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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 2283–2292

Specific inhibitors of *Plasmodium falciparum* thioredoxin reductase as potential antimalarial agents

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Received 12 November 2005; revised 5 January 2006; accepted 6 January 2006
Available online 3 February 2006

Abstract—*Plasmodium falciparum* thioredoxin reductase (PfTrxR: NADPH + $Trx(S)_2$ + $H^+ \leftrightarrow NADP^+$ + $Trx(SH)_2$) is a high M_r flavin-dependent TrxR that reduces thioredoxin (Trx) via a CysXXXXCys pair located penultimately to the C-terminal Gly. In this respect, PfTrxR differs significantly from its human counterpart which bears a Cys-Sec redox pair at the same position. PfTrxR is essentially involved in antioxidant defense and redox regulation of the parasite and has been previously validated by knock-out studies as a potential drug target for malaria chemotherapy. Moreover, human TrxR is present in most cancer cells at levels tenfold higher than in normal cells. Here we report the discovery of a series of potent inhibitors of PfTrxR. The three most promising inhibitors, **3** ($IC_{50}^{PfTrxR} = 2\,\mu M$ and $IC_{50}^{hTrxR} = 50\,\mu M$), **7** ($IC_{50}^{PfTrxR} = 2\,\mu M$ and $IC_{50}^{hTrxR} = 140\,\mu M$), and **11** ($IC_{50}^{PfTrxR} = 0.5\mu M$ and $IC_{50}^{hTrxR} = 4\,\mu M$) were selective for the parasite enzyme. Detailed mechanistic characterization of the effects of these compounds on the PfTrxR-catalyzed reaction showed clear uncompetitive inhibition with respect to both substrate and cofactor. For the most specific PfTrxR inhibitor **7**, an alkylation mechanism study based on a thiol conjugation model was performed. Furthermore, all three compounds were active in the lower micromolar range on the chloroquine-resistant *P. falciparum* strain K1 in vitro.

The protozoan parasite *Plasmodium falciparum* is responsible for up to three million deaths per year with Africa having more than 90% of this burden.[1] The high rate of failure of the currently available malaria prophylactics, and the increasing resistance to nearly all the available antimalarial drugs, strongly indicate that a new generation of drugs is urgently needed.[2]

Plasmodium falciparum does not efficiently develop in glucose-6-phosphate dehydrogenase deficient erythrocytes and is sensitive to reactive oxygen species (ROS) resulting from the host immune system, as well as from hemoglobin breakdown.[3] The small amount of free heme escaping the various detoxification pathways causes oxidative damage to host proteins and membranes, inhibits parasite enzymes, and finally lyses erythrocytes.[3,4] Two systems interact to protect malarial parasites against ROS: the glutathione system, comprising glutathione, glutathione reductase (GR), glutathione *S*-transferase, and different glutaredoxin-like proteins, and the thioredoxin system, which is

Abbreviations: CTC, charge transfer complex; DTNB, dithiobisnitrobenzoate; ESI-MS, electrospray ionization mass spectrometry; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; SAR, structure–activity relationships; Trx, thioredoxin; $TrxS_2$, oxidized thioredoxin; TrxR, thioredoxin reductase.

Keywords: Enzyme inhibition; Drug development; Malaria; *Plasmodium falciparum*; Thioredoxin reductase.

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doi:10.1016/j.bmcl.2006.01.027

based on thioredoxin (Trx), thioredoxin reductase (TrxR), and thioredoxin-dependent peroxidases.[5–7] Both systems use NADPH as a reductant.

The redox-active small protein Trx is involved in ribonucleotide reduction and thus DNA synthesis as well as in the redox control of many cellular processes, including transcriptional control, protein folding, and enzyme regulation.[8,9] Trx is reduced by thioredoxin reductase (TrxR: $NADPH + Trx(S)_2 + H^+ \leftrightarrow NADP^+ + Trx(SH)_2$), a homodimeric FAD-dependent protein. In nature, TrxR is found in two forms. The low M_r form, with a subunit of 35 kDa, is present in prokaryotes and lower eukaryotes including yeast, and has one FAD and one redox-active disulfide–dithiol in each monomer. The high M_r form, with a subunit of ca. 55 kDa, contains one FAD and two redox-active disulfide–dithiols per monomer and is found in higher eukaryotes including human and *P. falciparum*.[10,11] The flow of reducing equivalents in these proteins is from NADPH to the flavin, from the reduced flavin to the adjacent disulfide, and from the nascent dithiol to the second redox-active disulfide located on a flexible C-terminal arm of the protein. This nascent dithiol is the reductant of thioredoxin. In human TrxR (hTrxR) and other mammalian TrxRs, this third redox center is represented by a Cys-selenocysteine (Sec) pair. The accessibility and reactivity of this redox pair is presently exploited as a target for chemotherapeutic agents—an approach that is supported by the fact that TrxR is found at tenfold higher levels in most cancer cell lines than in normal cells.[12–15] In PfTrxR, the C-terminal redox pair is represented by a CysXXXXCys sequence. This difference in an essential structural and functional motif of host cell and parasite enzyme represents a most promising starting point for the development of specific PfTrxR inhibitors.[16–18] An X-ray structure of PfTrxR is not yet available, however, the structures of the isofunctional rat TrxR and of the closely related malarial glutathione reductase can serve as models.[19,20] Based on these structures, the dimer interface region and the subunit cavity might be specifically targeted in these large disulfide reductases in addition to the substrate binding sites.[20] The inhibition of PfTrxR is most likely to result in enhanced oxidative stress, in ineffective DNA synthesis and cell division, and in disturbed redox regulatory processes in the parasites. Knock-out studies on *P. falciparum* have shown that PfTrxR is essential for the parasite, thus representing a validated drug target.[21]

The approach of our collaborative effort to develop a new generation of antimalarial drugs has been two-pronged. The first approach was based on a high-throughput screen (HTS) of 350,000 compounds in a Pfizer library that yielded 114 PfTrxR inhibitors using a standard biochemical assay. Further screening identified 15 compounds that selectively inhibited the parasite TrxR, 13 of which were Mannich bases, 4 saturated and 9 unsaturated.[18] Based on these compounds we synthesized a series of unsaturated Mannich bases that acted as mechanism based inhibitors.[18] In the present paper, representing our second research approach, we have used structure–activity relationships (SARs) studies to develop a novel series of inhibitors that act from the micro- to nanomolar range and show high selectivity against the *P. falciparum* enzyme. A detailed mechanism for PfTrxR inactivation was investigated with the most specific PfTrxR inhibitor **7**. The inhibitors were evaluated on isolated *P. falciparum* and human TrxR, as well as on *P. falciparum* in cell culture.

Plasmodium falciparum TrxR represents a validated and highly interesting drug target to develop new antimalarial drugs. Inhibition of this enzyme is likely to affect the parasite at several vulnerable points in parallel, acting to impair its antioxidant defense, to disturb its redox regulation, and to decrease DNA synthesis. The fact that PfTrxR harbors a solvent-exposed and highly reactive redox center, which is essential for catalysis and differs from the host enzyme, is a promising starting point for the development of selective inhibitors.

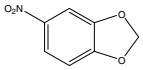
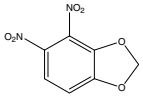
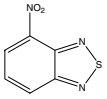
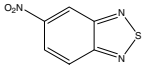
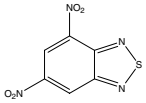
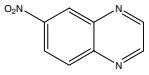
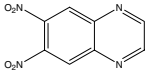
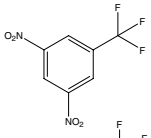
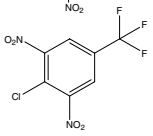
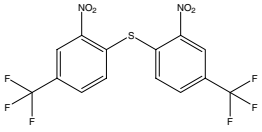
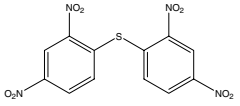
The high-throughput screening of 350,000 compounds has identified nitrophenyl compounds as *P. falciparum* TrxR inhibitors. The lead structure is compound **1** from which we developed our strategy based on SAR.[18] Because the methylendioxyphenyl group could have undesirable metabolic interactions with cytochrome P_{450} in vivo, we applied the elegant strategy from the Merck group which first discovered the 2,1,3-benzothiadiazole as a methylendioxyphenyl bioisoster.[22–24] The surrogate not only mimics both the shape and the molecular electronic properties of the methylendioxyphenyl group but also shows high bioavailability. Three 2,1,3-benzothiadiazoles differently substituted by nitro groups were investigated as TrxR inhibitors. We furthermore evaluated nitroquinoxaline and nitrophenyl derivatives as PfTrxR inhibitors, the structures and corresponding IC_{50} values of which are shown in Table 1,[25,26] and determined their antimalarial properties.

Each compound was tested for its ability to inhibit PfTrxR and the isofunctional host cell enzyme hTrxR in direct comparison.[27] The respective IC_{50} values determined under quasi-physiological conditions, that is, in phosphate buffer, pH 7.4, in the presence of 100 μM NADPH and 20 μM of the corresponding thioredoxin, are shown in Table 1. Since wild type hTrx tends to form dimers in solution, which disturbs the determination of exact kinetic constants, we employed the hTrx mutant C72S for the studies. Affinity and turnover rates of this mutant with respect to hTrxR are equivalent to those of the wild type.[28] The percentage of inhibition was calculated according to the following equation:

$$\% \text{ inhibition} = 100 - (V_i/V_0 \times 100), \qquad (1)$$

where V_i and V_0 are the activities determined in the presence and in the absence of inhibitor, respectively. Compounds **2**–**5**, **7**, **9**, and **11** inhibited PfTrxR with IC_{50} values $\leqslant 10$ μM. The most potent inhibitor was compound **11** with an IC_{50} of 500 nM. With the exception of compounds **1** and **5**, all inhibitors were more effective on the parasite enzyme than on hTrxR. Compounds **3** and **7** were by a factor of 25 and 70, respectively, more effective on PfTrxR, whereas compounds **6** and **9** were more potent on the parasite enzyme by factors between

Table 1. Structures of compounds **1**–**11** and IC_{50} values on *Plasmodium falciparum* TrxR and human TrxR

Compound	Structure	IC_{50} for PfTrxR (μM)	IC_{50} for hTrxR (μM)	Selectivity for PfTrxR/hTrxR
1		<10% inhibition at 100	> 200	—
2		10	80	8
3		2	50	25
4		10	90	9
5		10	~2	~0.2
6		40	>200	>5
7		2	140	70
8		30	>200	>6
9		8	100	12
10		20	>150	>7
11		0.5	4	8

5 and 12, respectively. This is a most important aspect since the development of specific PfTrxR inhibitors is a difficult task when considering the presence of a highly reactive selenocysteine residue at the C-terminal end of hTrxR, in contrast to the CysXXXXCys motif in PfTrxR. The high reactivity of the selenocysteine towards electrophilic compounds, such as gold- or platinum-containing inhibitors, is currently used as a chemotherapeutic approach to human cancer.[15,28–31] The fact that, in spite of this high reactivity of the hTrxR, a selective PfTrxR inhibition could be achieved points to the involvement of other binding moieties than the C-terminal redox center.

To explore the mechanism of inhibition in more detail, we employed two different approaches. First, for those compounds with the lowest IC_{50} values for PfTrxR, **3**, **7**, and **11**, we examined the inhibition of the closely related enzyme, glutathione reductase. Second, we determined K_i values and the type of inhibition with respect to the physiological substrates PfTrx and NADPH (Table 2 and Fig. 1). GR is a homodimeric FAD-dependent enzyme which is structurally and mechanistically closely related to TrxR. The most prominent difference between the two enzymes is the absence of the second, the C-terminal, redox-active disulfide in GR. In GR, the substrate glutathione is directly reduced at the

Table 2. K_i values and effected glutathione reductase inhibition of the three most potent PfTrxR inhibitors **3**, **7**, and **11**. Values represent means of two independent experiments which differed by less than 10%

Compound	hGR inhibition at IC_{50} for PfTrxR (%)	PfGR inhibition at IC_{50} for PfTrxR (%)	K_i for PfTrxR using PfTrx as varied substrate (μM)	K_i for PfTrxR using NADPH as varied substrate (μM)
3	81	69	0.65	1
7	27	39	n.d.	n.d.
11	18	45	0.2	0.2

n.d, not determined.

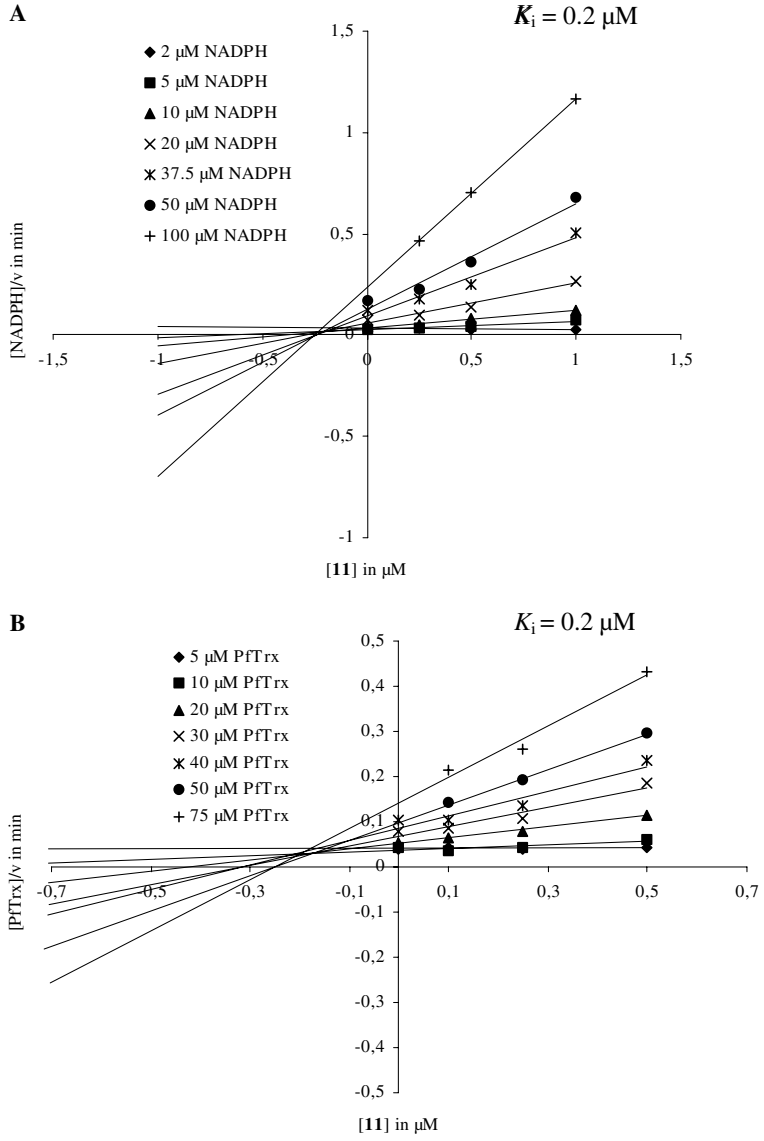
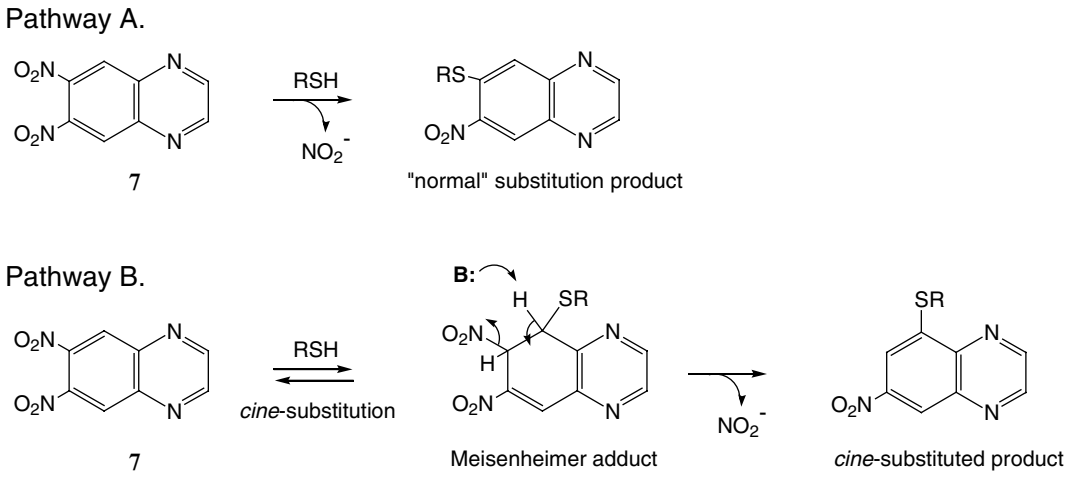


Figure 1. Cornish–Bowden plots showing that compound **11** inhibits PfTrxR uncompetitively with respect to both substrates, NADPH (A) and PfTrx (B).

N-terminal redox-active dithiol. PfGR has been well characterized and closely resembles its human counterpart.[32] High-resolution X-ray structures of both hGR and PfGR are available.[20,33]

The results in Table 2 indicate that, at inhibitor concentrations leading to 50% PfTrxR inhibition (namely 2, 2, and 0.5 μM), compounds **3**, **7**, and **11** lead to efficient inhibition of GR, between 18% and 81% for hGR, and between 39% and 69% for PfGR. Furthermore, the inhibition of PfTrxR was found to be uncompetitive with respect to both substrates Trx and NADPH (see below). These results support the notion that the inhibitors do not significantly interact with the C-terminal redox center of TrxR nor with the NADPH binding site. Rather they are likely to have affinity to other structural motifs or amino acid residues, possibly the intersubunit region which is present both in PfTrxR and GRs.

K_m values in the presence of varying inhibitor concentrations were determined on PfTrxR for both substrates, PfTrx and NADPH. For all three inhibitors uncompet-



Scheme 1. Postulated reactivity of 6,7-dinitroquinoxaline **7** toward nucleophilic attack by thiols.

itive inhibition was clearly observed as K_m and V_{max} values decreased with increasing inhibitor concentrations. This indicates that the inhibitors bind to the enzyme–substrate complex. Moreover, Cornish–Bowden plots showed intercepts of all lines (obtained at different substrate concentrations) in the upper left quadrant. Data for the most potent PfTrxR inhibitor, **11**, are shown in Figure 1. K_i values obtained for this uncompetitive inhibition were in the (sub)micromolar range for compounds **3** and **11** and are shown in Table 2. This indicates that the inhibitors bind to one of the enzyme–substrate intermediate complexes that occur during catalysis, as previously observed for 1-chloro-2,4-dinitrobenzene with hGR.[34]

To characterize the inhibition of *P. falciparum* TrxR by the most specific compound **7**, we investigated the reactivity of 6,7-dinitroquinoxaline with glutathione (GSH) as a potential irreversible component model for PfTrxR inactivation (Scheme 1).[35] Nitro aromatics, including 6,7-dinitroquinoxaline and various nitro-2,1,3-benzothiadiazoles, are known to be substituted by secondary amines[36] and alkoxides,[37] giving rise to a mixture of 'normal'- and cine-substitution products. In the latter, the nucleophilic group takes up the position adjacent to that occupied by the leaving (nitro) group. The intermediate, a Meisenheimer adduct formed between the nucleophile and the nitro aromatic ring, is not always detected. Thiols as nucleophilic species were studied in cine-substitution of 4-halogenobenzofurazans.[38] When 1:2 concentrations of **7** and GSH were allowed to react at neutral pH, the reaction mixture became yellow within a few seconds. After 27 h, HPLC analysis revealed two new peaks (t_R = 16.8 and 16.9 min) attesting the generation of two glutathione-adducts following 'normal' and cine-substitution. The ESI-MS analysis of the reaction mixture showed three molecular mass peaks at *m*/*z* 307.9 for glutathione, at *m*/*z* 483.8 for the glutathione-**7** adducts, and at *m*/*z* 612.8 for glutathione disulfide. The process is accompanied by the slow production of a stoichiometric amount of nitrite as confirmed by azo dye formation at 540 nm in two independent experiments using 2 equiv (Fig. 2) and 5 equiv GSH at pH 8.0.[39] No nitrite formation was detected from solutions of **7** in the absence of glutathione.

During our studies, we also observed a slow time-dependent inactivation of *P. falciparum* TrxR by the most specific inhibitor, the 6,7-dinitroquinoxaline **7**. To characterize the irreversible inhibition, 20 μM PfTrxR was incubated for 15 min with 300 μM NADPH and 100 μM compound **7**. In direct comparison with a control, ca. 15% of TrxR was modified irreversibly. This irreversible inhibition of TrxR is a slow process, as judged from the second-order rate constant k_2 evaluated as 120 M^{-1} s^{-1} determined from the equation $d[E]/dt = k[E][I]$ by using 5-fold excess of inhibitor (100 μM) over enzyme (20 μM). It should be noted that reduction

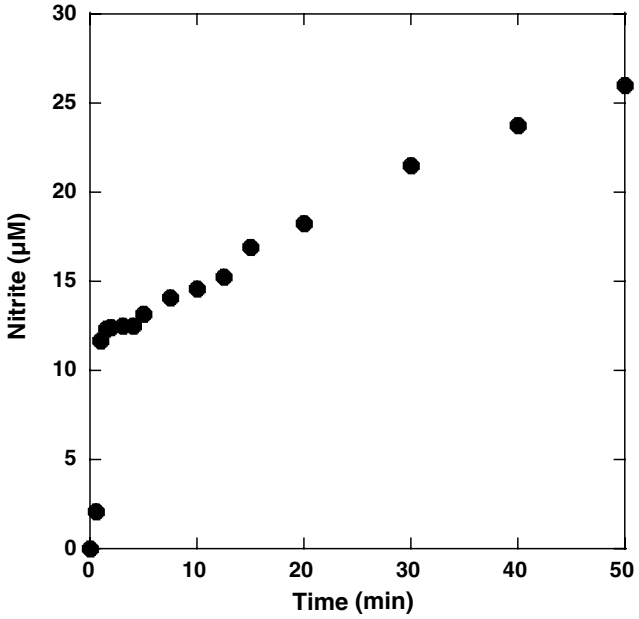


Figure 2. Kinetics of nitrite formation in the course of glutathionylation of 6,7-dinitroquinoxaline (**7**). 6,7-Dinitroquinoxaline (**7**) (500 μM, 1 equiv), 500 mM NEt_3 in 150 mM phosphate buffer, pH 8.0, were allowed to react with GSH (1000 μM, 2 equiv). Denitration occurred as a function of time.

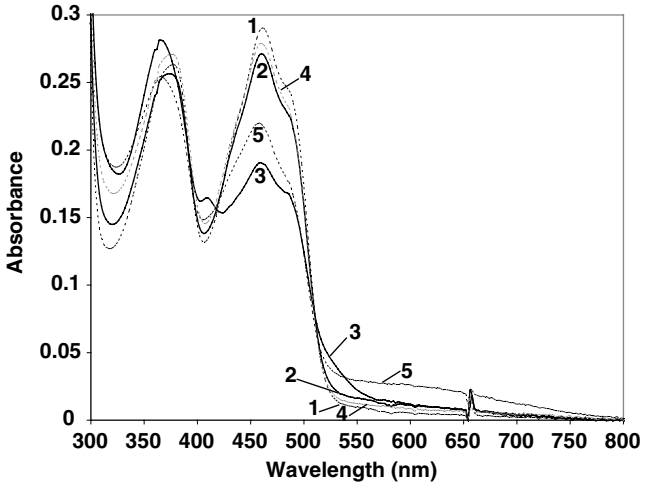
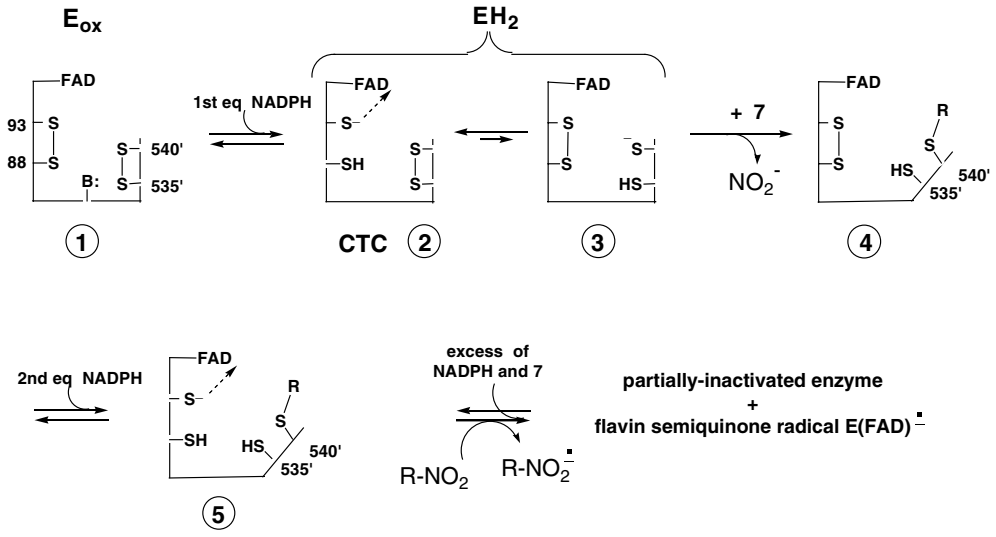


Figure 3. Reaction of 1 equiv NADPH-prereduced PfTrxR (EH_2) with 6,7-dinitroquinoxaline (**7**). Oxidized PfTrxR (curve 1) was reduced with 1 equiv NADPH per FAD giving rise to the charge transfer complex (CTC). Reaction of 6,7-dinitroquinoxaline (**7**) with reduced PfTrxR leads to apparent partial reoxidation of the protein over a 60 min period (curves 2–4). Addition of further NADPH (curve 5, 2.25 equiv NADPH total) restored the CTC at 540 nm. This behavior of the CTC absorbance makes reactions of the N-terminal thiols unlikely.

of the enzyme by NADPH is a prerequisite for this irreversible inhibition. Residual TrxR activity was also measured in the DTNB reduction assay. As an additional control, we added 2 μM inhibitor (appr. IC_{50}) directly to the assay and observed a 70% inhibition. When incubating the enzyme with NADPH and 2 μM inhibitor in the assay for 30 min, the inhibition increased to 85%. Thus, appr. 15% (irreversible) enzyme inhibition occurred in addition to the 70% reversible uncompetitive inhibition within 30 min.

In glutathione conjugate addition, following the rate-limiting attack of a nucleophile, reactions are normally considered to be essentially irreversible processes as evidenced by denitration; this last event results in either the normal substituted product (Scheme 1, pathway A) or in the final cine-substituted product following deprotonation of the Meisenheimer complex intermediate (Scheme 1, pathway B). Regarding the reversible nature of any covalent enzyme–inhibitor adducts formed, it must be assumed that within the PfTrxR-active site, the Meisenheimer adduct intermediate generated from the bound inhibitor **7** might be insufficiently stabilised and/or not well-oriented such that base-catalyzed proton subtraction is disfavored and the reverse reaction may occur (Scheme 1, pathway B). In a complete time-dependent PfTrxR inactivation, when TrxR is inactivated with increasing concentrations of **7** as a function of time, the residual steady-state rate did not decrease to a large extent nor tend to zero (data not shown). This does not support a truly irreversible inactivation mechanism as shown in glutathionylation mechanism (Scheme 1) but rather is consistent with the proposed reversible disruption of the Meisenheimer complex intermediate in a cine-substitution mechanism (Scheme 1, pathway B), giving back to the starting 6,7-dinitroquinoxaline **7**. Noteworthy is to mention that the resulting PfTrxR inhibition is very complex because the irreversible component resulting from preincubation of enzyme, NADPH and compound **7** is masked by the redox activity of the compound and the instability of PfTrxR in the presence of NADPH. The latter was also reported for the related *P. falciparum* glutathione reductase.[40]

As shown in Figure 3, the spectrum of the oxidized form (E_{ox}) of the recombinant enzyme, with a principal flavin peak at 460 nm (curve 1), is characteristic of other enzymes in the disulfide reductase family (Scheme 2, enzyme species 1). Formation of the 2-electron-reduced enzyme (EH_2) is accompanied by characteristic partial bleaching of the oxidized flavin spectrum and formation of a long-wavelength band, best measured at 540 nm due to the reduction of the disulfide bond between



Scheme 2. Proposed mechanism for TrxR irreversible inactivation by 6,7-dinitroquinoxaline **7**. Compound **7** reacts with one of the EH_2 species reduced at the C-terminus, species (**2**) after base-catalyzed denitration.

Cys93 and Cys88, and the formation of a thiolate–flavin charge transfer complex (CTC) between the flavin and the flavin-interacting thiol, Cys93. In this preliminary experiment for technical reasons, 1.0 equiv of NADPH and an excess of compound **7** were added together as described in the Ref. 41 (curve 2); it can be seen that most of the flavin is oxidized and there is very little CTC. Subsequent steps will show that the NADPH has reduced the flavin and reducing equivalents have passed to the N-terminal disulfide and then to the C-terminal disulfide (Scheme 2, enzyme species 2 and 3), which interpretation is reinforced by a previous work on the reaction of EH_2 with unsaturated Mannich bases.[18] The C-terminal thiolate has attacked compound **7** to form enzyme species 4. An additional 0.75 equiv of NADPH was added (curve 3) and the long-wavelength band indicates CTC formation. The shape of the CTC band is distorted somewhat due to the presence of a small amount of the flavin semiquinone as evidenced by the peak at 410 nm. Over the period of 1 h, further reaction between EH_2 and compound **7** leads to the loss of CTC (curve 4) and flavin reoxidation confirming the interpretation of the first step in the experiment. Addition of a further 0.5 equiv NADPH (curve 5) results again in CTC formation, in this case without distortion by the flavin anion radical (Scheme 2, enzyme species 5).

These data indicate that compound **7** reacts with EH_2, most likely with a C-terminal thiolate.[18] As shown in Scheme 2, EH_2 is an equilibrium mixture of the CTC and an enzyme form having the C-terminal thiols reduced (Scheme 2, species 3). This enzyme form can attack compound **7** catalyzed by a nearby base in the enzyme-active site (Schemes 1 and 2). The experiment was continued with further addition of NADPH and compound **7** (data not shown). The reoxidized enzyme had a normal spectrum and was found active in the NADPH oxidase assay. The oxidase activity of the native enzyme is low but has been increased by reaction with compound **7** (Schemes 1 and 2).

In an independent experiment, fresh PfTrxR (20 μM) was incubated with 300 μM NADPH and 100 μM compound **7** for 2.5 h at 25 °C. This resulted in a nitrite production of ca. 6 μM (in the enzyme solution, this means that appr. 6 μM SH-groups—of the 20 μM enzyme—are likely to have reacted). Based on the nitrite production—assuming that only one Cys residue is modified and leads to complete enzyme inhibition—we calculate a k_2 of 24 M^{-1} min^{-1}.

The effects of the three most potent PfTrxR inhibitors, **3**, **7**, and **11**, on the growth of malarial parasites were examined by testing on the chloroquine-resistant *P. falciparum* strain K1 in cell culture.[42]

IC_{50} values—indicating the drug concentration required for 50% parasite growth inhibition—were determined to be 11, 15, and 18 μM, respectively (Fig. 4). Taking into account activity losses due to incomplete uptake and metabolic turnover of the inhibitor, values are in good agreement with the kinetic data.

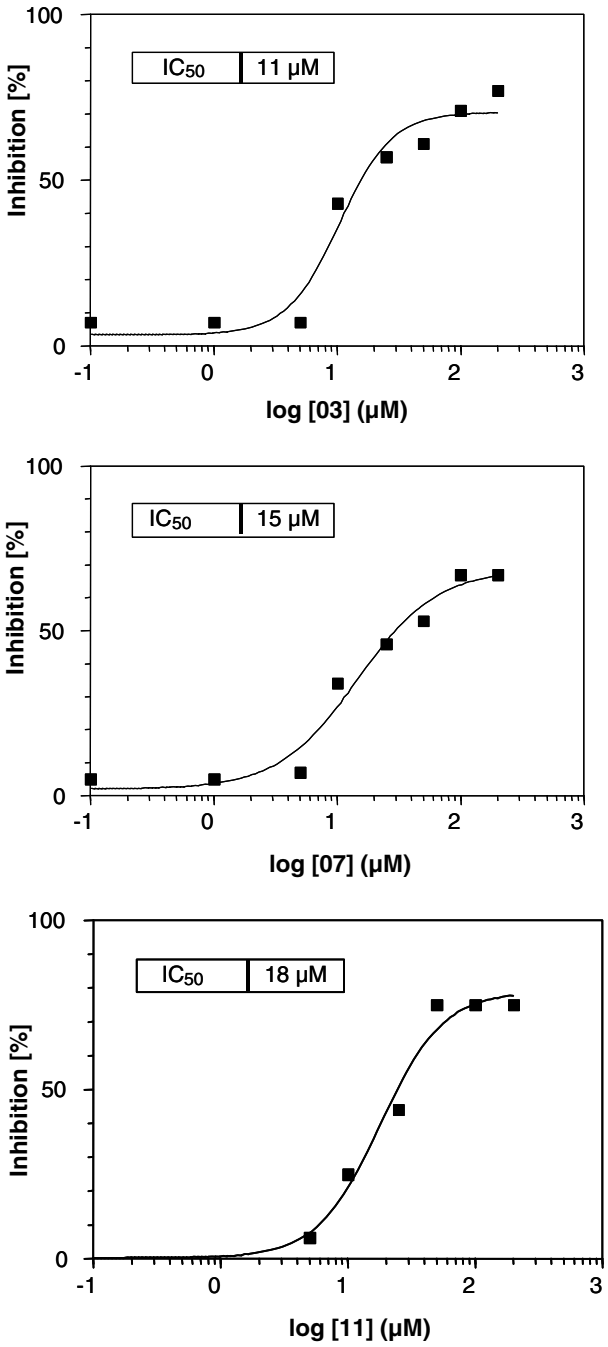


Figure 4. Effects of the most potent and selective PfTrxR inhibitors, **3** (top), **7** (middle), and **11** (bottom), on the growth of the *Plasmodium falciparum* strain K1 in cell culture. Inhibitors were applied to synchronized ring stage parasites. Parasitemia was assessed under the microscope after 48 h.

The most effective compound on cell culture, compound **3**, was also studied using the incorporation of [^{3}H]-hypoxanthine as a metabolic parameter to determine growth inhibition. In this assay, an IC_{50} value of 2.9 ± 0.82 μM was determined. This result is in good agreement with the fact that most drugs exhibit lower IC_{50} values when assessed with this metabolic method rather than with the determination of parasitemia. Along these lines, the IC_{50} for chloroquine determined in parallel was between 350 and 450 nM when counting

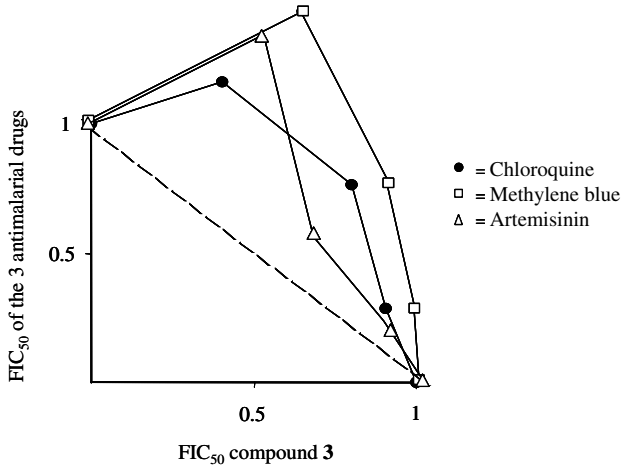


Figure 5. Interaction of compound **3** with clinically used antimalarial drugs as determined on the chloroquine-resistant *Plasmodium falciparum* strain K1. FIC = fractionary inhibitory concentration.

parasites and 120 ± 28 nM when using [^{3}H]-hypoxanthine incorporation. Furthermore, combinations of compound **3** with the clinically used antimalarials chloroquine, methylene blue, and artemisinin were tested using [^{3}H]-hypoxanthine incorporation in combination with the isobologram method.[43,44] Data obtained for compound **3** are shown in Figure 5. They clearly demonstrate that the PfTrxR inhibitor acts antagonistically with all three established antimalarial drugs.

In our earlier attempt to selectively inhibit PfTrxR with Mannich bases, we postulated irreversible inactivation of the PfTrxR by bisalkylation of α,β-unsaturated Mannich bases by the C-terminal thiols after observation of competitive inhibition with DTNB.[18] The inhibitors in the present study, have been shown to act uncompetitively, and to an approximately equal extent, in the presence of both physiological substrates of PfTrxR. All compounds tested were active on malarial parasites in the low micromolar range in both chemical and biological assays. Taken together our data indicate that specificity for PfTrxR can be achieved by inhibitors that bind differentially the to enzyme–substrate complex. A possible binding site of these inhibitors is the interface between the two subunits of the enzyme—a hypothesis we will try to verify and utilize for further inhibitor development.

Acknowledgments

The authors thank Elisabeth Fischer and Marina Fischer for their excellent technical assistance. Our work was supported by the Deutsche Forschungsgemeinschaft (SFB 535 'Invasion Mechanisms and Replication Strategies of Pathogens', project A12 to K.B. and SFB 544 'Control of Tropical Infectious Diseases' project B14 to E.D.C.), by the Centre National de la Recherche Scientifique (E.D.C.), by the NIGMS (GM11106) from the National Institute of General Medical Sciences, by the DAAD (scholarship to MBA), and by the OVPR at the University of Michigan.

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25. *Materials and analytical methods.* NADPH was obtained from BioMol and GSSG from Sigma. All starting materials and solvents were purchased from Aldrich, Fischer Scientific or Roth at the highest commercially available quality and were used without further purification. Melting points were recorded with a Fisher Scientific melting point apparatus and are uncorrected. Elemental analyses were performed at the University of Michigan Elemental Analysis Laboratory. Analytical HPLC was run on a

Nucleosil 100-5 C18 column (Macherey & Nagel, 4× 300 mm, 100 μm, 300 Å) using a Merck-Hitachi L-6200 instrument with an L-4000 UV detector set at 254 nm. The reaction mixtures were directly injected into the HPLC apparatus. The components were separated at a flow rate of 1.0 mL/min using the following conditions: 100% solvent A (0.05% TFA in H_2O) for 5 min, followed by a linear gradient from 100% solvent A to 100% solvent B (0.05% TFA in (v/v) 80% acetonitrile–20% H_2O) over 15 min and 100% B for another 10 min. The retention times (t_R) are given in min. ESI-MS of the glutathionylation products was carried out in Johannes Lechner's group at the Biochemie-Zentrum der Universität Heidelberg.

26. *Chemistry*. Compounds **1**, **8**, and **9** were purchased from Aldrich Chemical Co., while compound **2** was obtained from the Interbioscreen Compound Library. 4-Nitro-2,1,3-benzothiadiazole (**3**): mp 107–108 °C (mp 107 °C);[37] 5-nitro-2,1,3-benzothiadiazole (**4**): mp 130–131 °C (mp 133 °C);[45] 4,6-dinitro-2,1,3-benzothiadiazole (**5**): mp 135–136 °C; 6-nitroquinoxaline (**6**): mp 175–176 °C (mp 177–179 °C);[46] 6,7-dinitroquinoxaline (**7**): mp 195–196 °C (mp 193–194 °C);[47] bis-(2-nitro-4-trifluoromethylphenyl) sulfide (**10**): mp 145–146 °C (mp 146–147 °C);[48] bis-(2,4-dinitrophenyl) sulfide (**11**): mp 193–194 °C were synthesized according to reported procedures. The purity of compounds **5** and **11**—for which the melting point was not reported in the literature—was controlled by elemental analyses with data that agreed with the calculated values within 0.4%. Compound (**5**): Anal. calcd for $C_6H_2N_4O_4$ S: C, 31.86; H, 0.89; N, 24.77; S, 14.18. Found: C, 32.04; H, 0.80; N, 24.87; S, 14.04. Compound (**11**): Anal. calcd for $C_{12}H_6N_4O_8S$: C, 39.35; H, 1.65; N, 15.30; S, 8.75. Found: C, 39.77; H, 1.59; N, 15.45; S, 8.99.
27. *Enzyme inhibition.* PfTrxR and PfTrx were prepared as described by Kanzok et al.,[5] purification of the human enzyme from placenta carried out according to Gromer et al.[49] with slight modifications. PfGR was produced as described by Färber et al.,[50] hGR according to Nordhoff et al.,[51] both enzymes kindly provided by Prof. Heiner Schirmer, Biochemie-Zentrum, Heidelberg University. The human thioredoxin mutant C72S was prepared as described by Irmler et al.[25]
    For comparative purposes, the inhibitory potency of the compounds against both *P. falciparum* and human TrxR was assessed by determining the IC_{50} values under standard conditions. TrxR was assayed in a total volume of 500 μL in 100 mM potassium phosphate, 2 mM EDTA, pH 7.4, with 100 μM NADPH and appr. 10 mU/mL TrxR. The reaction at 25 °C was monitored by the decrease in absorbance at 340 nm and was started without preincubation (<30 s) with 20 μM PfTrx or hTrx C72S. Since all inhibitors were dissolved in DMSO, controls containing the respective DMSO concentration (max 2%) were measured in parallel and subtracted.GR assays were carried out at 25 °C in 50 mM potassium phosphate, 200 mM KCl, and 1 mM EDTA, pH 6.9. The NADPH concentration was 100 μM, appr. 1 nM PfGR or hGR was used for the 1 mL assays. In the presence of inhibitor concentrations resulting in 50% inhibition of PfTrxR, NADPH consumption was assessed immediately after starting with 1 mM GSSG. Compounds, which showed good selective inhibition of PfTrxR—in comparison with hTrxR—as indicated by their IC_{50} values, were tested further with varying inhibitor and substrate concentrations to determine the inhibition type.
    To test time-dependent enzyme inhibition, *P. falciparum* TrxR (20 μM) was allowed to react for 15 min with 300 μM NADPH in the presence or absence of 100 μM compound **7** in a final volume of 50 μL at 25 °C. All reaction mixtures contained 2% DMSO. Five microliters of the reaction mixture was removed and the residual activity was measured in the Trx standard assay (see above). Both unreacted and **7**-reacted enzymes were dialyzed overnight and then tested for residual activity in the Trx assay.
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35. *Glutathionylation of 6,7-dinitroquinoxaline (**7**).* The glutathionylation product of compound **7** was prepared by mixing 736 μL of a 10 mM solution of **7** in DMSO (442 μM final concentration) with an equal volume of 20 mM GSH in water (884 μM final concentration), in the presence of a 9.7 mM triethylamine solution in water (final pH 7.4). The suspension was allowed to react at 25 °C. The glutathionylation products were analyzed by HPLC (Nucleosil C-18) by injecting 30 μL aliquots after different times (0 min to 27 h) and by electrospray mass spectroscopy (ESI-MS). The retention times were 16.8 and 16.9 min for the glutanionylated-**7** products ('normal'- and cine-substitution products) and 21.3 min for the starting 6,7-dinitroquinoxaline **7**. The percentage of the starting materials converted with time was calculated from the peak area ratio. The reaction mixture was also analyzed by ESI-MS after completion.
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39. *Nitrite formation.* The rate of denitration during glutathionylation and enzymatic reactions was determined spectrophotometrically at 540 mM by monitoring the concentrations of nitrite following formation of the red azo dye in the presence of sulfanilamide, naphthylethylenediamine dihydrochloride, and the fourfold diluted reaction mixture as described.[52] Nitrite solutions (10–100 μM) were used for the calibration curve.
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41. *Anaerobiosis spectroscopy, NADPH titration, and reaction of 6,7-dinitroquinoxaline (**7**) with prereduced PfTrxR, EH_2.* The reductive titration was performed under anaerobic conditions at 25 °C. Anaerobiosis was achieved by alternately degassing under vacuum and equilibration with ultrapure argon for eight cycles as described previously.[53] The enzyme solution was protected from light during anaerobiosis and reductive titration. The NADPH stock solution was prepared in 40 mM Tris base solution (pH 9.0) and was made anaerobic by argon flushing for 10 min

prior to use. The concentration of the anaerobic NADPH solution was determined spectrophotometrically at 340 nM ($\varepsilon_{340nm} = 6.22\ mM^{-1}\ cm^{-1}$). Absorption spectra were recorded on a HP 8452A spectrophotometer. All spectra were corrected for slight turbidity by subtracting the absorbance between 800 and 820 nm. A typical experiment proceeded in three phases: first, 25.7 μM TrxR (in a cuvette with two sidearms similar to those described previously in Ref. 53) was reduced anaerobically in 100 mM sodium phosphate buffer, pH 7.0, by 1.1 equiv NADPH (contained in a 100 μL gastight Hamilton syringe) forming the FAD-thiolate CTC; next, 3 equiv of compound **7** per FAD-containing subunit (20 mM inhibitor solution in 100% DMSO contained in one sidearm of the cuvette) was added and the effect on the CTC was followed. In the described experiment, an undefined trace amount of compound **7** was already present in the enzyme solution before NADPH addition; this is, likely due to volatility of the compound revealed during the eight vacuum-argon cycles. Finally, the protein modified by 3 equiv of compound **7** was titrated with additional NADPH and again the effect of enzyme modification on the FAD-thiolate CTC was examined. The enzyme was reoxidized by opening the cuvette to air and tested for residual activity. In parallel, two controls were prepared according to the same titration conditions over the same period of time and assayed for residual Trx reduction at the end of the titration. The first control, that is, 'control TrxR-NADPH', consisted of TrxR incubated with the same amount of NADPH as in the titration. The second control, that is, 'control TrxR inhibitor', consisted of TrxR incubated with inhibitor but in the absence of NADPH. This study was performed using a recombinant enzyme with a specific activity of 18 U/mg which was >95% pure as determined by SDS–polyacrylamide gel electroporesis (data not shown). The specific activities of wild type TrxR and inhibitor-reacted enzyme from titration experiments were determined in the Trx reduction assay. It was carried out at 25 °C in a 1 mL cuvette containing 100 mM phosphate buffer, pH 7.0, in the presence of 100 μM NADPH, 0.01 to 0.1 μM TrxR (unreacted and inhibitor-reacted TrxR), and 50 μM Trx. The change in absorbance was measured at 340 nm.

42. *Cultivation of malarial parasites and inhibitor testing in cell culture.* For testing the effects of the three most potent PfTrxR inhibitors (compounds **3**, **7**, and **11**) on the growth of malarial parasites, we employed the chloroquine-resistant *P. falciparum* strain K1 cultivated according to Trager and Jensen,[54] with slight modifications. The parasites were synchronized to the ring stage and parasitemia was adjusted to 1–1.5% and hematocrit to 3.3%. For each inhibitor, 12 wells and 2 control wells were prepared. The inhibitors were dissolved and diluted in DMSO, sterile filtered, and added to the cultures resulting in final concentrations of 100, 10, 1, 0.1, and 0.01 μM, respectively, and maximal DMSO concentrations of 1%. Cultures were incubated at 37 °C, 5% O_2 and 3% CO_2 for 48 h. Blood smears were prepared after 24 and 48 h, and parasitemia was determined by counting Giemsa-stained parasites under the microscope. Percentage growth inhibition was plotted against the logarithmic concentration of the inhibitor and the IC_{50} values were calculated using the software Prism 3.0.
43. *Testing of drug combinations in cell culture.* The isotopic drug sensitivity assay by means of the semiautomated microdilution technique[55] was employed to study drug combination effects[44] with chloroquine, methylene blue, and artemisinin. This method is based on the incorporation of radioactive (^{3}H)-hypoxanthine by the malarial parasite after one growth cycle in hypoxanthine-free medium. In a 96-well microtiter plate (Nunc^R), a twofold serial dilution of four times the starting concentration of each drug to be tested (100 μL) was carried out from the upper wells to the lower wells of the plate. As such, the last lower wells contained 64 times more diluted drug as the initial upper wells. Parasites were incubated in these serial drug dilutions at a parasitemia of 0.125% and a hematocrit of 1.25% in hypoxanthine-free medium in a total volume of 200 μL. After 48 h, 50 μL of ^{3}H hypoxanthine (0.5 μCi) was added to each well and incubated for another 24 h. Each well was harvested, dried, and the viability of the parasites in each well, which is proportional to the radioactive counts, was assessed. Fractional inhibitory drug concentrations were obtained with FIC_{50} A = (IC_{50} of A + B)/(IC_{50} of A), FIC_{50} B = (IC_{50} of B + A)/(IC_{50} of B), and FIC_{50} of the drug combination = FIC_{50} A + FIC_{50} B. If this FIC_{50} value is <1, the two drugs act synergistically, if it is equal to 1 the two drugs act additively, and if it is >1 the drugs act antagonistically.
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