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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 (*TgDXR*). 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 *TgDXR* and several potent inhibitors were identified with K_i values as low as 48 nM. Analysis of the results as well as those of *E. coli* and *Plasmodium falciparum* DXR enzymes revealed a different structure-activity relationship profile for the inhibition of *TgDXR*.

Keywords

Toxoplasma gondii; 1-Deoxy-D-xylulose-5-phosphate Reductoisomerase; Protein expression; Enzyme inhibition; Structure-activity relationship

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

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~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 Mg²⁺ and NADPH as enzyme cofactors (Figure 1). Fosmidomycin (Figure 2), a naturally occurring antibiotic, has been found to be a potent inhibitor of DXR⁶ 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 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 (*TgDXR*) 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 *TgDXR* as a target for developing novel anti-toxoplasmosis drugs.²² Here, we report the expression, purification and biochemical characterization of recombinant *T. gondii* DXR (*TgDXR*). The inhibitory activity as well as structure activity relationships of *TgDXR* inhibitors are also presented.

First, we performed multiple protein alignments of the putative *TgDXR* (NCBI Reference Sequence: XP_002370806.1) with *E. coli* (*Ec*) and *P. falciparum* (*Pf*) DXRs, and the result is shown in Figure 3. Similar to *PfDXR*, *TgDXR* 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, *TgDXR* 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 *EcDXR* and *PfDXR*, 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 (Figure 3) as well as the structural information from *EcDXR* 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 kD (Supplemental Data on-line Figure 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 μ M), NADPH (100 μ M), 50 μ g/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 (Figure 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 μ M) and, as shown in Figure 4d, the K_m value of *Tg*DXR for DXP was determined to be 25.5 ± 3.7 μ M when fitted into Michaelis–Menten equation. This is comparable to K_m values of *Ec*DXR (99 μ M),¹³ *Pf*DXR (106 μ M)¹⁵ and *Mycobacterium tuberculosis* DXR (47 μ M).²³

Upon optimization of the *Tg*DXR enzyme assay conditions, the inhibitory activity of compounds **1** – **11** (Figure 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. Fosmidomycin (**1**) and FR900098 (**2**) are highly polar phosphonohydroxamic acids, while compounds **3** – **9** possess more lipophilic properties. Compounds **3** and **4** are phosphonate DXR inhibitors with a pyridine-containing, lipophilic side chain, which was found to be essential for inhibition.¹² Hydroxypyridinone compound **5** is the only potent DXR inhibitor without a phosphonate/phosphate group, which also exhibits broad antibacterial activity.¹¹ Pyridine-containing fosmidomycin derivatives **6** – **9** were recently found to have considerably higher activity against *Pf*DXR as well as the proliferation of *P. falciparum*,¹⁴ as compared to fosmidomycin. Analogous compounds **10** and **11** possess a 3,4-dichlorophenyl substituent at the α -position, which were also reported to possess potent antimalarial activities.¹⁶

Table 1 summarizes the K_i values of compounds **1** – **11** against the DXR enzymes of *T. gondii*, *E. coli* and *P. falciparum*. Fosmidomycin (**1**) and FR900098 (**2**) are very strong inhibitors of the *T. gondii* enzyme with K_i values of 90 and 48 nM. Compounds **3** and **4** without a hydroxamate as metal-binding group are considerably less active, with their K_i values being in the low μ M range. The inhibitory activities of the above four compounds against *Tg*DXR are generally in line with those against *Ec*DXR and *Pf*DXR (Table 1). However, the non-phosphonate compound **5** exhibits only very weak inhibitory activity against *Tg*DXR (as observed for *Pf*DXR, the other eukaryotic species) with a K_i value of

25.6 μM . This could explain that despite its high lipophilicity, compound **5** does not block proliferation of *T. gondii* using our previous method,²² although it possesses broad antibacterial activity including *E. coli* presumably due to its strong activity against *EcDXR*.¹¹ Pyridine-containing compounds **6** and **7** with a formyl group are potent inhibitors of *TgDXR* with K_i values of 55 and 79 nM, respectively, being more active than their parent compound fosmidomycin. This shows that an appropriate α -substituent may provide favorable interactions with the *T. gondii* enzyme, as also observed for *EcDXR* and *PfDXR*. Surprisingly, the acetyl analogs, compounds **8** and **9**, exhibit on average ~11-fold less activity than compounds **6** and **7**, suggesting that with an α -substituent, the terminal methyl group is disfavored on binding to *TgDXR*. This feature is quite different from those of *EcDXR* and *PfDXR*, for which **8** and **9** show similar or even higher activities as compared to their formyl analogs **6** and **7** (Table 1). The same SAR is observed for compounds **10** and **11** against *TgDXR*, with a formyl group ($K_i = 77$ nM) in **10** showing considerably more inhibitory activity than compound **11** with an acetyl moiety ($K_i = 220$ nM).

Figure 5 illustrates the plots of the inhibitory activities of compounds **1** – **11** against *TgDXR* with those against *EcDXR* and *PfDXR*. Although there are reasonable correlations between the $\text{p}K_i^{\text{TgDXR}}$ and the $\text{p}K_i^{\text{EcDXR}}$ and $\text{p}K_i^{\text{PfDXR}}$ values with R^2 of 0.67 and 0.65, respectively, the slope of 0.61 for *TgDXR* vs. *EcDXR* is far from the theoretic value of 1 and there are several obvious outliers (out of 11 inhibitors) in these two figures. In addition, the SARs described above also show a different profile for *TgDXR* inhibition. These comparisons suggest that more biochemical, structural and pharmacological studies of *TgDXR* are needed to develop effective anti-toxoplasmosis drugs. The methods reported here for expression and inhibition of recombinant *TgDXR* could therefore be useful for these studies as well as high-throughput screening for potent inhibitors of the enzyme.

In summary, this work is of interest for several reasons. Our previous studies validated DXR as a drug target for treating toxoplasmosis. We therefore expressed and purified recombinant *TgDXR*, which was found to be enzymatically active. Importantly, we directly support our previous assumption that *TgDXR* is fully susceptible to fosmidomycin.²² *TgDXR* was observed to exert maximal activity in the presence of 4 mM Mg^{2+} at pH 7.5 – 8.0. At these conditions, the K_m value for the substrate DXP was determined to be 25.5 μM . A collection of 11 compounds were tested against *TgDXR* and several potent inhibitors were identified with K_i values as low as 48 nM. Analysis of these results as compared to those of *EcDXR* and *PfDXR* revealed a different structure-activity relationship profile for the inhibition of *TgDXR*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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25. The plasmid was transformed into *E. coli* (BL21-CodonPlus strain from Agilent) and cultured in LB medium containing kanamycin (25 µg/mL) and chloramphenicol (34 µg/mL). Upon reaching an optical density of ~0.6 at 600 nm, *TgDXR* expression was induced by adding 0.25 mM isopropylthiogalactoside (IPTG) for 4 hours at 37°C. Cells were then harvested and resuspended in 50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl (buffer A) containing 20 mM imidazole. After addition of 0.2 mM phenylmethylsulfonyl fluoride and sonication at 0 °C, the lysate was centrifuged at 20,000 rpm for 25 min and the supernatant was collected and subjected to an affinity column chromatography using the Ni-affinity column chromatography (HiTrap IMAC FF from GE Healthcare). The resin was washed with 30 mM imidazole in buffer A and then the protein was eluted with 300 mM imidazole in buffer A. After desalting (HiTrap Desalting, GE Healthcare) to 20 mM Tris pH 7.5, 150 mM NaCl, 2% glycerol, the protein was concentrated and stored in small aliquots at -80 °C.
26. The enzyme activity was determined in 96-well microplates using purified *TgDXR* (100 nM), 4 mM MgCl₂, 100 µM DXP, 100 µM NADPH in 50 mM HEPES buffer (pH = 7.6) containing 50 µg/mL bovine serum albumin (BSA). For inhibition assays, compounds were incubated with

*Tg*DXR for 10 min at 30 °C before adding DXP to initiate the reaction. The reaction rate was monitored at 340 nm using a Beckman DTX-880 microplate reader. The initial velocities of wells containing increasing concentrations of an inhibitor were calculated and imported into Prism (version 5.0, GraphPad Software, Inc., La Jolla, CA). The IC₅₀ values were then obtained by using a standard dose response curve fitting. For less potent inhibitors (IC₅₀ > 500 nM), *K_i* values were calculated using the formula $K_i = IC_{50}/(1+[S]/K_m)$, where [S] is the concentration of DXP (100 μM) and *K_m* was determined to be 25.5 μM. For highly potent inhibitors (IC₅₀ ≤ 500 nM), the Morrison tight inhibition equation in Prism was used to calculate their *K_i* values.

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

Figure 2.
Representative DXR inhibitors.

Figure 3.
Clustal X alignment of *E. coli* (Ec), *T. gondii* (Tg) and *P. falciparum* (Pf) DXR.

Figure 4.

(A) Effects of $[\text{Mg}^{2+}]$ on *TgDXR* catalyzed reaction. See Ref. 26 for a general assay condition; (B) Effects of divalent metal ions on *TgDXR* catalyzed reaction; (C) Effects of pH on *TgDXR* catalyzed reaction ($[\text{Mg}^{2+}] = 2 \text{ mM}$); (D) Effects of $[\text{DXP}]$ on *TgDXR* catalyzed reaction ($[\text{Mg}^{2+}] = 4 \text{ mM}$).

Figure 5. Correlations between $TgDXR$ inhibition and that of (A) $EcDXR$ and (B) $PtDXR$.

Table 1

K_i values of **1** – **11** against three DXR enzymes.

Compound	<i>Tg</i> DXR (μ M)	<i>Ec</i> DXR (μ M) ^a	<i>Pf</i> DXR (μ M) ^a
1	0.090	0.027	0.021
2	0.048	0.019	0.011
3	4.1	2.3	3.3
4	2.1	0.42	1.1
5	25.6	0.70	14.6
6	0.055	0.087	0.013
7	0.079	0.035	0.0089
8	0.97	0.042	0.0019
9	0.53	0.082	0.013
10	0.077	0.058	0.015
11	0.22	0.036	0.025

^aData were from Refs. 13, 14 and 15.