Structural insights into thioredoxin-2: a component of malaria parasite protein secretion machinery

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Thioredoxins are vital components of Plasmodium proteome and act as both reducing agents and protein disulfide reductases. The malaria parasite P. falciparum thioredoxin-2 (PfTrx-2) is part of the multi-protein complex embedded within the parasite parasitophorous vacuolar membrane (PVM) which purportedly directs protein secretion. We have characterized structural and enzymatic features of PfTrx-2, and we show that PfTrx-2 adopts a canonical thioredoxin fold but with significant structural differences in its N-terminus. Our confocal localization data suggest distinct PVM residency of PfTrx-2. Based on the crystal structure of PfTrx-2, we screened and tested small molecule drug-like libraries for compounds which target unique structural features of PfTrx-2. Disruption of PfTrx-2 interactions using specific inhibitors may result in a dysfunctional parasite translocon that is rendered unable to secrete pathogenic proteins into hosts. This approach therefore offers a new focus for anti-malarial drug development.

Malaria is one of the world's most important infectious diseases and is responsible for enormous mortality and morbidity1. The unicellular parasite Plasmodium falciparum causes most aggressive form of this disease, leading to severe malaria1. Malaria parasites are constantly exposed to high fluxes of toxic reactive oxygen species (ROS) and to counter this they have developed elaborate redox systems comprising of thioredoxins, peroxiredoxins, glutathiones and superoxide dismutases amongst others2. Maintenance of proper redox balance is therefore clearly vital for erythrocytic stages of Plasmodium, as has been demonstrated by malaria protective effects of glucose-6-phosphate deficiency3. A tight control on parasitic redox balance is also important due to the different intra- and extracellular environments the parasite is exposed to. Oxidative stress is induced within the parasite due to its high metabolic and replicative rates, due to the constant reliance on host haemoglobin digestion, and due to ROS produced by the host2,4–6. Plasmodium apparently lacks some important components of redox systems viz., catalase and a genuine glutathione peroxidase5,7. It was recently shown that the parasite imports human peroxiredoxin-2 for peroxide detoxification7–11. These unique features of Plasmodium redox biology can provide valuable targets for development of specific anti-malarials.

Plasmodium falciparum contains three thioredoxins - Trx1, Trx2, Trx3 and two thioredoxin-like proteins (Tlp1 and Tlp2)12–13. Trx1 and Tlp1 are cytosolic, Trx3 is localized to the endoplasmic reticulum, while Tlp2 is mitochondrial13. Thioredoxin 2 (PfTrx-2 from hereon, MAL13P1.225), the focus of this present work, is a 19 kDa protein with an ER signal sequence. Significantly, PfTrx-2 was recently shown to be part of the so-called translocon complex (PTEX) which is a parasite derived multi-protein machinery resident in parasite parasitophorous vacuolar membrane (PVM), responsible for protein secretion into host cells14. Plasmodium parasite remodels the red blood cells by exporting, 5% of its encoded genome (~300 proteins)15–18. These exported proteins are critical for parasite survival in host cells as they play important roles in nutrient uptake, immune evasion and virulence associated functions of the parasite19–22. Protein translocation is a complex process as it involves crossing three membranes viz. endoplasmic reticulum, parasite plasma membrane and parasitophorous vacuolar membrane. A hydrophobic signal sequence at the N-terminus of proteins is usually sufficient to target them to the ER and subsequent secretion to the parasitophorous vacuole. However, how proteins are transported out of PVM to the host cytoplasm is not fully understood23–25. The situation is even more complex for proteins which are destined for red blood cell plasma membrane. A pentapeptide motif (RxLxE) named Plasmodium export element (PEXEL) has been identified in proteins secreted out of the parasite to the host cell14. PEXEL motif is present in both the proteins lacking a signal peptide sequence such as PIEMP1 family as well as those having one like Stevor family proteins25. However, many proteins lacking a PEXEL motif are also secreted out by the parasite
into the host cell by some unknown mechanism. PEXEL motif is a protease recognition site, cleaved by Plasmapsin V in the ER. It is proposed that Plasmapsin V is more than a signal peptidase and it targets PEXEL containing proteins to the PTEX translocon by channeling the cleaved proteins to HSP101 chaperon. It is proposed that Plasmapsin V is more than a signal peptidase and it targets PEXEL containing proteins to the PTEX translocon by channeling the cleaved proteins to HSP101 chaperon. It is further hypothesized that at the PTEX proteins are translocated through a membrane pore formed by EXP2 protein. Other components of PTEX include PTEX150, PTEX88 and HSP101 which together enable secretion of parasite proteins that contain the PEXEL motif.

PfTrx-2 is a component of the PTEX, where it likely plays role of a protease disulfide isomerase, assisting in protein unfolding before translocation. PfTrx-2 was previously shown to be mitochondria localized, but recent studies have shown it to be PVM localized as well as to an organelle of unknown identity, again based on GFP-fusion constructs.

Here, we present crystal structure of Plasmodium falciparum Trx2 (PfTrx-2) at 2.9 Å resolution. Evidently, there is only partial conservation in the PfTrx-2 active site residues, and PfTrx-2 structure reveals several unique features when compared to its human counterpart (HuTrx-2). We also show that disabling PfTrx-2 function by small molecule inhibitors is likely to block or reduce processing/export of hundreds of parasite proteins. This may be a novel strategy to combat parasite growth.

**Results**

**PfTrx-2 expression, purification, antibody generation and localization.** Recombinant PfTrx-2 was very prone to precipitation, and to reduce protein precipitation buffers were maintained in 5 mM DTT while precipitates were pelleted out. The remaining PfTrx-2 in supernatants was found to be fully active and was used for all biochemical and crystallization experiments. PfTrx-2 was previously shown to be localized to the parasite mitochondrion using GFP-PfTrx2 constructs. More recently, it was shown to be resident in the parasite PVM and to an unknown parasite organelle. We generated antibodies against recombinant PfTrx-2 in rabbit to verify localization of PfTrx-2 at different stages of parasite life cycle. We confirm that PfTrx-2 is localized to the PVM (only) in different asexual stages of Plasmodium (Fig. 2, supplementary movies 1, 2 and 3).

**Crystallization and crystal structure of PfTrx-2.** Hexagonal-shaped crystals were obtained using hanging drop vapor diffusion method by incubating 1 μl of protein with 1 μl of buffer containing 2.2 M ammonium sulfate and 0.125 mM bis-tris (pH 5.5) at 20°C over a period of ~5 days. The crystals belong to space group P321 with two monomers of PfTrx-2 in the asymmetric unit and a solvent content of ~71%. Attempts to solve the crystal structure of PfTrx-2 by molecular replacement failed and PfTrx-2 was solved by MIRAS techniques using native data along with platinum and mercury derivatives. Overall structure of PfTrx-2 consists of four β strands (β1, β2, β3 and β4), three α helices (α1, α2 and α3) and an extended N-terminus named L1 (residues 56–71) (Fig. 3a and 3b). In PfTrx-2 crystal structure, 25 residues (amino acid numbers 29–53) are disordered as no clear electron density was obvious for them in either 2Fo-Fc or Fo-Fc maps. No clear side chain density was observed for residues from 54 to 68. The overall fold of PfTrx-2 is similar to those of other thioredoxins except for the L1 segment. This extended coil region in PfTrx-2 contacts the other monomer of PfTrx-2 in asymmetric unit (Fig. 3a and 3b). The resulting monomer - monomer contacts are not extensive (total surface area per PfTrx-2 is ~5100 Å²; buried surface area per PfTrx-2 ~134.5

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**Figure 1 | PfTrx-2 activity and structure.** (a) Insulin reduction assay of PfTrx-2 activity. (b) A cartoon representation of the crystal structure of PfTrx-2. α helices are colored magenta, β sheets are colored yellow while β turns and loop regions are colored blue. Active site cystines are shown as sticks at the end of α helix. (c) A domain diagram of PfTrx-2 representing the structural elements identified in the crystal structure. The color coding is the same as in (b). Residues 1–24 forms the signal sequence region, amino-acids 24–53 are disordered while residues 54–157 form the thioredoxin fold.
oxidized PfTrx-2. As expected, PfTrx-2 Cys 85 (pKa state; despite repeated efforts we were unable to obtain crystals of absent. We therefore conclude that PfTrx-2 structure is in a reduced inside the protein while Cys 82 (pKa, sulfurs in disulfide bridges are in chains respectively (Fig. 3c and 3d)).

Comparison with human mitochondrial thioredoxin. Reduced form of human mitochondrial thioredoxin (PDB code 1UVZ) was superimposed on PfTrx-2 using Chimera (Fig. 3a and 3b). The active site residues of PfTrx-2 (WCQAC) are present at beginning of α1 helix (Fig. 3c). Both molecules of PfTrx-2 are in reduced form with distances between S\(^{-}\) atoms of Cys 82 and Cys 85 of ~3.7 Å and 3.9 Å for A and B chains respectively (Fig. 3c and 3d). Typical distances between sulfurs in disulfide bridges are in ~2 Å range, and a distance of ~3.6 Å represents the minimum distance when a disulfide bridge is absent. We therefore conclude that PfTrx-2 structure is in a reduced state; despite repeated efforts we were unable to obtain crystals of oxidized PfTrx-2. As expected, PfTrx-2 Cys 85 (pKa ~11) is buried inside the protein while Cys 82 (pKa ~8) is exposed in the loop region at α1 helix terminus. Cys 82 is the active cysteine that likely makes the first nucleophilic attack on substrate disulfides leading to formation intermolecular disulfide bond. This link between PfTrx-2 and its substrate is then likely attacked by Cys 85 which acts as the nucleophile and releases the substrate. The active site Trp 81 is stabilized by an intramolecular hydrogen bond with Asp 111 (Fig. 3d) and is solvent exposed.

Surface features of PfTrx-2. The electrostatic surface of PfTrx-2 seems positively charged in contrast to the other thioredoxins (including HuTrx-2) that display negatively charged surfaces (Fig. 5a and 5b). Binding of HuTrx-2 with electron acceptor proteins is predicted to involve an acidic cavity near the active site on HuTrx-2 surface – complemented by a basic protuberance due to Lys49 of electron acceptor protein hPDX31–34. Residues in this region are conserved in both human and Plasmodium Trx-2 (highlighted in the former results in ~18 Å and ~80° variation relative to HuTrx-2, exposes PfTrx-2 β strands beneath (Fig. 4a) and creates a groove on the surface of PfTrx-2 (Fig. 4c).

PfTrx-2 Activity inhibition by Aurothiomalate (ATM). ATM is known to inhibit the thioredoxin system. Although, ATM was tested for treatment of malaria, its mechanism of action is only partially explained by stimulation of eryptosis. Moreover, oxidative stress is known to stimulate eryptosis. Therefore, eryptosis might be an indirect outcome of the action of ATM on thioredoxin system leading to oxidative stress. To investigate this hypothesis, we tested
ATM for inhibition of PfTrx-2 activity in an insulin based assay. ATM was able to inhibit the PfTrx-2 activity in a dose dependent manner with an IC\textsubscript{50} of \(20\, \mu\text{M}\) (Fig. 6a).

In-silico docking of drug-like libraries on PfTrx-2 crystal structure. PfTrx-2 structure shows many unique features which seem useful for selective targeting by drug like compounds. Three regions on the protein surface were selectively targeted - Region 1, Region 2 and Region 3 (Fig. 7a). Region 1 is the area on the protein surface surrounding the active site. We selected this region as residues in the active site are only partially conserved between humans and \textit{Plasmodium} (Pf: CQAC, Hu: CGPC). Region 2 is the extended L1 region unique to PfTrx-2, while region 3 is the region on one side of the L1 region (Fig. 7a). Interestingly, top hits docked mostly to L1 (region 2) with a few compounds docking to regions 1 and 3. We tested top 29 inhibitors which together docked to all three regions on PfTrx-2 surface in parasite viability assays (Fig. 6b). Of the 29, four inhibitors were able to inhibit parasite growth in micromolar ranges and gave reasonable values for IC\textsubscript{50} (Fig. 6c).

Inhibitor 18 and 20 docked (Fig. 7a and 7d). Despite having different skeletal types (the SPECS diversity set library was used), these inhibitors seem to make similar interactions with residues on surface of PfTrx-2. They therefore can serve as chemical starting points for development of more specific and potent Trx2 inhibitors.

Table 1 | X-Ray Data Processing, Structure Solution and Refinement Statistics Values in the parentheses are for the highest resolution shell.

| Data collection | Native | Pt-Derivative | Hg-Derivative |
|----------------|--------|---------------|---------------|
| Wavelength (Å) | 1.07   | 1.07          | 1.07          |
| Space group    | P3\(_2\) 21 | P3\(_2\) 21 | P3\(_2\) 21 |
| a, b, c (Å)    | 86.61, 86.61, 90.86 | 86.71, 86.71, 90.14 | 87.05, 87.05, 90.9 |
| α, β, γ        | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 |
| Resolution (Å) | 100–2.9 (3.00–2.90) | 50.0–3.5 (3.63–3.50) | 100–3.2 (3.31–3.20) |
| R\textsubscript{merge} | 0.040(0.36) | 0.083(0.569) | 0.064(0.463) |
| I/σ(I)         | 29.23(2.97) | 30.89(5.0) | 66.05(6.87) |
| Completeness (%)| 98.2(99.1) | 100(100) | 99.9(100) |
| Redundancy     | 3.7(3.7) | 14.4(14.9) | 21.6(20.6) |
| Unique reflections | 8947(877) | 5222(511) | 6929(672) |
| AutoSol        | 4358 | 5877 |
| Bijvoet pairs  | 0.0661 | 0.0388 |
| Mean anomalous signal (\(<\Delta F>/<\sigma F>\)) | 0.06 | 0.06 |
| SKEW           | 0.89 | 0.89 |
| CORR_RMS       | 37.8 +/− 33.3 | 37.8 +/− 33.3 |
| Bayesian CC     | 0.53 | 0.53 |
| FOM            | 3 | 4 |
| Sites used for phasing | 3 | 4 |
| Refined sites  | 3 | 4 |
| AutoBuild      | 178 |
| Residues built | 178 |
| Side chains placed | 27 |
| R\textsubscript{work}/R\textsubscript{free} | 0.34/0.41 |
| Model Map CC   | 0.71 |
| Refined        | 39.26 – 2.90 |
| Resolution (Å) | 8635 |
| No. reflections | 0.25/0.29 |
| Number of atoms | Protein 1599 |
|                | Water 33 |
|                | Rmsd |
|                | Bond 0.02 |
|                | Angle 1.68 |
|                | B-factors |
|                | Protein 90.89 |
|                | Water 58.73 |

Discussion

Malaria parasites have evolved clever strategies for survival in multiple hosts. One such facet to malaria parasites is their ability to extensively remodel host cells by exporting numerous parasite proteins\textsuperscript{15–18}. PfTrx-2 is a component of the translocon of exported proteins (PTEX), located on the parasite PVM where it purportedly helps in protein folding/unfolding before substrate secretion. We recombinantly expressed PfTrx-2 in \textit{E. coli} and purified the protein to homogeneity by conventional chromatographic techniques. Protein activity was tested by an insulin reduction assay which suggested enzymatically competent PfTrx-2 (Fig. 1a). In agreement with earlier studies, we confirmed PVM localization of PfTrx-2 using polyclonal antibodies specifically generated against recombinant PfTrx-2 (Fig. 6b). We could not confirm localization of PfTrx-2 to any organelle of unknown identity inside the parasite as has been reported earlier\textsuperscript{13}. Confocal imagery-based movies which show distinct localization of PfTrx-2 in the erythrocytic stages accompany this manuscript (supplementary data).
Aurothiomalate is a known inhibitor of thioredoxin reductase in rat and is used for the treatment of rheumatoid arthritis and cancer. Interestingly, aurothiomalate also reduces *Plasmodium* parasitemia both *in vitro* and in infected mice. However, the mechanism of anti-parasitic action for aurothiomalate could only be partially explained—entailing stimulation of eryptosis in infected RBCs.

Figure 3 | Two molecules of PfTrx-2 in the crystallographic asymmetric unit. (a) A surface representation of PfTrx-2. (b) A cartoon representation showing L1 regions of the two PfTrx-2 contacting each other. (c) Active site of PfTrx-2 showing two cysteines in a reduced state and (d) Trp 81 interacts with the Asp 111 through a hydrogen bond.

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Figure 4 | Comparision of human and *P. falciparum* thioredoxin structures. (a) A structural superposition of PfTrx-2 (green) and HuTrx-2 (magenta). L1 region is displaced by ~18 Å and ~80 degrees in PfTrx-2. (b) A structure based sequence alignment of PfTrx-2 and HuTrx-2. (c) Electrostatic surface representation of PfTrx-2. A basic groove (marked by black arrow) is evident on the surface of PfTrx-2 where acidic regions of PTEX proteins can dock.
Here, we show that aurothiomalate inhibits PfTrx-2 activity in a dose dependent manner with an IC$_{50}$ of $\sim20$ μM (Fig. 6a). The growth inhibitory effects of aurothiomalate might therefore be due to blockage of protein secretion via the translocon where PfTrx-2 resides. Besides three thioredoxins and two thioredoxin-like proteins, *Plasmodium falciparum* additionally encodes a thioredoxin reductase.

Figure 5 | Comparison of surface electrostatics of (a) PfTrx-2 and (b) HuTrx-2. Each view is rotated by 180 degrees with respect to the previous view.

Figure 6 | PfTrx-2 inhibition assays. (a) Aurothiomalate inhibition assay. (b) Parasite growth inhibition assay (PIA) performed using 100 μM of different inhibitors. (c) PIA performed with four best inhibitors selected from (b) using a concentration range from 0.001 μM to 10000 μM.
which shows a dual localization to both cytoplasm and mitochondrion of the Plasmodium parasites\(^1\). One or several of these parasite proteins could therefore be the target of aurothiomalate action.

Structural analysis of PfTrx-2 highlights unique features in the Plasmodium enzyme when compared to its human counterpart (Fig. 1b). The N-terminal α helix and β strand in HuTrx-2 are replaced by an extended coil (L1 region) in PfTrx-2 structure. L1 is stabilized by interactions with another PfTrx-2 protomer in the crystal asymmetric unit. This L1 region of PfTrx-2 is largely basic in nature, and could potentially form interaction interfaces for the numerous acidic regions in PTEX150 and EXP2 (Fig. 4c). The strikingly different electrostatic surface properties of PfTrx-2 and HuTrx-2 suggest different modes of interaction, with possibly different protein partners (Fig. 5). Opposite locations for PfTrx-2 active site and its L1 region suggests that the spatial segregation may be biologically relevant. For example, one outcome of distal locations of PfTrx2 active site and L1 region may be to allow simultaneous engagement of PfTrx2 with PTEX components on one side and substrates on the other side. This unique architectural feature therefore presents opportunities for targeting PfTrx-2 with small molecular inhibitors directed against one or both sites. Our crystallographic analysis therefore supports targeting PfTrx-2 for structure guided drug design. Interestingly, our in silico docking studies with drug-like libraries show that most of the top docking poses localize within the L1 region of PfTrx-2 (region 2 in Fig. 6b, Fig. 7a). In summary, our structural-functional and inhibitor discovery data together provide a platform for development of more potent PfTrx-2 inhibitors that can impede protein export from malaria parasites by interfering with the interaction interfaces of PfTrx-2.

**Methods**

Molecular cloning, Expression and Purification. PfTrx-2 gene contains a predicted ER signal sequence at the N-terminus. Gene construct of PfTrx-2, without its signal sequence from amino acids 28–157, was designed for optimal expression in E. coli. The construct was sub-cloned in pGEX4T1 vector with BamH1 and Sal1 restriction sites and protein was expressed as a GST fusion. For overproduction of the fused protein, Escherichia coli Lemo21 DE3 competent cells were freshly transformed, and a single colony was grown in 10 ml of LB media containing 100 μg/ml ampicillin. After 12 hours of growth, this seed culture was used to inoculate 1 liter of LB media that was grown for 3.5 hours at 37 °C. Protein expression was induced by adding 0.25 mM isopropyl β-D-thiogalactopyranoside, and the culture was grown for 20 hours post-induction at 16 °C. Cells were harvested by centrifugation at 5000 g for 15 minutes. The resulting bacterial pellet was suspended in a buffer containing 1XPBS, 5 mM DTT and protease inhibitor cocktail (Roche). Cells were lysed by sonication and cleared by centrifugation at 20,000 X g for 45 minutes. The cleared supernatant was applied to 5 ml GST FF column (GE Biosciences). The GST-Trx-2 fusion protein was purified by elution with a buffer containing 1XPBS, 5 mM DTT and 20 mM reduced glutathione on AKTA automated protein purifier (GE Biosciences). Best fractions were checked by SDS-PAGE and those containing pure PfTrx-2 were pooled and buffer-exchanged in 20 mM Tris containing 5 mM DTT using a desalting column and the GST tag was removed by incubating with thrombin protease in 1XPBS (GE Biosciences) at 20 °C overnight. The cleaved protein was concentrated by using 3 KDa cutoff centricron centrifugal device (Millipore) and purified by gel filtration chromatography on S75 gel-filtration column (GE Biosciences). Best fractions were checked by SDS-PAGE and those containing pure PfTrx-2 were pooled and buffer-exchanged in 20 mM Tris pH 8.0, 20 mM NaCl and 10 mM DTT. The protein was concentrated to 5 mg/ml using a 3 KDa cutoff cenricron centrifugal device (Millipore) and stored at −80 °C.

Immunofluorescence assays using confocal microscopy. Anti-PfTrx-2 antibodies raised in a rabbit were used in immunofluorescence assays for localization of native PfTrx-2 within malaria parasites. The assay was performed as described earlier\(^4\). Cells were washed in PBS and fixed in solution using 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 25 minutes. After a PBS wash, cells were permeabilized by using 0.1% Triton X-100 in PBS for 10 minutes. After another PBS wash, cells were treated with 0.1 mg/ml sodium borohydride in PBS for 10 minutes. Cells were washed again with PBS, blocked in 3% BSA/PBS for 1 hour and incubated overnight with protein-A column purified rabbit anti-protein IgG antibody (1:250 dilution) at 4 °C. Cells were washed three times with PBS for 10 minutes each and incubated with Alexa488-tagged anti-rabbit secondary antibody for 1 hour at room temperature and allowed to settle onto coverslips coated with poly-L-lysine (100 mg/ml). Finally, the coverslips were washed three times in PBS, mounted in antifade with DAPI reagent (Invitrogen) and sealed. Confocal microscopy was performed on a Nikon eclipse TE2000U microscope. Pre-immune sera were taken as negative controls.

Crystallization, Data Collection and Structure Determination. PfTrx-2 crystals were obtained at 20 °C by hanging drop vapor diffusion method using 1 μl of PfTrx-2 (5 mg/ml) and 1 μl of 0.125 M Bis-tris pH 5.5, 2.2 M ammonium sulfate and 5 mM DTT. Single, hexagonal shaped crystals were added to a cryoprotectant solution containing 0.125 mM Bis-tris pH 5.5, 3.0 M ammonium sulfate and 20% glycerol for 1 minute before flash freezing in cooled nitrogen gas at 100 K. For phasing, crystals were soaked in 100 mM mercury (II) potassium iodide in cryoprotectant solution.
before flash freezing. For Pt derivatives, crystals were soaked in 100 mM D-ribois(ethylenediamine) diplatinum (II) nitrate in cryoprotectant solution before flash freezing. X-ray diffraction data were collected on a MARCCD detector at BM14 beam line of European Synchrotron Radiation Facility at Grenoble, France. Pt(II) and Hg(II) derivative datasets were collected along with native data in frozen conditions. The diffraction images were processed and scaled with HKL2000 suite program. Four Hg and two Pt complexes were found by AutoSol in PHENIX and by SHELXE++. PtTrx-2 crystals are trigonal and belong to the space group $P3_121$ (Table 1). The structure was solved by MIAS procedures using native and derivative datasets in PHENIX. Initial model was build by AutoBuild in PHENIX, which was subsequently rebuilt manually using COOT. Model refinement was performed using phenix.refine in PHENIX.

Activity assay and Inhibition of PTTrx-2 activity. Enzymatic activity of recombinant PTTrx-2 was determined by insulin inhibition assay. Briefly, 2.0 - 8.0 μg of recombinant PTTrx-2 was used in 1 ml of 0.1 M potassium phosphate (pH 7.0), 2 mM EDTA, 0.1% bovine insulin, and 1 mM DTT. The same buffer without DTT was used as a control. Protein concentration was determined by the Bio-Rad protein assay kit with BSA as standard. Thioredoxin activity was measured at 25°C and defined as the maximal increase rate of turbidity at O.D. at 650 nm due to inhibition of protein precipitation. For inhibition of activity assay, varying amounts of inhibitors were added in the reaction mixtures and decrease in turbidity at O.D. 650 nm due to inhibition of insulin precipitation was measured.

In silico screening of PTTrx-2 inhibitors. Molecular docking and in silico screening were carried using PTTrx-2 structure in docking program AutoDock vina with a SPECS ‘Species world diversity’ library. The library was prepared using Ligprep module in Schrodinger software suite to ensure proper partial charges and protonation states. Physicochemical properties were predicted using Qikprop module in Schrodinger suite. For in silico screening, we applied 10 lead-like filters derived from Lipinski’s rule of five: 1< QPlogP< 4; 100 < molecular weight < 350; H donor < 5; H acceptor < 10; rotatable bounds < 15; 5 < φ < 90; 100 A < θ < 120; A < FISA < 300 A, FISA < FOQA. After an initial lead-like filtering process, 900 compounds were collated for screening. The top docking poses were isolated from each output file for further analysis. Top ligands were selected based on docking scores and H-bond interactions with residues in PTTrx-2.

Parasite Growth Inhibition Assays. Top inhibitors selected via above procedure were tested on malaria parasite P. falciparum D7 strain. Parasites were cultured using sorbitol and starting with ring stages these parasites were cultured in 96 well plates at 1% parasitemia and 2% hematocrit. Initial screening of all top compounds was performed by growing parasites at 100 μM inhibitor concentration. After 48 hours of growth, parasitemia was measured by fluorescence assay as described earlier. Briefly, culture was lysed by adding 100 μl of lysis buffer [Tris (20 mM, pH 7.5), EDTA (5 mM), saponin (0.008%, w/v) and Triton X-100:0.08%, w/v], SYBR green I was added to each well (0.2 μl of SYBR Green in 1 ml of lysis buffer), and mixed until no erythrocyte sediments were visible. After 1 hour incubation in dark at room temperature, fluorescence was measured with multi-well plate reader (Victor3, Perkin Elmer) at excitation and emission wavelength centered at 485 nm and 530 nm, respectively. To access the effect of selected inhibitors, fluorescence readings were compared and subtracted from fluorescence values at 100% parasite inhibition - fluorescence value at 100 μM chloroquine was taken as 100% parasite growth. We then calculated percentage parasitemia for each inhibitor. To further validate the inhibitory activity of top compounds from this first screen, experiments were repeated with a broader range of inhibitor concentrations ranging from 1 mM to 1 mM. Each experiment was repeated three times and each value is average of four measurements. These data were used to calculate IC50 values for each inhibitor.

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Author contributions
Ashwani Sharma purified, crystallized and solved PfTrx-2 structure. Sameer Dixit assisted with protein work while Arvind Sharma performed confocal studies and parasite growth assays. Ashwani Sharma and Amit Sharma conceived the study, designed all experiments and wrote the manuscript.

Additional information
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