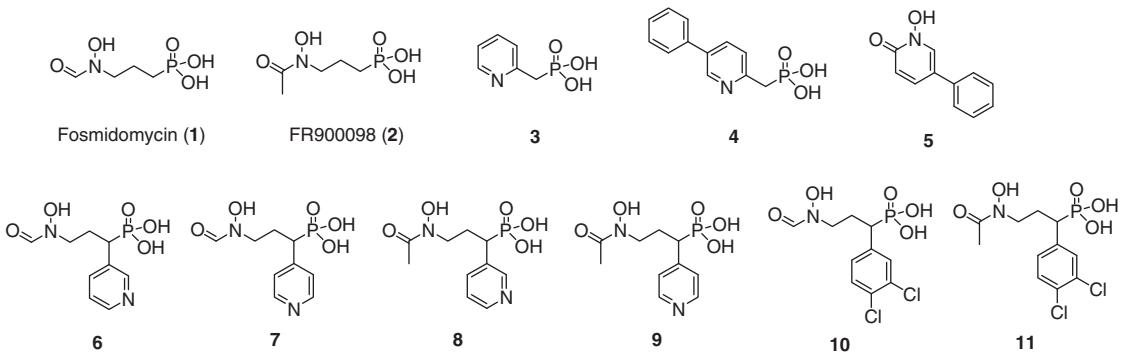

Figure 2. Representative DXR inhibitors.