Research Article

Thianthrene is a novel inhibitor of *Leishmania donovani* pteridine reductase 1 (PTR1)

Jaspreet Kaur[1], Divya Dube[2], Ravishankar Ramachandran[2], Prashant Singh[3] and Neeloo Singh[1]

[1]Drug Target Discovery & Development and [2]Molecular & Structural Biology Division, Central Drug Research Institute (CSIR), Chattar Manzil Palace, Lucknow, India; [3]Department of Chemistry, D.A.V. (P.G.) College, Dehradun-248001, India

Received on November 18, 2011; Accepted on April 30, 2012; Published on June 16, 2012

Correspondence should be addressed to Neeloo Singh; Phone: +91 9415002065, Fax: +91 522 22623405, E-mail: neeloo888@yahoo.com

Abstract

Pteridine reductase 1 (PTR1) from *Leishmania donovani* is a short chain reductase that catalyses the NADPH-dependent reduction of folates and pterins. It has gained attention as a therapeutic target because it acts as a metabolic bypass for dihydrofolate reductase (DHFR) targeting drugs and is thought to be responsible for the failure of conventional therapies against the trypanosomatids. In the present study, we report the identification of thianthrene as a potent inhibitor of *L. donovani* PTR1 (*Ld*PTR1) based on both structure-based virtual screening and experimental verification. Thianthrene displayed uncompetitive mixed type inhibition in a recombinant enzyme inhibition assay. In addition, cell based assays and flow cytometry showed that the intracellular amastigotes were inhibited by thianthrene *in vitro*. The results of our study could be considered for the development of novel therapeutics based on PTR1 inhibition.

Introduction

Leishmaniasis is caused by protozoan parasites of the *Leishmania* genus. The disease can lead to severely disfiguring mucocutaneous manifestations and cause lethal visceral infection. Visceral leishmaniasis (VL) or kala-azar is a vector-borne tropical disease that infects half a million people every year. The disease is strongly linked to poverty and 90% of the cases are found in the poorest areas of Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan (WHO 2012). In India, the state of Bihar alone contains ~50% of the world's cases of VL. No effective vaccines are available against *Leishmania* infection (Carter *et al.* 2007, Handman 2001) and chemotherapy remains the only treatment option for controlling infection.

The identification of novel drug targets can help develop new therapeutic strategies against VL. The parasites exhibit many atypical features in the pteridine metabolic pathway which are essential for growth; these could prove to be excellent targets for chemotherapeutic treatment. *Leishmania* and other trypanosomatid protozoans are auxotrophs for reduced pteridines (pterins and folates) which are required for critical cellular pathways like nucleic acid and protein biosynthesis. Thus, they rely on the uptake of pterin compounds, such as biopterin or folate, from the host. These then undergo two successive reductions to generate the active tetrahydro-species. Two enzymes carry out these reactions in the protozoans, namely bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) and pteridine reductase (PTR1). The former is the major enzyme known to reduce folate and 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) (Nare *et al.* 1997a). PTR1 is responsible for the NADPH-dependent reversible reduction of oxidized pterins to dihydrobiopterin (DHB) as well as of tetrahydrobiopterin (THB) and folates to DHF and THF (Nare *et al.* 1997b) (Figure 1).

The PTR1 enzyme was discovered in *Leishmania* several years ago (Gourley *et al.* 1999). Studies indicate that the primary role of PTR1 is to salvage oxidized pterins and its secondary role is to reduce folates (Bello *et al.* 1994, Nare *et al.* 1997b, Wang *et al.* 1997). It is the only enzyme that has been reported to reduce biopterin in *Leishmania* parasites and has

been shown to be essential for growth *in vivo* (Bello *et al.* 1994, Nare *et al.* 1997b, Sienkiewicz *et al.* 2010). Interestingly, PTR1 is much less susceptible to inhibition by clinical DHFR inhibitors like methotrexate (IC50 of 1.1µM, 0.005µM and 0.04µM for *L. major* PTR1 (*Lm*PTR1), *Lm*DHFR-TS and human DHFR (*h*DHFR), respectively) while it catalyzes the same reaction as that of DHFR. It is therefore likely to be responsible for the failure of antifolate therapeutic strategies targeted against DHFR by acting as a metabolic bypass (Hardy *et al.* 1997, Nare *et al.* 1997a). In this regard, PTR1 presents an attractive drug target for the development of novel therapeutic tools.

High throughput virtual screening has been applied extensively in modern drug discovery (Tulloch *et al.* 2010). Potent DHFR inhibitors are already known, and we have worked towards designing novel PTR1 inhibitors based on the enzyme identified from the clinical isolate of *L. donovani* PTR1 (*Ld*PTR1). Using structural analysis combined with biochemical verification, we propose a structure-function model of this important enzyme. The results of our study could lay the foundation for the design of novel vaccination and anti-PTR1 drug-like agents.

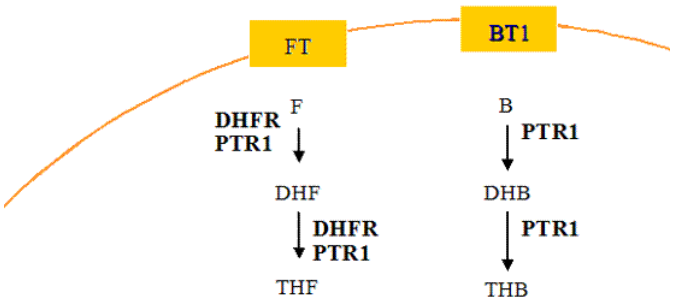


Figure 1. Folate and pterin transport and processing**.** Folate and pterin enter the cell by the folate (FT) and biopterin (BT1) transporters, respectively. Folate is then reduced to 7,8-dihydrofolate (DHF) and 5,6,7,8-tetrahydrofolate (THF) by dihydrofolate reductase (DHFR) and pteridine reductase (PTR1). Similarly, biopterin is reduced to dihydrobiopterin (DHB) and tetrahydrobiopterin (THB) by PTR1.

Materials and Methods

Macrophage culture

The J774A.1 mouse (BALB/c) macrophage cell line was obtained from the National Centre for Cell Science (NCCS Pune, India) and used as a cellular host for the *in vitro* intracellular test of antileishmanial activity against amastigotes. Cells were maintained at 37°C, 5% CO_2, 95% air. They were cultured in RPMI 1640 medium (Gibco-BRL) containing 2 g/L sodium bicarbonate, 6 g/L HEPES, 10% (v/v) heat inactivated fetal bovine serum (HIFBS; Gibco, Germany), 100 U penicillin and 100 µg/mL streptomycin.

Routine *L. donovani* parasite culture and counting

Green fluorescent protein (GFP) transfected *L. donovani* were prepared as described previously (Singh & Dube 2004) and cultured in Medium 199 (pH 7.2) (Sigma), supplemented with Hank's salts, 2.05 mM L-glutamine, 12 mM HEPES buffer (Sigma), 10% (v/v) HIFBS, 100 units/mL penicillin, 100 µg/mL streptomycin and 150 µg/mL geneticin sulfate (G418). They were grown in vented T25 tissue culture flasks and maintained at 25°C. Promastigote cultures were initiated at 106 parasites per ml and subcultured every 3-4 days. Parasite counts were performed in duplicate using a hemocytometer and a particle counter (Beckman Coulter, Fullerton, CA).

In silico docking studies

For the identification of inhibitors against *Ld*PTR1, *de novo* design of ligands was performed in a virtual screening strategy. The CAP Database (Chemicals Available for Purchase) by Accelrys Inc., consisting of approximately 75,000 compounds, was used as the ligand source in the virtual screening experiments.

A recent report on the structure of recombinant *Ld*PTR1 revealed a disordered active site (Barrack *et al.* 2010). In this regard, a homology model of *Ld*PTR1 was built using the resolved crystal structure of *Lm*PTR1 (PDB code: 1E92) as a template (Gourley *et al.* 1999), using the homology modelling tool Modeller 8v1 (Martí-Renom *et al.* 2000). More specifically, comparative homology modelling started with the searching of the query protein sequence against the Protein Data Bank using BLAST (Basic Local Alignment Search Tool). The highest scoring sequence was identified as *Lm*PTR1 and chosen as a template to build the homology model of *Ld*PTR1. The *L. major* and *L. donovani* enzymes share 91% sequence identity (Kumar et al., 2004) and the catalytic residues Asp181, Tyr191, Tyr194, Lys198 are conserved; therefore, the details of the catalytic mechanism are expected to be identical between them. The active site residues are shown in Figure 2 and the sequence alignment between the two species is shown in Figure 3. The model was further optimized by adding all hydrogens and subjected to 100 steps of minimization with Tripos Forcefield of the SYBYL 7.1 Molecular Modeling Suite (Tripos Inc., St. Louis, MO). Its structural quality was then verified using tools available at the Procheck and the Verify3D server (Laskowski *et al.* 1996). Ramachandran plot calculations showed that 95.3% of the residues are in favored and 4.7% exist in allowed regions (Figure 4). Analysis using the Verify3D pro-

gram showed 82.81% of the residues having an average 3D-1D score > 0.2 (Lüthy *et al.* 1992). These analyses indicate that the model has a good quality. The binding site was further modelled with the cofactor $NADP^+$ from the crystal structure of *Lm*PTR1 (Pdb: 1E92) (Accelrys 11, San Diego, CA).

Ludi, a *de novo* structure based drug design tool (Bohm 1992) employing the InsightII interface was then used to perform the virtual screening experiments. This software uses a systemic search algorithm with either a linking or growing strategy for ligand conformational structure generation. In this study, Ludi parameters were assigned using standard default values and ligand library as specified in Ludi/CAP. The default parameters include Linkages (set as none), Max RMSd (set between 0.3-0.5) and Rotatable bonds (set to One_At_A_Time). In addition, the Min Separation parameter was kept between 3.0 and 3.5, the Dens L and Dens P parameters were set to 25, the Min Surf parameter was set to 50 and The Max Unfilled Cavity parameter was set to 0. The Centre of Search was defined by choosing the PTR1 active site residue A194: OH with a search sphere radius of 7 Å. The virtual screening was performed using the targeted search mode. This allows the software to specify the receptor atoms that fragments are required to interact with. Results obtained were analyzed and prioritized based on the Energy_estimate_3 scoring function. This function was chosen in order to evaluate the change in free energy upon binding contributions made by the polar as well as the hydrophobic and aromatic-aromatic interactions.

Enzyme expression, purification and activity assays

To establish the targeted enzymatic reaction system, the recombinant enzyme *Ld*PTR1 was expressed in *E. coli* and purified based on its N-terminal His6 tag by affinity chromatography using a Ni^{2+}-IDA Hi-Trap chelating sepharose column in AKTAprime plus (GE Healthcare, CA) (Kumar *et al.* 2004). Reductase activity (*Ld*PTR1) was assayed as described previously (Kaur *et al.* 2010). *K*m and *V*max values for biopterin were determined using a Lineweaver-Burk plot.

Flow cytometry based growth inhibition assay

The J774A.1 mouse (BALB/c) macrophage cell line was used for the *in vitro* intracellular drug efficacy test. The assay was performed as described in the protocol (Kaur *et al.* 2010).

Statistical analysis

The data are presented as mean±SD. The statistical analysis was performed by one-way ANOVA using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

Results and Discussion

Molecular modelling and docking of thianthrene

Enzymes of folate metabolism are proven targets for

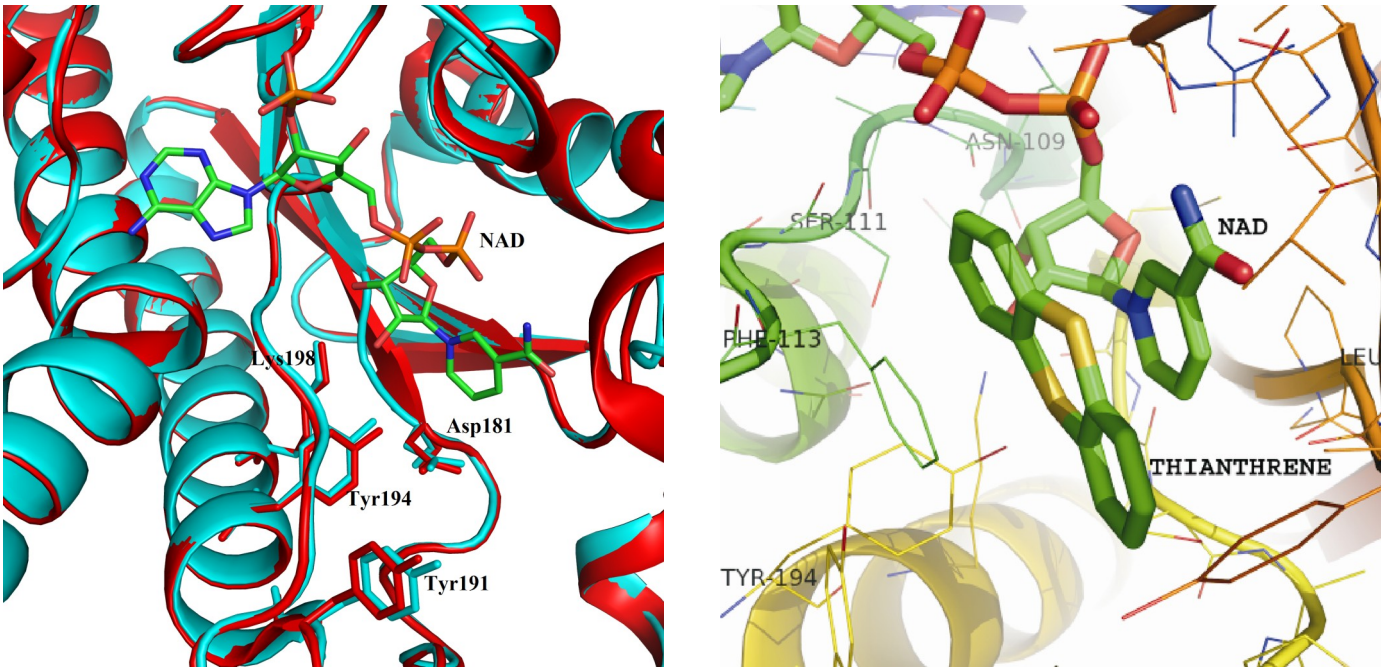


Figure 2. Superposition of modeled *Ld*PTR1 (cyan) onto the *Lm*PTR1 template (red). (A) The active site of PTR1 is shown together with the bound cofactor NAD and the conserved active site residues are labelled. (B) The inhibitor thianthrene is shown docked into the binding site of the *Ld*PTR1. The contacting active site residues are labelled along with the bound cofactor NAD. The sulphur atom of thianthrene is involved in Sulphur……π interactions with F113 and nicotinamide.

```
LmPTR1   GSHMTAPTVPVALVTGAAKRLGRSIAEGLHAEGYAVCLHYHRSAAEANALSATLNARRPN 60
LdPTR1   ---MTAPTVPVALVTGAAKRLGSGIAEGLHAEGYAVCLHYHRSAAEANTLAATLNARRPN 57
            ******************* .************************:*:*********

LmPTR1   SAITVQADLSNVATAPVSGADGSAPVTLFTRCAELVAACYTHWGRCDVLVNNASSFYPTP 120
LdPTR1   SAIPVQADLSNVAKAPAGGADGAAPVTLFKRCADLVAACYTHWGRCDVLVNNASSFYPTP 117
         ***.*********.**..****:******.***:**************************

LmPTR1   LLRNDEDGHEPCVGDREAMETATADLFGSNAIAPYFLIKAFAHRVAGTPAKHRGTNYSII 180
LdPTR1   LLRKDEDGHVPCVGDREAMEAAAADLFGSNAMAPYFLIKAFAHRVADTPAEQRGTNYSIV 177
         ***:***** **********:*:********:**************.***::*******:

LmPTR1   NMVDAMTNQPLLGYTIYTMAKGALEGLTRSAALELAPLQIRVNGVGPGLSVLVDDMPPAV 240
LdPTR1   NMVDAMTSQPLLGYTIYTMAKGALEGLTRSAALELAPLQIRVNGVGPGLSVLADDMPPAV 237
         *******.*****************************************.*******

LmPTR1   WEGHRSKVPLYQRDSSAAEVSDVVIFLCSSKAKYITGTCVKVDGGYSLTRA 291
LdPTR1   REDYRSKVPLYQRDSSAAEVSDVVIFLCSSKAKYVTGTCVKVDGGYSLTRA 288
          *.:*******************************:****************
```

Figure 3. Sequence alignment of the modeled *Ld*PTR1 onto the *Lm*PTR1 template. The * indicates the fully conserved residues while : and . indicate conservation between groups of strongly and weakly similar properties, respectively.

the treatment of several bacterial and parasitic infections (Webster 1990) and antifolate-based drugs, such as methotrexate, are widely used in such cases. However, in the case of trypanosomatids, resistance is mediated principally by amplification of a trypanosomatid specific PTR1 (Nare *et al.* 1997a). PTR1 catalyses the same reaction as DHFR but is less susceptible to known antifolates, providing a metabolic bypass to alleviate DHFR inhibition. Inhibition of PTR1 would facilitate the exploitation of DHFR-specific antifolates and provide an efficient therapeutic approach.

In drug discovery, the 3D conformational arrangement of the active site determines the likelihood of finding a molecule with the right properties. We have applied computational methods (virtual screening, modelling and chemical similarity searches) for ligand identification. The co-crystal structure of *Lm*PTR1 is available with the bound substrate DHB: PTR1−$NADP^+$−DHB (Pdb: 1E92), the bound methotrexate: PTR1−NADPH−MTX (Pdb: 1E7W) and the bound inhibitor 2,4,6-triaminoquinazoline (TAQ): PTR1-NADP-TAQ (Pdb: 1W0C). As a large part of the pterin binding site interacts with nicotinamide, the substrate or inhibitor can only bind effectively after formation of the protein-cofactor complex. In this regard, the PTR1 binding site was modelled with the cofactor $NADP^+$, in order to obtain a more realistic virtual screening model. The inhibitors against PTR1 were identified using Ludi. The control docking calculations were performed using DHB as the known substrate. The resultant scores for binding affinities calculated by Ludi are shown in Table 1.

The screening returned thianthrene and methotrexate as the top best hits currently available in chemical stock databases. The flat aromatic ring of methotrexate is sandwiched between the nicotinamide and Phe113 aromatic rings whilst methotrexate is making hydrogen bonded interactions with Ser111 and

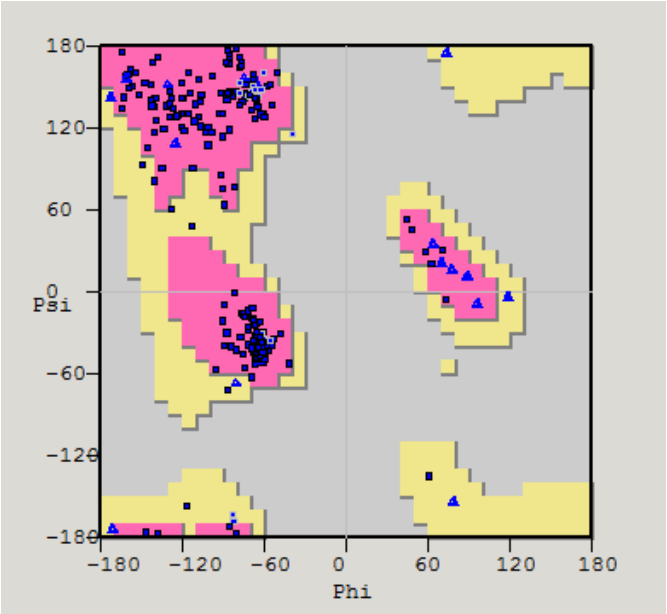


Figure 4. Ramachandran Plot showing the different regions of the modeled *Ld*PTR1. The plot was generated using Procheck.

Tyr194. The docking conformation for thianthrene as predicted by Ludi is shown in Figure 2. The inhibitor mimics the pterin head group of the prototypic antifolate drug methotrexate in the spatial disposition and exploits similar sandwiched hydrophobic stacking to bind to the PTR1 active site (McLuskey *et al.* 2004). Thianthrene is stacked between Phe113 and the nicotinamide ring of the cofactor by using parallel-displaced and face-to-face aromatic-aromatic interactions within the active site of PTR1 (Figure 2) (Gallivan & Dougherty 2000). Such stacking interactions between substrate and nicotinamide are exclusive to PTR1 amongst all SDR family members (Gourley *et al.* 2001). The terminal oxygen for Y194 is also making an aryl O-H type of stacking interactions with the aromatic ring of thianthrene (Perutz *et al.* 1986).

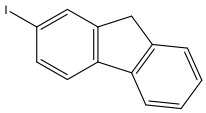
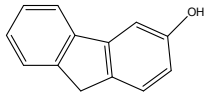
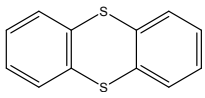
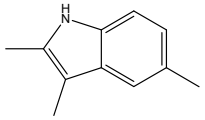
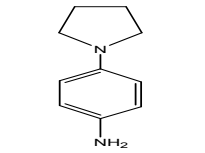
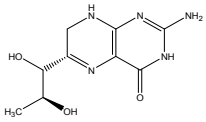
No	Ligand CAP ID	Structure	Ludi Score
1	154861 2-Iodo-9H-fluorene		751
2	161750 9H-Fluoren-3-ol		767
3	10177 Thianthrene		686
4	250024 2,3,5-Trimethyl-1H-indole		600
5	19302 4-Pyrrolidin-1-yl-phenylamine		659
6	DHB 2-Amino-6-(1,2-dihydroxy-propyl)-7,8-dihydro-3H-pteridin-4-one		684

Table 1. Predicted Ludi Scores for the selected compounds with PTR1 along with the compound structures. DHB refers to the Control Docking with 7, 8 Dihydrobiopterin.

More than 20 complexes are available in the protein data bank that fit the criteria for pteridine reductase inhibition. Hydrophobic stacking interactions between the nicotinamide and Phe113 is an important aspect for substrate recognition and catalysis in the pteridine reductase catalytic mechanism. The docking studies show that the inhibitor thianthrene is able to bind at the same catalytic center. Like thianthrene, the PTR1 natural substrates are pterin and folates which also contain a flat ring system. Perhaps the addition of a polar side-chain to the basic aromatic backbone of thianthrene could further enhance the specificity towards the enzyme.

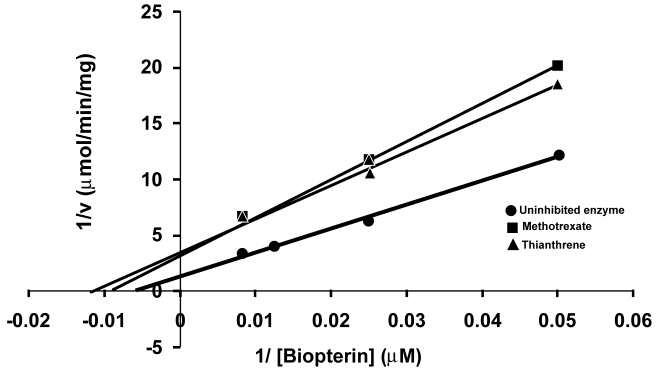


Figure 5. Lineweaver–Burk plot for methotrexate and thianthrene inhibition of *Ld*PTR1. Circle: uninhibited enzyme; square: assay in the presence of methotrexate; triangle: assay in the presence of thianthrene.

Kinetics of *L. donovani* PTR1

Recombinant enzyme inhibition was performed to confirm the target specificity of thianthrene to *Ld*PTR1. The enzyme concentration was optimized at 0.34µM and the pH at 4.8 for *Ld*PTR1 activity (Kaur *et al.* 2010). PTR1 has a greater affinity for biopterin than dihydrobiopterin. *Km* and *Vmax* values were derived using the Lineweaver-Burk plot and were found to be 5.85±1.02µM and 0.13±0.05µmol/min/mg in the case of biopterin and 19.4±1.7µM and 2.8±0.5µmol/min/mg in the case of dihydrobiopterin, respectively (Singh *et al.* 2010). The properties of the recombinant *Ld*PTR1 were similar to native *Lm*PTR1 (Nare *et al.* 1997b). The latter has a 91% sequence identity to *Ld*PTR1 while the active and NADPH binding sites are highly conserved in these two distantly related species (Kumar *et al.* 2004). Using methotrexate, a known antifolate inhibitor of *Plasmodium falciparum* DHFR (*Pf*DHFR) (Shallom *et al.* 1999), the *Ki* value was found to be 1.2µM for *Ld*PTR1 against the biopterin substrate (Figure 5). The *K*i for methotrexate inhibition against the biopterin substrate reactions performed by *Ld*PTR1 did not significantly alter at pH 4.8 (Figure 5).

Overexpression of PTR1 could also contribute to relieving the inhibition of DHFR-TS, by increasing

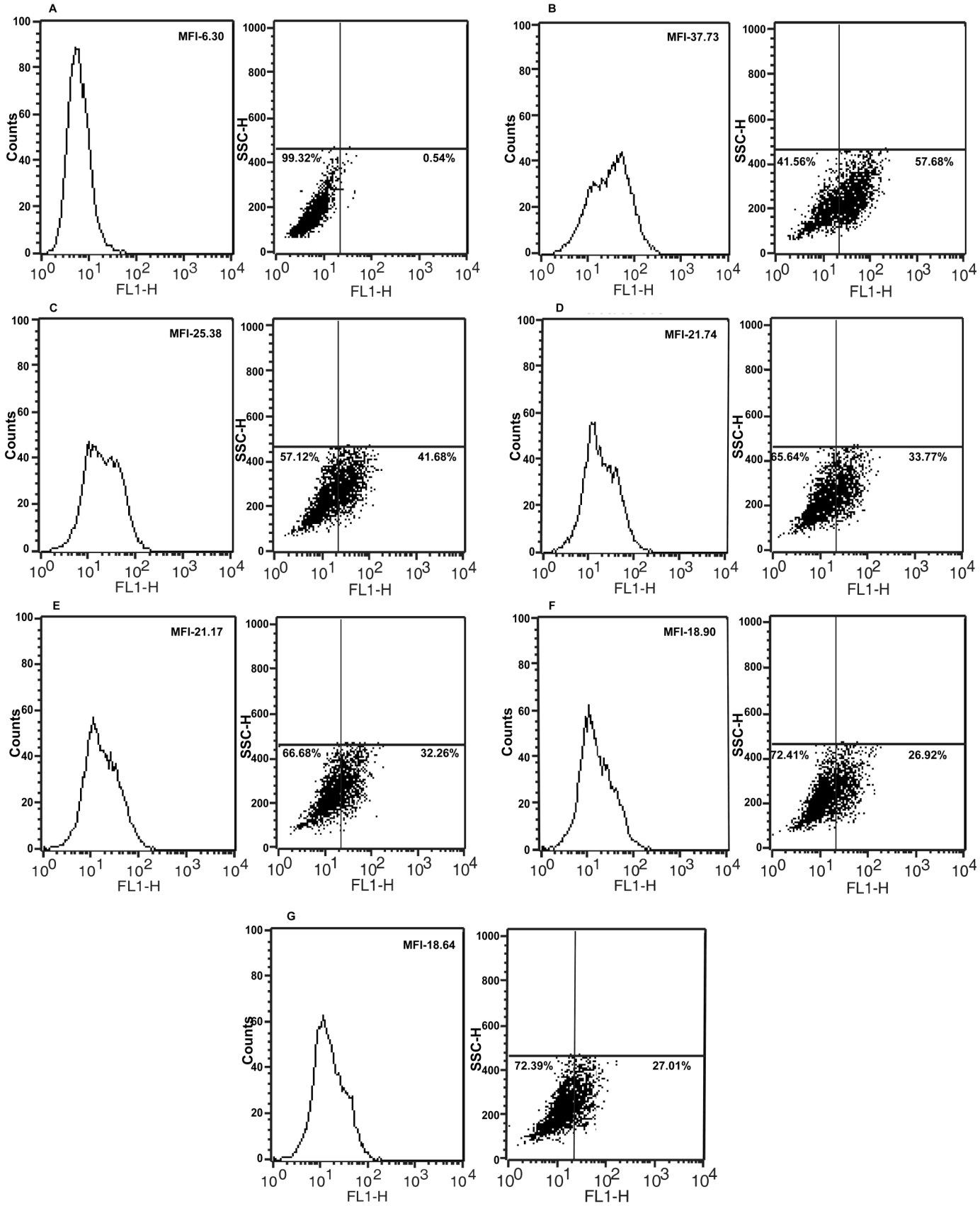


Figure 6. Representative histograms and dot plots of (A) Macrophages, (B) Macrophages infected with promastigotes expressing GFP (C-G) Infected macrophages with 2, 3, 4, 5 and 6 µg/mL of thianthrene, in that order.

the H_2folate pools indirectly through increased utilization of biopterin or directly by reduction of folate. In this manner, PTR1 provides a metabolic by-pass of DHFR-TS inhibition. The *Ki* value for *Ld*PTR1 was found to be 3 times less than *Lm*PTR1 (Cavazzuti *et al.* 2008). *Ld*PTR1 with the biopterin substrate exhibits uncompetitive mixed type of inhibition, indicating that thianthrene binds specifically to the PTR1 cofactor rather than the naked enzyme. Inhibition studies of *Ld*PTR1 with thianthrene showed a *Ki* value of 1.0μM (Figure 5). Despite being a far smaller molecule, thianthrene displays a similar inhibition constant to that of methotrexate.

In vitro efficacy of thianthrene against the *L. donovani* intracellular amastigotes

As the macrophage-amastigote model is considered as the gold standard (Singh & Dube 2004) for establishing the drug sensitivity profile of an antileishmanial compound, promastigotes expressing GFP were used to infect J774A.1 macrophage cells. Our *Leishmania* promastigote transfectants proliferated and were infective to macrophages resulting in fluorescent amastigotes, this way maintaining the characteristics of the parental wild-type. The infection rate of macrophages was measured by using the MFI of the FL1 histogram for the uninfected and infected cultures. As there is no fluorescence calibration data available for the macrophage population, the MFI was found to be equal to 6.30, according to the histogram shown in Figure 6. The maximum cell population (99.32%) is shown in the lower left (LL) quadrant of Figure 6. Furthermore, MFI was found to be 37.73 in the histogram of macrophages infected with GFP-expressing promastigotes. Cells (57.68%) from the LL quadrant were shifted to lower right (LR) quadrant. In addition, the MFI decreased from 25.38 to 21.74 to 21.17 to 18.90 and 18.64 at 2, 3, 4, 5 & 6μg/mL of thianthrene, respectively. In line with this, the number of cells from the LR quadrant decreased from 41.68% to 33.77% to 32.26% to 26.92% and 27.01% at 2, 3, 4, 5 & 6μg/mL of thianthrene, respectively (Figure 6).

Flow cytometry results indicated that the intracellular amastigotes of *L. donovani* were inhibited by thianthrene. The IC_{50} of thianthrene was found to be 23μM. The thianthrene was also checked against the J774A.1 cell line to determine whether the doses used for IC_{50} on intramacrophage amastigotes were toxic to the cells themselves. The experimental results indicated that the CC_{50} value was 2-3 times higher (87μM) than the IC_{50} dose (23μM) for intracellular amastigotes.

In conclusion, the antileishmanial activity of thianthrene was identified by structural modeling studies as well as cell and enzyme inhibition assays. Currently, microarray analysis on intracellular *Leishmania* treated with thianthrene is carried out in an effort to identify the genes that are differentially expressed in intracellular thianthrene-treated *Leishmania* cells.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by the Department of Biotechnology, New Delhi, India (Grant No. BT/PR5452/BRB/10/430/2004) and the Council of Scientific and Industrial Research [Grant No. CMM0017].

References

Barrack KL, Tulloch LB, Burke LA, Fyfe PK & Hunter WN 2010. Structure of recombinant *Leishmania donovani* pteridine reductase reveals a disordered active site. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **67** 33-37.

Bello AR, Nare B, Freedman D, Hardy L & Beverley SM 1994. PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major. Proc Natl Acad Sci USA* **91** 11442-11446.

Bohm HJ 1992. LUDI: rule-based automatic design of new substituents for enzyme inhibitor leads. *J Comput Aided Mol Des* **6** 593-606.

Carter KC, Henriquez FL, Campbell SA, Roberts CW, Nok A, Mullen AB & McFarlane E 2007. DNA vaccination against the parasite enzyme gamma-glutamylcysteine synthetase confers protection against *Leishmania donovani* infection. *Vaccine* **25** 4502-4509.

Cavazzuti A, Paglietti G, Hunter WN, Gamarro F, Piras S, Loriga M, Allecca S, Corona P, McLuskey K, Tulloch L, Gibellini F, Ferrari S & Costi MP 2008 Discovery of potent pteridine reductase inhibitors to guide antiparasite drug development. *Proc Natl Acad Sci U S A* **105** 1448-1453.

Gallivan JP & Dougherty DA 2000 A Computational Study of Cation−π Interactions vs Salt Bridges in Aqueous Media: Implications for Protein Engineering. *J Am Chem Soc* **122** 870-874.

Gourley DG, Luba J, Hardy LW, Beverley SM & Hunter WN 1999 Crystallization of recombinant *Leishmania major* pteridine reductase 1 (PTR1). *Acta Cryst D Biol Crystallogr* **55** 1608-1610.

Gourley DG, Schüttelkopf AW, Leonard GA, Luba J, Hardy LW, Beverley SM & Hunter WN 2001 Pteridine reductase mechanism correlates pterin metabolism with drug resistance in trypanosomatid parasites. *Nat Struct Biol* **8** 521-525.

Handman E 2001 Leishmaniasis: Current Status of Vaccine Development. *Clin Microbiol Rev* **14** 229-243.

Hardy LW, Matthews W, Nare B & Beverly SM 1997. Bio-

chemical and genetic tests for inhibitors of *Leishmania* pteridine pathways. *Exp Parasitol* **87** 157-169.

Kaur J, Sundar S & Singh N 2010 Molecular docking, structure-activity relationship and biologic evaluation of the anticancer drug monastrol as pteridine reductase inhibitor in *Leishmania donovani* clinical isolate. *J Antimicrob Chemother* **65** 1742-1748.

Kumar P, Kothari H & Singh N 2004 Overexpression in *Escherichia Coli* and purification of pteridine reductase 1 (PTR1) from a clinical isolate of *Leishmania donovani*. *Protein Expr Purif* **38** 228-236.

Laskowski RA, Rullmannn JA, MacArthur MW, Kaptein R, & Thornton JM 1996 AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J Biomol NMR* **8** 477-486.

Lüthy R, Bowie JU & Eisenberg D 1992 Assessment of protein models with three-dimensional profiles. *Nature* **356** 83-85.

Martí-Renom MA, Stuart AC, Fiser A, Sánchez R, Melo F & Sali A 2000 Comparative protein structure modeling of genes and genomes. *Annu Rev Biophys Biomol Struct* **29** 291-325.

McLuskey K, Gibellini F, Carvalho P, Avery MA & Hunter WN 2004 Inhibition of *Leishmania major* pteridine reductase by 2,4,6-triaminoquinazoline: structure of the NADPH ternary complex. *Acta Crystallogr Sect D Biol Crystallogr* **60** 1780-1785.

Nare B, Hardy L & Beverley SM 1997b The roles of pteridine reductase 1 and dihydrofolate reductase-thymidylate synthase in pteridine metabolism in the protozoan parasite *Leishmania major*. *J Biol Chem* **272** 13883-13891.

Nare B, Luba J, Hardy LW & Beverley SM 1997a New approaches to *Leishmania* chemotherapy: pteridine reductase 1 (PTR1) as a target and modulator of antifolate sensitivity. *Parasitology* **114** S101-110.

Perutz MF, Fermi G, Abraham DJ, Poyart C & Busaux E 1986 Hemoglobin as a receptor of drugs and peptides: x-ray studies of the stereochemistry of binding. *J Am Chem Soc* **108** 1064-1078.

Shallom S, Zhang K, Jiang L & Rathod PK 1999 Essential protein-protein interactions between *Plasmodium falciparum* thymidylate synthase and dihydrofolate reductase domains. *J Biol Chem* **274** 37781-37786.

Sienkiewicz N, Ong HB & Fairlamb AH 2010 *Trypanosoma brucei* pteridine reductase 1 is essential for survival *in vitro* and for virulence *in mice*. *Mol Microbiol* **77** 658-671.

Singh N & Dube A 2004 Fluorescent *Leishmania*: application to antileishmanial drug testing. *Am J Trop Med Hyg* **71** 400-402.

Singh N, Kumar P & Kaur J 2009 Pteridine reductase 1 as an antileishmanial drug target: Antifolate chemotherapy in leishmaniasis. VDM Verlag Dr. Müller.

Tulloch LB, Martini VP, Iulek J, Huggan JK, Lee JH, Gibson CL, Smith TK, Suckling CJ & Hunter WN 2010 Structure-Based Design of Pteridine Reductase Inhibitors Targeting African Sleeping Sickness and the Leishmaniases. *J Med Chem* **53** 221-229.

Wang J, Leblanc E, Chang CF, Papadopoulou B, Bray T, Whiteley JM, Lin SX & Ouellette M 1997 Pterin and folate reduction by the *Leishmania tarentolae* H locus short-chain dehydrogenase/reductase PTR1. *Arch Biochem Biophys* **342** 197-202.

Webster LT 1990 Goodman & Gilman's The Pharmacological Basis of Therapeutics, edn 8, pp 954-1017. Eds LL Brunton, BA Chabner, BC Knollmann. New York: Pergamon Press.

WHO 2012 Leishmaniasis Epidemics. In *Programmes and projects.* Retrieved from http://www.who.int/leishmaniasis/epidemic/en/