The unicellular protozoan parasite *Toxoplasma gondii*, the causative agent of toxoplasmosis, is an important human pathogen. 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 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%. 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 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* 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 *TgDXR*.

**Keywords:**
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1-Deoxy-D-xylulose-5-phosphate reductoisomerase
Protein expression
Enzyme inhibition
Structure–activity relationship

**A R T I C L E   I N F O**

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**A B S T R A C T**

The apicomplexan parasite *Toxoplasma gondii*, the causative agent of toxoplasmosis, is an important human pathogen. 1-Deoxy-o-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* 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 *TgDXR*.

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Here, we report the expression, purification and biochemical characterization of recombinant T. gondii DXR and in-
teractors are also presented.

First, we performed multiple protein alignments of the putative TgDXR (NCBI Reference Sequence: XP_002370806.1) with Esche-
richia coli (Ec) and Plasmodium 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 Ec enzyme. This sequence likely represents the bipartite apicoplast targeting peptide, 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 (Fig. 3) as well as the structural information from EcDXR and PfDXR suggest the segment 224–285 of TgDXR 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 TgDXR 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. His6-
tagged recombinant TgDXR 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 Mg2+ and NADPH. 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 TgDXR (100 nM), DXP (100 μM), NADPH (100 μM), 50 μg/mL BSA (bovine serum albumin) and varying concentrations of MgCl2. As shown in Figure 4a, the activity of TgDXR is dependent on Mg2+. The enzyme is completely inactive in the absence Mg2+ and activity increases with higher [Mg2+] until reaching a maximum at 4 mM Mg2+. We noted that activity of the enzyme can also be supported by Mn2+ and Co2+, two additional commonly used divalent metal ions, as illustrated in Figure 4b. In the presence of Mn2+ (2 mM) TgDXR exhibits essentially the same activity as with Mg2+, and shows approximately half of the activity with Co2+ (2 mM). In addition, we measured the pH-dependence of TgDXR 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 TgDXR 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 TgDXR 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 EcDXR (99 μM)13 and PfDXR (106 μM)14 and Mycobacterium tuberculosis DXR (47 μM).23

Upon optimization of the TgDXR enzyme assay conditions, the inhibitor activity of compounds 1–11 (Fig. 2) was determined in order to explore their structure–activity relationships (SAR) for this enzyme.16 These selected compounds represent a broad structural diversity and are particularly suited for the initial SAR study. Fosm-

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

Figure 2. Representative DXR inhibitors.

Idomycin (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 Hydroxy pyridinone compound 5 is the only potent DXR inhibitor without a phosphate/phosphate group, which also exhibits broad antibacterial activity.11 Pyridine-containing fosmidomycin derivatives 6–9 were recently found to have considerably higher activity against PDXR as well as the proliferation of P. falciparum,13 as compared to fosmidomycin. Analogous compounds 10 and 11 possess a 3,4-dichlorophenyl substituent at the β-position, which were also reported to possess potent antimalarial activities.16

Table 1 summarizes the Ki 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 Ki values of 90 and 48 nM. Compounds 3 and 4 without a hydroxamate as metal-binding group are considerably less active, with their Ki values being in the low μM range. The inhibitory activities of the above four compounds against TgDXR are generally in line with those against EcDXR and PDXR (Table 1). However, the non-phosphonate compound 5 exhibits only very weak inhibitory activity against TgDXR (as observed for PDXR, the other eukaryotic species) with a Ki 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 Ki 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 PDXR. 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 PDXR, 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 (Ki = 77 nM) in 10 showing considerably more inhibitory activity than compound 11 with an acetyl moiety (Ki = 220 nM).

Figure 5 illustrates the plots of the inhibitory activities of compounds 1–11 against TgDXR with those against EcDXR and PDXR. Although there are reasonable correlations between the pKᵢTgDXR and the pKᵢEcDXR and pKᵢPDXR values with R² of 0.67 and 0.65, respectively, the slope of 0.61 for TgDXR versus 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 de-

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**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>TgDXR (μM)</th>
<th>EcDXR (μM)</th>
<th>PDXR (μM)</th>
</tr>
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<tr>
<td>1</td>
<td>0.090</td>
<td>0.027</td>
<td>0.021</td>
</tr>
<tr>
<td>2</td>
<td>0.048</td>
<td>0.019</td>
<td>0.011</td>
</tr>
<tr>
<td>3</td>
<td>4.1</td>
<td>2.3</td>
<td>3.3</td>
</tr>
<tr>
<td>4</td>
<td>2.1</td>
<td>0.42</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>25.6</td>
<td>0.70</td>
<td>14.6</td>
</tr>
<tr>
<td>6</td>
<td>0.055</td>
<td>0.087</td>
<td>0.013</td>
</tr>
<tr>
<td>7</td>
<td>0.009</td>
<td>0.013</td>
<td>0.0025</td>
</tr>
<tr>
<td>8</td>
<td>0.0042</td>
<td>0.0019</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.053</td>
<td>0.082</td>
<td>0.013</td>
</tr>
<tr>
<td>10</td>
<td>0.077</td>
<td>0.058</td>
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</tr>
<tr>
<td>11</td>
<td>0.22</td>
<td>0.036</td>
<td>0.025</td>
</tr>
</tbody>
</table>

* Data were from Refs. 13–15.
scribed 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. The methods reported here for expression and purification of 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 Mg2+ at pH 7.5–8.0. At these conditions, the IC50 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 Ki 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.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.01.097.

References and notes

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 h 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 (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 TgDXR 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 using a standard dose response curve fitting. For less potent inhibitors (IC50 >500 nM), Ki values were calculated using the formula K = IC50/[1 + [I]IC50], where [I] is the concentration of DXP (100 μM) and Ki was determined to be 23.5 μM. For highly potent inhibitors (IC50 ≤500 nM), the Morrison tight inhibition equation in Prism was used to calculate their Ki values.