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Discovery and Biochemical Characterization of *Plasmodium* Thioredoxin Reductase Inhibitors from an Antimalarial Set

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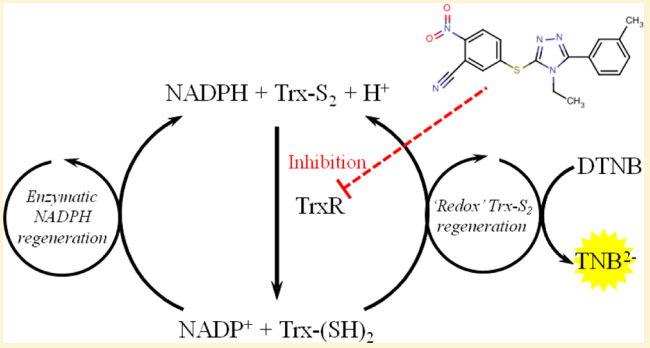
Supporting Information

ABSTRACT: *Plasmodium falciparum* is the most prevalent and deadly species of the human malaria parasites, and thioredoxin reductase (TrxR) is an enzyme involved in the redox response to oxidative stress. Essential for *P. falciparum* survival, the enzyme has been highlighted as a promising target for novel antimalarial drugs. Here we report the discovery and characterization of seven molecules from an antimalarial set of 13533 compounds through single-target TrxR biochemical screens. We have produced high-purity, full-length, recombinant native enzyme from four *Plasmodium* species, and thioredoxin substrates from *P. falciparum* and *Rattus norvegicus*. The enzymes were screened using a unique, high-throughput, in vitro native substrate assay, and we have observed selectivity between the *Plasmodium* species and the mammalian form of the enzyme. This has indicated differences in their biomolecular profiles and has provided valuable insights into the biochemical mechanisms of action of compounds with proven antimalarial activity.



Malaria is a major threat to human health, with approximately 215 million cases in 2010.[1] A recent study has shown the disease to be the underlying cause of death in 1.24 million patients in 2010, which is considerably higher than previous estimates.[2] Responsible for approximately 90% of the mortality, *Plasmodium falciparum* is the most prevalent and deadly form of the causative human parasite, whereas *Plasmodium vivax* represents the species with the greatest morbidity.[3] Pathogenic strains are able to infect only the human host. There are significant challenges to studying *P. falciparum* and especially *P. vivax* in vivo,[4] so strains such as rodent *Plasmodium berghei* and *Plasmodium yoelii* are the standard animal models used for antimalarial drug discovery.[5]

The erythrocytic stage of the *Plasmodium* life cycle is responsible for the pathology of malaria in humans and is the target of most antimalarials currently in use. During this phase, the parasite conducts an extensive degradation of erythrocyte hemoglobin and is particularly vulnerable to attack by reactive oxygen species. One of the parasite's primary defenses against this oxidative stress is the enzyme thioredoxin reductase (TrxR).[6−10] TrxR provides protection by reducing thioredoxin (Trx) from its inactive disulfide state to an active dithiol form, which acts as a donor of electrons to a wide range of molecules.[8,11−14] TrxR is essential in *P. falciparum* as demonstrated in knockout studies, where viable parasites harboring a disrupted *trxR* gene cannot be generated unless an additional wild copy of the gene is present.[15]

Protective antioxidant/redox systems are enhanced during the erythrocytic stage, and disruption of these has been proposed as a potential method of inhibiting parasitic growth to halt progression of the disease.[6,7,15] The active center of mammalian TrxR contains selenocysteine (Sec), which is absent in *P. falciparum* TrxR, providing an attractive target for selectivity and rational drug design.[6,8,9,12,14,16,17]

Drug resistance in *P. falciparum* is rapidly increasing, even against the latest front line antimalarials, and this spread of resistance may soon compromise current treatments.[18] The efficacy of the artemisinin combination therapies (ACTs) is beginning to be curtailed by the emergence of resistance to the endoperoxide component of the combination.[19,20] Challenges, both scientific and financial, in the discovery of new antimalarial drugs have resulted in a 15 year gap since the last major breakthrough.[21] Recently, thousands of new molecules with

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proven activity against *P. falciparum* have been identified, and the details have been publicly released.[22−24] These compounds, identified through whole cell screens, can be a valuable source of new chemical starting points for antimalarial lead identification. Indeed, phenotypic screening has recently led to the discovery of one of the most promising novel antimalarial classes, the spiroindolones.[25] Inhibitors identified using this methodology may act on more than one molecular target, resulting in favorable clinical profiles, and may exhibit reduced susceptibility to target-associated resistance in contrast to classical enzyme inhibitors that interdict single targets or a single point in a cellular pathway.[26,27]

However, the modes of action of these molecules are largely unknown, and understanding their mechanisms of action is key, particularly when validating and prioritizing scaffolds for chemical optimization and progression toward clinical development.[28] Single-target screens remain one of the most accurate ways to identify the mechanism of action. Ensuring the phenotypic activity properties of inhibitors before using them as chemical starting points to elucidate the mode of action in a specific biochemical assay represents a significant shift in the modern drug discovery paradigm.

As an example of this model, we have generated a toolbox of novel, recombinant reagents and assays to explore inhibition of TrxR as the potential mechanism of action of one of the subsets of compounds recently identified through phenotypic screening, the Tres Cantos antimalarial set (TCAMS), which comprises 13533 molecules that are highly potent growth inhibitors of the *P. falciparum* parasite.[22,29] We report the results of these studies here and are making these unique resources available to the malarial community as a continued open source approach, so that they may be used to realize progress in antimalarial drug discovery in the future.

EXPERIMENTAL PROCEDURES

Unless stated otherwise, all Gateway cloning reagents, vectors, and *Escherichia coli* cells were purchased from Invitrogen, synthetic genes were produced by GeneArt, KOD DNA polymerase was purchased from Novagen, Turbo Broth was purchased from Stratech Scientific Ltd., isopropyl β-D-thiogalactosidase (IPTG) was purchased from Calbiochem, Protease Inhibitor Cocktail Set III EDTA-Free (PIC), used at a 1:1000 dilution, was purchased from Merck Biosciences, and all other reagents were purchased from Sigma-Aldrich. Purification columns and media were purchased from GE Healthcare. The final expression vectors were sequenced to verify the integrity of the inserts. The purities of the final purified proteins were determined by gel densitometry scanning (SynGene, GeneTools).

Cloning and Expression. The gene sequences for TrxRs from *P. falciparum*, *P. vivax*, *P. berghei*, and *P. yoelii* and Trx from *P. falciparum* and *Rattus norvegicus* were codon optimized and synthesized for expression in *E. coli* (Figures 1−6 of the Supporting Information). The sequence for TrxR from *P. falciparum* was amplified by polymerase chain reaction (PCR) using primers 1 (ggggacaagtttgtacaaaaaagcaggcttcgaaggagatagaaccATGTGCAAAGATAAAAACG) and 2 (ggggaccactttgtacaagaaagctgggtcTTAGCCGCATTTTCCGCCACC) to generate the shorter 1626 bp isoform. *P. vivax* TrxR was amplified using primers 3 (ggggacaagtttgtacaaaaaagcaggctccgaaggagatagaaccATGAGCGGCACCGAAAGCGG) and 4 (ggggaccactttgtacaagaaagctgggttTTAACCACATTTACCACCACC), and *P. falciparum* Trx was amplified by PCR using primers 5 (ggggacaagtttgtacaaaaaagcaggcttcgaaggagatagaaccATGGTGAAAATTGTGACCAGCC) and 6 (ggggaccactttgtacaagaaagctgggtcTTACGCCGCATAAATTTCAATCAGC). Gateway *att*B sites are underlined, and the gene sequence is shown in uppercase. These PCR products were inserted into pDONR221 by Gateway BP Clonase II reactions. The gene sequences for TrxRs from *P. berghei* and *P. yoelii* and Trx from *R. norvegicus* were synthesized directly into the pDONR221 vector. All genes were shuttled into pDEST14 by Gateway LR Clonase II reactions to produce plasmids suitable for protein expression. The vectors were transformed into *E. coli* BL21 Star (DE3), and cells were grown at 37 °C in either LB medium (*P. vivax* TrxR and *P. falciparum* Trx) or Turbo broth, supplemented with either 1% (v/v) glucose (*P. falciparum* and *P. berghei* TrxR) or glycerol to an A_{600} between 0.9 and 3.4 before induction with 0.5 mM IPTG. The temperature of the cultures was reduced to either 18 °C (*P. falciparum* and *P. berghei* TrxR and *R. norvegicus* Trx) or 25 °C for 20 h before they were harvested.

Purification of TrxR Enzymes. Cell pastes were lysed by sonication in buffer A for TrxR from *P. falciparum* and *P. vivax* [50 mM Tris, 50 mM NaCl, PIC, 0.1% (w/v) lysozyme, and 1 unit/mL benzonase (pH 6.5)], buffer B for *P. berghei* TrxR [25 mM Tris, 25 mM NaCl, and PIC (pH 9.0)], or buffer C for *P. yoelli* TrxR [25 mM Tris and PIC (pH 7.0)] followed by centrifugation at 108000*g*. Clarified supernatants were loaded onto HiTrap Blue HP columns, washed to baseline with the appropriate buffer, and eluted with buffer containing 1 M NaCl. The HiTrap Blue nonadsorbed fraction for *P. vivax* TrxR was diluted with buffer C (pH 9.0) to a conductivity of 4.5 mS and further purified using a 1 mL Resource Q anion exchange column. The partially purified preparations of each TrxR enzyme were passed down a Superdex 200 prep grade size exclusion column equilibrated in buffer D [50 mM Tris, 150 mM NaCl, 10% (v/v) glycerol, and PIC] at pH 7.0 (*P. falciparum* and *P. yoelii* TrxR) or pH 8.0.

Purification of Trx from *P. falciparum* and *R. norvegicus*. Cells were lysed by sonication in buffer A (without lysozyme at pH 8.0) and buffer D [50 mM Tris, 150 mM NaCl, and PIC (pH 7.0)] for each species. The lysates were passed down a Superdex 75 prep grade size exclusion column, the eluted fractions were concentrated using a 3 kDa NMWL Ultracel YM membrane in a Stirred Cell and again passed down the size exclusion column. HiTrap Blue and Resource Q columns were washed to baseline and eluted in a linear gradient over 15 column volumes. Superdex size exclusion columns were run at flow rates between 8 and 23 cm/h. The intact molecular weight of each purified protein was determined by liquid chromatography−mass spectrometry (LC−MS) analysis and the identity of each by peptide mass fingerprinting.

Free Thiol Capping. A 10-fold molar excess of *N*-ethylmaleimide (NEM) (Thermo Fisher Scientific) was added to each Trx and each mixture incubated for 1 h at room temperature. The samples were dialyzed against buffer D (pH 8.0) to remove excess reagent.

Enzyme assays were conducted at room temperature in 384- or 1536-well clear-bottom microplates (Greiner Bio-One). *R. norvegicus* TrxR was purchased from IMCO. All solutions were prepared using 50 mM Tris and 2 mM EDTA (pH 7.5) using reagents from Sigma-Aldrich. All concentrations are final unless otherwise stated. Reactions were performed using NADPH and Trx substrates together with 1 mM glucose 6-phosphate and at

least 50 μM insulin and 33.3 units/mL glucose-6-phosphate dehydrogenase. The reactions were quenched with a stop solution containing 0.03% (v/v) SDS and 100 μM DTNB, unless otherwise stated. Absorbance was read following a 1 h preincubation at 412 nm using a Tecan Safire 2 plate reader (Tecan Group Ltd.).

NADPH and Trx K_m Determinations. For *Plasmodium* TrxRs, serial dilutions of NADPH from 25 μM were added to coupling reagents containing 15 μM *P. falciparum* Trx and incubated with either 7.5 nM *P. falciparum* TrxR, 1.5 nM *P. vivax* TrxR, 4 nM *P. berghei* TrxR, or 7.5 nM *P. yoelii* TrxR. Reactions were quenched at discrete intervals upon addition of stop solution and mixtures left for 30 min before being read.

Serial dilutions of *P. falciparum* Trx from 25 μM were added to a solution containing 200 μM NADPH and 200 μM insulin. Reactions were initiated upon addition of 15 nM *P. falciparum* TrxR, 3 nM *P. vivax* TrxR, 8 nM *P. berghei* TrxR, or 15 nM *P. yoelii* TrxR and monitored kinetically as a decrease in absorbance at 340 nm for 40 min using a Tecan Safire 2 instrument. For *R. norvegicus* TrxR, serial dilutions of NADPH from 200 μM or *R. norvegicus* Trx from 25 μM were added to cosubstrate/coupling reagent solutions containing either 10 μM *R. norvegicus* Trx or 200 μM NADPH and incubated with either 20 or 25 nM *R. norvegicus* TrxR, respectively. Reactions were quenched and mixtures left for 30 min before the absorbance at 420 nm was read using an EnVision plate reader (PerkinElmer, Waltham, MA). In all cases, initial rate data were fit to the Michaelis–Menten equation using GraFit version 5.0.12 (Erithacus Software Ltd.).

High-Throughput Screening of the Tres Cantos Antimalarial Compound Set. Compounds were tested at 10 μM or in dose response from 100 μM (11-point, 3-fold serial dilution). They were preincubated for 10 min with an enzyme solution containing either 10 nM *P. falciparum* TrxR, 2 nM *P. vivax* TrxR, 12 nM *P. berghei* TrxR, or 10 nM *P. yoelii* TrxR. Reactions were initiated upon addition of a substrate solution containing 1 μM *P. falciparum* Trx and 1 μM NADPH. Fully coupled assays were quenched upon addition of the stop solution. Screening was performed in 1536-well plates, with a final assay volume of 8 μL. Conditions for the *R. norvegicus* assay were identical but used 20 nM *R. norvegicus* TrxR and 0.5 μM *R. norvegicus* Trx. Absorbance was recorded using an EnVision plate reader (PerkinElmer) at 420 nm and normalized to control values of the uninhibited and fully inhibited enzyme. Hits were identified as absorbance values statistically greater than the mean value of the fully inhibited control and progressed to dose–response experiments. The concentration of the compound required to inhibit the enzyme by 50% (IC_{50}) was calculated using XLfit (IDBS). pIC_{50} values are defined as the negative log of IC_{50}.

Mode of Inhibition. Compounds were tested in dose response from 25 μM (16-point, 1.5-fold serial dilution). They were added to substrate solutions containing either 0.3, 1, 3, and 9 μM NADPH in the presence of 9 μM *P. falciparum* Trx or 0.1, 0.3, 1, and 3 μM *P. falciparum* Trx in the presence of 9 μM NADPH. Reactions were started by the addition of 10 nM *P. falciparum* TrxR. Coupling and quenching reagents were included at concentrations previously described. Initial rates were fit to the equation for noncompetitive inhibition using GraFit.

RESULTS

Generation of Enzymes and Substrates. The codon usage of the gene sequences for TrxR enzymes and Trx substrates were specifically optimized for overexpression in *E. coli*. Although this approach is not always successful, it can lead to substantially higher yields of expressed proteins.[30] To reproduce the most physiologically relevant context for the activity of the recombinant molecules in vitro, the full-length, untagged proteins were expressed.

A HiTrap Blue HP column was shown to be a highly effective initial purification step for the TrxRs from *P. falciparum*, *P. berghei*, and *P. yoelii*, probably because of the interaction of the blue sepharose affinity matrix with the nicotinamide binding site.[31] Interestingly, the enzyme from *P. vivax* did not display affinity for the blue sepharose, and a Resource Q ion exchange column was identified as an alternative. In each case, the retention volume of each species on a size exclusion column indicated an apparent molecular mass approximately equivalent to that of a homodimeric species. The two-step purification protocol developed for each TrxR construct yielded 2.4, 0.1, 0.2, and 1 mg of the enzymes per gram of *E. coli* cell paste from *P. falciparum*, *P. vivax*, *P. berghei*, and *P. yoelii* with purities of 80, 88, 81, and >95%, respectively, when the final reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (Figure 1) was assessed by densitometric scanning.

The relatively small sizes of the Trx substrates (11.7 kDa) were successfully exploited, allowing the proteins to be purified directly from crude lysates using a Superdex 75 size exclusion column. To reduce the background signal in the enzymatic assay, the free thiol groups on the substrate proteins were

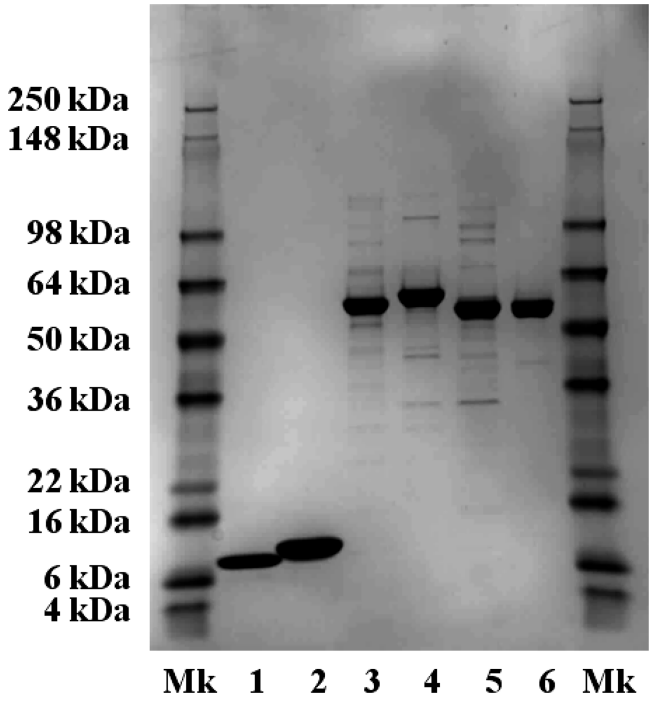

Figure 1. Tris-glycine reducing SDS–PAGE analysis (4 to 20%) of 2.5 μg total protein loadings of thioredoxin (Trx) and thioredoxin reductase (TrxR) final purified products: (1) *P. falciparum* Trx, (2) *R. norvegicus* Trx, (3) *P. falciparum* TrxR, (4) *P. vivax* TrxR, (5) *P. berghei* TrxR, and (6) *P. yoelii* TrxR. Mk lanes contained SeeBlue Plus2 Pre-Stained standards (Invitrogen).

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chemically modified (capped) by incubation with NEM.[32] These processes yielded 15.9 and 0.7 mg of capped Trx per gram of *E. coli* cell paste from *P. falciparum* and *R. norvegicus*, respectively, with both substrates present at >95% purity.

Excision of gel bands from all the final samples, followed by tryptic digestion and peptide mass fingerprinting analysis, confirmed the identity of each protein. Molecular masses determined by LC−MS were found to be consistent with the expected masses for the TrxRs from *P. falciparum*, *P. vivax*, and *P. berghei*, and both of the NEM-capped Trx substrates. The apparent molecular mass of TrxR from *P. yoelii* determined by reducing SDS−PAGE appeared to be less than the expected value of 71.0 kDa. LC−MS analysis revealed multiple species with molecular masses ranging between 57.5 and 59.3 kDa. Edman sequencing determined N-terminal sequences of XNDKKNNTQ~ and XDKKNNTQ~. These data were consistent with *P. yoelii* TrxR species with putative N-termini beginning at amino acids 99, 100, 113, and 114. While the exact nature of this processing is unknown, the excluded N-terminal region of amino acids 1−99 is not conserved in any of the three other *Plasmodium* TrxR enzymes (Figure 7 of the Supporting Information) and is coincident with the second methionine of the protein sequence at position 98. With the two conserved redox active disulfide bonds and the FAD binding site still present,[17] the expressed protein demonstrated activity equivalent to that of the TrxR enzymes from *P. falciparum* and *P. berghei*. This missing N-terminal region is therefore thought to be nonfunctional, and the biological properties of the expressed protein are fully retained.

Enzyme Assays. To develop a suitable screening assay, it was necessary to establish that the recombinant TrxR enzymes produced were catalytically active with their Trx and NADPH substrates. Two functional properties characterize the thioredoxin family of proteins. First, reduced Trx (active site dithiol) catalyzes protein disulfide reductions, and oxidized Trx (active site disulfide) is reduced by TrxR, which acquires electrons from NADPH. Assay formats that can operate at K_m for both substrates offer the best opportunities to discover enzymatic inhibitors of TrxR.[33] However, with the affinities for both Trx and NADPH being similar and in the same low micromolar range as the detection limit for $NADP^+$, this poses a challenge for configuring an appropriate assay.[8,34−36] We therefore looked to develop a novel assay that would allow the use of both substrates at K_m and provide a miniaturized, robust, and sensitive detection format for supporting a high-throughput screen.

TrxR was used to catalyze the NADPH-dependent reduction of oxidized Trx that was subsequently regenerated using insulin as the protein substrate, because it can be rapidly reoxidized by insulin disulfides.[37] This reaction was monitored kinetically as a decrease in absorbance at 340 nm resulting from the concomitant oxidation of NADPH to $NADP^+$,[38] making it ideal for reagent evaluation but unsuitable for high-throughput screening because of its absorption wavelength. Consequently, the screening format combined the insulin redox regeneration to maintain the Trx concentration at K_m and introduced a second enzymatic regeneration reaction comprising glucose-6-phosphate dehydrogenase and glucose 6-phosphate. These were both present in excess to regenerate NADPH from $NADP^+$ and also to allow the NADPH concentration to be maintained at K_m. Reactions were subsequently quenched upon the addition of SDS containing excess 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). DTNB reacts with free thiols, primarily on the reduced insulin chains, resulting in the generation of 2-nitro-5-thiobenzoate (TNB^{2-}) that can be monitored as an increase in absorbance at 412 nm (Figure 2 and Figure 8 of the Supporting Information).

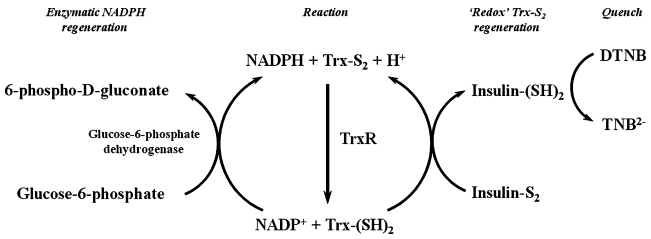

Figure 2. Assay schematic of the complete redox−enzymatic coupled screening assay depicting the reaction of glucose-6-phosphate dehydrogenase and glucose-6-phosphate that regenerated reduced NADPH and the insulin disulfides that regenerated the oxidized thioredoxin substrate. Following the addition of the quench solution, the insulin disulfides resulting from the reoxidation of thioredoxin react with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to form 2-nitro-5-thiobenzoate (TNB^{2-}), which can be quantified by its absorbance at 412 nm.

Because of the nature of the detection, it was also necessary to block any potential free thiols in the Trx substrate as these would contribute significantly to a background signal. Modification using NEM effectively reduced the concentration of free thiol from 40 to 4 μM per 140 μM protein without affecting its ability to act as a substrate of TrxR (Figure 9 of the Supporting Information). It also eliminated any background signal, significantly improving the sensitivity and robustness of the final assay.

Utilizing the insulin and glucose-6-phosphate dehydrogenase regeneration pathways, a panel of novel assays was configured to maintain either Trx, NADPH, or both substrates at K_m concentrations. These robust, uniquely sensitive assays were ideal for use in validating the protein reagents, determining the kinetic parameters of the TrxR enzymes, identifying inhibitors by miniaturized high-throughput screening, and subsequently exploring their enzymatic modes of inhibition.

Kinetic Characterization. Using the assays outlined above, the kinetic parameters were reconfirmed for each of the TrxR enzymes (Figure 3C and Figure 10 of the Supporting Information) and agreed well with previously published data.[8,34−36,39] All TrxR enzymes tested exhibited NADPH- and Trx-dependent reductase activities, both of which were hyperbolically dependent on substrate concentration.

The reduction of oxidized *P. falciparum* Trx by NADPH yielding $NADP^+$ and reduced *P. falciparum* Trx is a typical bisubstrate/biproduct reaction. Its mechanism, with respect to order of substrate binding and product release, can be explored by classical steady-state analysis. Double-reciprocal plots of initial velocities as a function of *P. falciparum* Trx concentration at discrete concentrations of NADPH returned a series of parallel lines (Figure 11 of the Supporting Information), indicative of a ping-pong bi-bi mechanism in which NADPH binds first and *P. falciparum* Trx binds second.[40] This is in agreement with literature data for TrxR from *P. falciparum*[41] and other species.[42,43]

This detailed characterization of the enzymes demonstrated the recombinant molecules were functionally active and were suitable for use in high-throughput screening and detailed mechanistic studies.

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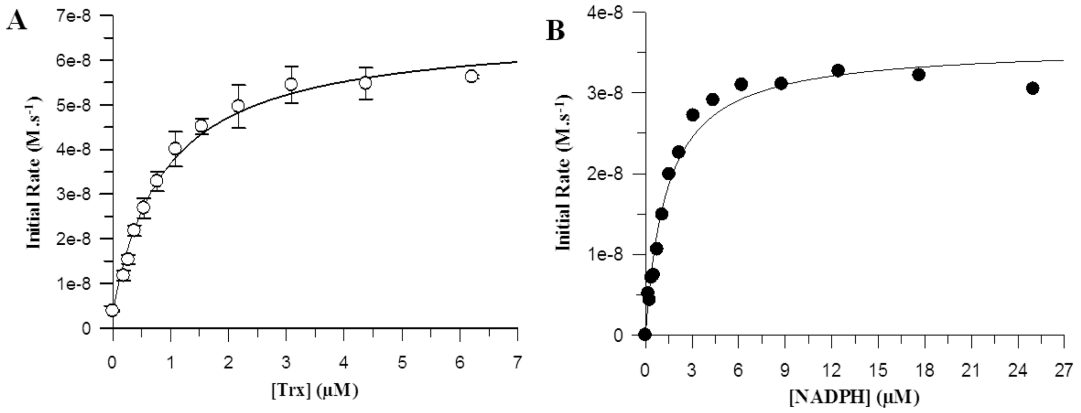

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TrxR species	Substrate	K_m (μM)	k_{cat}^{app} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
P. falciparum	*P.falciparum* Trx	0.90 ± 0.08	4.2 ± 0.1	4.7
	NADPH	1.39 ± 0.16	4.8 ± 0.1	3.4
P. vivax	*P.falciparum* Trx	1.61 ± 0.21	25.0 ± 1.0	15.5
	NADPH	1.96 ± 0.27	37.3 ± 1.5	19.0
P. berghei	*P.falciparum* Trx	2.19 ± 0.23	9.6 ± 0.3	4.4
	NADPH	1.80 ± 0.20	8.1 ± 0.3	4.5
P. yoelii	*P.falciparum* Trx	2.09 ± 0.20	7.9 ± 0.2	3.8
	NADPH	1.71 ± 0.17	6.2 ± 0.2	3.6
R. norvegicus	*R. norvegicus* Trx	0.25 ± 0.03	2.3 ± 0.1	9.0
	NADPH	0.24 ± 0.04	1.9 ± 0.1	8.1

Figure 3. *P. falciparum* TrxR exhibits NADPH- and Trx-dependent reductase activities, both of which are hyperbolically dependent on Trx (A) and NADPH (B) concentration. A comparison of both activities determined in equivalent assays with other *Plasmodium* and mammalian species is summarized in panel C. The NADPH K_m value was generated using the redox/enzymatic coupled screening assay under conditions where the titrated NADPH was not depleted. Trx K_m value was determined at saturating NADPH concentrations using the redox only regeneration assay to generate initial rates. Consequently, there are no error bars on the NADPH plot, although data are representative of three independent tests. As the affinities of the most potent inhibitors were significantly weaker than the protein concentrations required for assay, it was not possible to accurately determine active site concentrations; therefore, all catalytic rate constants (k_{cat}) are quoted as "apparent" values (k_{cat}^{app}).

High-Throughput Screening and Mode of Inhibition. Single-shot screening of the TCAMS identified 506 compounds that returned >40% inhibition of either *P. falciparum* or *P. vivax* TrxR. A total of 235 compounds were reconfirmed in full curve mode and were profiled against our panel of TrxRs. These results were combined with whole cell potencies[22] and cytotoxicity data, generated using a human cell line, HepG2, as a surrogate for cytotoxicity[44] (Table 1 of the Supporting Information). The structures and activities of the seven compounds that met our criteria of potency (pIC_{50} > 5 against *P. falciparum* TrxR), specificity (no activity against *R. norvegicus* TrxR), and cytotoxicity (<20% at 10 μM) are listed in Table 1 and can be grouped into two distinct chemotypes.

To explore further the mechanism of inhibition of *P. falciparum* TrxR by these compounds, we varied the concentration of each inhibitor as a function of substrate concentration. All of them behaved as noncompetitive inhibitors with respect to both substrates (Figure 12 of the Supporting Information), with K_i values ranging from 1.3 to 4.1 μM (Table 2). The lack of competition of *P. falciparum* TrxR inhibitors with either substrate has already been previously described,[45] suggesting that the interactions between the inhibitor and the enzyme are outside the substrates' binding sites, possibly in the intersubunit region.

DISCUSSION

Phenotypic screening is one of the preferred approaches currently employed by the malarial community to discover new potent chemotypes, and identifying new targets to initiate target-based drug discovery approaches is an ongoing challenge. Knowledge of the molecular target responsible for the antimalarial mode of action of a phenotypically active compound is clearly beneficial in the chemical optimization process, as it allows the opportunity to rationalize the in vivo activity and specificity from simplified chemical models and assists in establishing a clearer safety profile for the future drug.

Antimalarial lead discovery has produced disappointing results over the past few years, and the recent publication of compounds identified through whole cell screening opens up a unique opportunity to fill this gap.[22−24] Initial chemo- and bioinformatic analyses with available retrospective biochemical assay data can allow the generation of putative target hypotheses for these molecules. However, only experimental verification by testing the compounds in an appropriate biochemical screen of an essential target can provide a definitive link between the observed whole cell inhibition and a specific antimalarial mode of action.

We have applied this approach to TrxR, an essential enzyme in the erythrocytic phase of *P. falciparum*.[15] Here we report the first published target-based high-throughput screen of the

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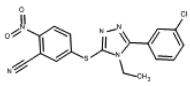
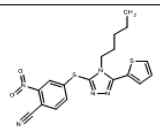
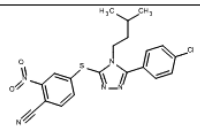
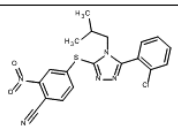
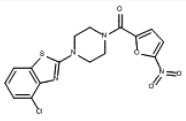
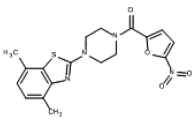
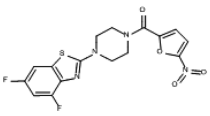
Table 1. Structures and Activities for the Seven Molecules That Met Our Criteria of Specificity[a]

Compound	Structure	pIC50 (TrxR enzymatic assay)					pIC50 (*P. falciparum* whole cell assay)	% Inhibition @10μM (Human cell cytotoxicity assay)
		P. falciparum	*P. vivax*	*P. berghei*	*P. yoelii*	*R. norvegicus*		
TCMDC-135234		5.2	4.8	4.7	4.8	< 4.0	6.1	7.5
TCMDC-135585		5.2	4.5	4.8	4.8	< 4.0	6.1	18.5
TCMDC-135596		5.6	4.6	< 4.0	< 4.0	< 4.0	6.1	17.5
TCMDC-135609		5.4	4.7	< 4.0	< 4.0	< 4.0	6.0	16.5
TCMDC-124316		5.4	5.2	5.0	5.0	< 4.0	6.0	-19.6
TCMDC-124315		5.2	< 4.0	< 4.0	< 4.0	< 4.0	6.1	-2.4
TCMDC-125149		5.0	< 4.0	< 4.0	< 4.0	< 4.0	6.0	-2.8

[a]In all cases, the purity exceeded 80%, as determined by UPLC-MS.

Table 2. K_i Values for Inhibition of *P. falciparum* TrxR with Respect to Substrates NADPH and *P. falciparum* Trx

Compound	K_i (μM)	
	Saturating *P. falciparum* Trx, variable NADPH	Saturating NADPH, variable *P. falciparum* Trx
TCMDC-135234	1.4 ± 0.1	3.4 ± 0.4
TCMDC-135585	1.5 ± 0.1	2.2 ± 0.3
TCMDC-135596	1.4 ± 0.1	1.8 ± 0.2
TCMDC-135609	2.7 ± 0.1	3.4 ± 0.4
TCMDC-124316	1.8 ± 0.1	4.1 ± 0.6
TCMDC-124315	1.3 ± 0.1	2.3 ± 0.4
TCMDC-125149	1.3 ± 0.1	2.8 ± 0.3

TCAMS, a subset of the GlaxoSmithKline (GSK) screening collection that demonstrated >80% inhibition of *P. falciparum* growth in a whole cell assay.[22] The recombinant, full-length, untagged enzymes from *P. falciparum*, *P. vivax*, *P. berghei*, and *P. yoelii* have been produced at high purity, as well as the Trx substrates from *P. falciparum* and *R. norvegicus*. A novel, robust, and miniaturized assay that maintains the native substrates Trx and NADPH at K_m concentrations has been developed to identify inhibitors of these enzymes in a high-throughput mode. Comparison of these data from the *Plasmodium* and mammalian enzymes allows the identification of potent and *Plasmodium*-selective inhibitors. A simple and straightforward modification of the assay has allowed the rapid characterization of the enzymatic mode of inhibition of the most promising compounds. In addition, reported *P. falciparum* whole cell activity and cytotoxicity data from a hepatoma HepG2 assay can be used for the selection of compounds with a suitable in vitro therapeutic index.

Screening of the TCAMS in this unique assay has identified seven novel, potent, and specific inhibitors of *Plasmodium* TrxR, which are devoid of significant HepG2 toxicity. They are grouped into two novel chemical families not previously described to have antimalarial activity. In fact, until now, only three *P. falciparum* TrxR inhibitors with known antimalarial activity have been described.[45]

P. falciparum TrxR is a homodimeric enzyme with three redox centers, FAD, Cys-88/Cys-93, and Cys-535/Cys-540, which functions by transferring reducing equivalents from the NADPH-reduced FAD to the Cys-535/Cys-540 pair (at the C-terminal region of one subunit) through dithiol−disulfide exchange involving the intermediate Cys-88/Cys-93 pair[17] (at the N-terminal domain of the other subunit). The crystallographic structure of the parasitic enzyme is undetermined, but

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in mammalian enzymes, the terminal redox site, located in the flexible C-terminal domain of each subunit, is replaced with a Cys-Sec pair. This Cys-Sec pair is exposed to solvent upon reduction and engages in dithiol−disulfide exchange with the Trx substrate.[16,46,47] We have demonstrated that the reaction of the *P. falciparum* enzyme with the two substrates follows a ping-pong mechanism, in agreement with the structural evidence from human TrxR.[47]

All seven potent and selective molecules identified from the screen contain electrophilic moieties. Given the higher nucleophilicity of Sec versus that of Cys, the preferential binding of these compounds to the *Plasmodium* enzymes should discount inhibition by reaction with the solvent-exposed dithiol C-terminal redox site. This is in agreement with the noncompetitive behavior observed for these molecules with respect to Trx and NADPH and alludes to allosterism. Similar behavior was observed with the three previously described *P. falciparum* TrxR inhibitors, which bear a nitrophenyl moiety similar to one of the two electrophilic families in the TCAMS compounds.[45] The noncompetitive inhibition observed for these molecules, together with the ping-pong mechanism of the enzyme with its substrates, is compatible with binding in the intersubunit region. Here, the inhibitors could interfere with dithiol−disulfide exchange, thus preventing reduction of the C-terminal Cys pair and the solvent exposure required to reduce the Trx substrate.

The origin of the specificity of these new inhibitors remains unclear given the absence of an atomic-resolution structure of *P. falciparum* TrxR, either alone or in complex with one or more of them. The determination of this structure would allow the rationalization of the observed findings and is clearly the next step to follow.

In this work, we report the generation and characterization of an extensive toolbox of biochemical reagents and assays for exploring *Plasmodium* TrxR activity from multiple species in unprecedented detail. Using these tools, we have identified the first inhibitors within the TCAMS to be experimentally mapped to this essential target in *P. falciparum*. We anticipate that these findings will be of great benefit to the scientific community and will accelerate the discovery of new antimalarial drugs.

■ ASSOCIATED CONTENT

Supporting Information

Expressed gene sequences of TrxR and Trx proteins, an amino acid alignment of TrxR enzymes, assay linearity plots, effect of Trx-NEM capping on TrxR activity, enzyme kinetic parameters, kinetic mechanism of *P. falciparum* TrxR, mode of inhibition studies using exemplar compounds, and a pIC_{50} summary table of reconfirmed hits. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.J.T. and I. Caballero contributed equally to this work.

Author Contributions

A.J.T. designed and performed cloning, expression, and protein purification work and coordinated publication contributions. M.J.H. contributed to the design and strategic execution of cloning, purification, and publication strategies. A.L.B. produced *P. yoelii* TrxR. D.A.T. designed and developed the assays, validated initial protein reagents, performed enzyme kinetic characterization, and contributed expertise to mode of inhibition studies. I. Caballero and C.C. performed enzyme kinetic characterization, contributed experimental work and expertise during TCAMS screen data processing, and designed and performed compound mode of inhibition studies. I. Coma was the project leader and performed TCAMS screen data handling and analysis. F.-J.G. was the lead biologist and supported the design of protein constructs, biological assays, and mode of inhibition studies. G.C. performed chemoinformatic analysis. A.J.T., D.A.T., I. Caballero, C.C., and G.C. wrote the manuscript with the assistance of the other authors.

Notes

The authors declare no competing financial interests.

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