

High Throughput Screening against the Peroxidase Cascade of African Trypanosomes Identifies Antiparasitic Compounds That Inactivate Tryparedoxin^{*[S]}

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Background: Hydroperoxide detoxification in African trypanosomes relies on a cascade composed of trypanothione, trypanothione reductase, tryparedoxin, and tryparedoxin peroxidases.

Results: A library screening against the peroxidase system unraveled trypanocidal compounds that inactivate tryparedoxin *in vitro* and in the intact parasite.

Conclusion: Tryparedoxin is a druggable target.

Significance: Detection of novel target molecules is a crucial step to overcome the unsatisfactory chemotherapy of sleeping sickness.

In African trypanosomes, the detoxification of broad spectrum hydroperoxides relies on a unique cascade composed of trypanothione (T(SH)₂), trypanothione reductase, tryparedoxin (Tpx), and nonselenium glutathione peroxidase-type enzymes. All three proteins are essential for *Trypanosoma brucei*. Here, we subjected the complete system to a high throughput screening approach with nearly 80,000 chemicals. Twelve compounds inhibited the peroxidase system. All but one carried chloroalkyl substituents. The detailed kinetic analysis showed that two compounds weakly inhibited trypanothione reductase, but none of them specifically interacted with the peroxidase. They proved to be time-dependent inhibitors of Tpx-modifying Cys-40, the first cysteine of its active site WCPC motif. Importantly, gel shift assays verified Tpx as a target in the intact parasites. T(SH)₂, present in the *in vitro* assays and in the cells in high molar excess, did not interfere with Tpx inactivation. The compounds inhibited the proliferation of bloodstream *T. brucei* with EC₅₀ values down to <1 μM and exerted up to 83-fold lower toxicity toward HeLa cells. Irreversible inhibitors are traditionally regarded as unfavorable. However, a large number of antimicrobials and anticancer therapeutics acts covalently with their target protein. The compounds identified here also interacted with recombinant human thioredoxin, a distant relative of Tpx. This finding might even be exploited for thioredoxin-based anticancer drug development approaches reported recently. The fact that the T(SH)₂/Tpx couple occupies a central position within the trypanosomal thiol metabolism and delivers electrons also for the synthesis of DNA precursors renders the parasite-specific oxidoreductase an attractive drug target molecule.

Trypanosomes and *Leishmania* are the causative agents of a variety of tropical diseases. Chemotherapy of diseases caused by these parasitic protozoa can hardly be rated satisfactory. The very few drugs available suffer from high toxicity, the need of hospitalization, and increasing resistance development. One approach toward the development of novel antimicrobial agents is the identification of pathways that do not occur or are substantially different in the mammalian host. In this context, the unique hydroperoxide metabolism of trypanosomatids is an attractive target. *Trypanosoma brucei*, the causative agent of African sleeping sickness, lacks catalase and classical glutathione peroxidases. Detoxification of hydroperoxides relies on 2-Cys-peroxiredoxins (Prxs)² and nonselenium glutathione peroxidase-type (Px) enzymes, both of which act as tryparedoxin (Tpx) peroxidases (Scheme) (1, 2). The Prx-type enzymes exert a strong preference for hydrogen peroxide as substrate, whereas the Px-type peroxidases accept fatty acid-derived hydroperoxides and protect the parasite from membrane damage (3).

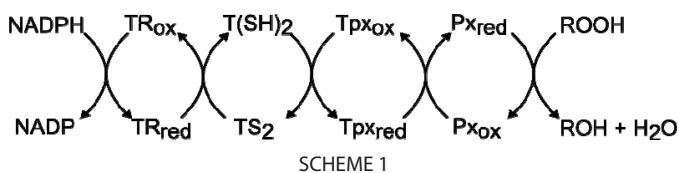
Trypanothione (T(SH)₂, bis(glutathionyl)spermidine) is the main low molecular mass thiol of these parasites. The dithiol is kept reduced by NADPH and the flavoenzyme trypanothione reductase (TR), the system replacing the glutathione/glutathione reductase couple of the mammalian host (4). TR is the most thoroughly studied enzyme of the trypanothione metabolism, and a large number of inhibitors has been identified (5–9). Tpx is a distant member of the thioredoxin (Trx) protein family. The parasite-specific protein has a WCPC active site motif instead of the canonical WCGPC sequence in Trxs, and with 16,000 Da, it is larger than human Trx (12,000 Da) (10, 11). Tpx is kept in the dithiol state by the spontaneous reaction with T(SH)₂ (11,

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[S] This article contains supplemental Figs. S1–S3 and Tables S1 and S2.

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² The abbreviations used are: Prx, 2-Cys-peroxiredoxin; HTS, high throughput screening; MAL-PEG, α-methoxy-ω-ethylmaleimide poly(ethylene glycol); Px, nonselenium glutathione peroxidase-type enzyme; t-bOOH, tertiary butyl hydroperoxide; TCEP, tris(2-carboxyethyl)phosphine; Tpx, tryparedoxin; TR, trypanothione reductase; Trx, thioredoxin; T(SH)₂, trypanothione.



12), whereas the host Trx is reduced by thioredoxin reductase, an enzyme also missing in trypanosomatid organisms. *T. brucei* Tpx plays a central role in most of the T(SH)₂-dependent parasite pathways (1). Examples are the detoxification of hydroperoxides and, as shown recently, the reduction of protein-bound methionine sulfoxide residues (13). Most importantly, the T(SH)₂/Tpx system delivers the reducing equivalents for the synthesis of DNA precursors catalyzed by ribonucleotide reductase and thus is involved in parasite replication (14).

Previous high throughput screening (HTS) approaches against the parasite trypanothione system mainly focused on the detection of TR inhibitors. Different chemotypes were identified that showed selectivity for TR over human glutathione reductase (15). Another approach revealed compounds with potent antiparasitic activity, but only moderate correlation with TR inhibition (6). All enzymes establishing the parasite peroxidase system, namely TR, Tpx, and both types of tryparedoxin peroxidases, have been shown to be essential for *T. brucei* (16–19) and thus fulfill a crucial prerequisite of a putative drug target molecule. Recently the effect of the antitumor quinol PMX 464 on the parasite peroxidase systems has been studied (20). In mammalian and yeast cells, the quinol inhibits thioredoxin (21–23). Toward *T. brucei*, PMX 464 showed cytotoxic activity and caused depletion of cellular T(SH)₂. *In vitro*, the compound interacted with T(SH)₂ and both the Prx- and Px-type enzymes but neither with TR nor Tpx (20). Here, we present an HTS approach toward the peroxidase system of African trypanosomes followed by the *in vitro* and *in vivo* identification of the target protein. Primary goal was to identify putative lead compounds for a drug design directed against the Px-type enzyme. Interestingly, the analysis resulted in compounds that specifically inactivated Tpx. Importantly, Tpx could be demonstrated to be targeted in the intact parasite.

EXPERIMENTAL PROCEDURES

Materials—NADPH was purchased from AppliChem; *t*-bOOH, TCEP, Tween 20 and DMSO were from Sigma; and MAL-PEG (5272 Da) was from Iris Biotech. Recombinant tag-free *T. brucei* Px (24), *T. brucei* Prx (3), wild type *T. brucei* His₆-Tpx, C40S-Tpx-His₆, and C43S-Tpx-His₆ (25), *T. cruzi* TR (26), T(SH)₂, and trypanothione disulfide (27) were prepared as described. To obtain tag-free Tpx, the coding region was amplified by PCR from pQE-60-*tpx* (11), cloned into the pETtrx_1b vector (kindly provided by G. Stier, EMBL), and overexpressed in *Escherichia coli*. The protein was purified following the procedure for Px (24). A sample of human Trx was a kind gift of Dr. R. H. Schirmer (Biochemie-Zentrum der Universität Heidelberg). The compound library used is a collection of commercial chemicals, purchased from three vendors (AMRI, ENAMINE, and ChemBridge). The compounds were selected around carefully picked scaffolds that represent a subset of analogs. The library represents an optimal coverage of

available chemical space around each scaffold, and the compounds fulfill the Lipinski criteria (28).

HTS Assay—The assays were performed in a total volume of 20 μ l of 20 mM Na-HEPES, 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20, pH 7.5 (screening buffer), in black 384-well plates with a round clear bottom (Corning Glass). The plates were prepared with 2 μ l of 400 μ M compounds (4% DMSO) as described by Sehr *et al.* (29). 10 μ l of 600 μ M *t*-bOOH in screening buffer was added. The reaction was started immediately by adding 8 μ l of a premixture containing NADPH, TR, T(SH)₂, Tpx, and Px, which resulted in a final concentration of 280 μ M NADPH, 40 nM TR, 100 μ M T(SH)₂, 8 μ M Tpx, 20 nM Px, 300 μ M *t*-bOOH, and 0.4% DMSO. After 4 min of centrifugation at 1000 \times *g* to remove air bubbles, the plates were transferred to an Envision multilabel plate reader (PerkinElmer Life Sciences), and NADPH consumption was recorded at 340 nm and 25 °C. The first data point was taken after 15 min. In total, nine reads (one data point every 19 min) were monitored. The absorption decrease between the second and seventh data point was used to calculate the peroxidase activity. Columns 1 and 2 of each plate contained 0.4% DMSO corresponding to full activity (0% inhibition). In columns 23 and 24, the reaction mixtures lacked Px and thus represented the spontaneous reaction of *t*-bOOH with T(SH)₂ (100% inhibition). The data were evaluated using Activity Base (IDBS). The percentage of inhibition was calculated as $100 \times (1 - ((x - \mu^-) / (\mu^+ - \mu^-)))$, where x is the slope of the absorption decrease/time in the presence of inhibitor; μ^- is the mean slope of the negative controls (0% inhibition), and μ^+ is the mean slope of the positive control (100% inhibition). The Z' factor as quality parameter of the assays was calculated from the controls in column 1, 2, 23, and 24 (30).

IC₅₀ Determinations—Compounds that in the HTS revealed >20% inhibition were re-ordered. The assays were conducted as described above using an 11-point titration from 200 μ M to 200 nM. The final concentration of DMSO was 2%. The percentage of inhibition was plotted against the compound concentration, and IC₅₀ values were calculated.

EC₅₀ Determinations—Bloodstream *T. brucei* (strain 449) were grown as described (18). 10 mM stock solutions of the compounds were prediluted to 500 μ M and then serially 1:5 (7 point titration) with DMSO. Aliquots of 10 μ l were spotted on a 24-well plate (Greiner), and 990 μ l of trypanosome culture (5×10^5 cells/well) was added. Cells cultured in the presence of 1 and 9% DMSO served as negative and positive control, respectively. After 24 h, living cells were counted using a hemocytometer. Cell density was plotted against the compound concentration, and EC₅₀ values were calculated. For the determination of EC₅₀ values after 72 h, the compound stock solutions were diluted with medium to 500 μ M and then 1:1 with 5% DMSO in HMI-9 medium (10-point titration). 10- μ l aliquots were spotted on a 96-well plate (PerkinElmer Life Sciences), and 90 μ l of trypanosome suspension (250 cells) was added resulting in 2500 cells/ml and a final concentration of 0.5% DMSO. HeLa (Kyoto) cells (provided by Dr. R. Pepperkok, EMBL) were cultured as described by Erfle *et al.* (31). 1000 cells/well (90 μ l) were seeded in a 96-well plate. The next day, 10 μ l of compound solution (diluted in DMEM) was added to the adherent cells. After 72 h, proliferation of triplicate cultures (*T. brucei* and HeLa cells) was

Antiparasitic Inhibitors of Tryparedoxin

quantified using ATPLite one-step (PerkinElmer Life Sciences) following the protocol of the manufacturer. The luminescence of each sample, expressed as percentage of that of the control cultures, was plotted against the compound concentration, and EC_{50} values were calculated. Columns 1 and 12 of each plate with 0.5 and 9% DMSO served as negative and positive controls, respectively.

TR Inhibition—Inhibition of TR was measured in a 384-well plate. Plates were prepared with 2 μ l of an 11-point titration of the compounds. 10 μ l of a premixture of NADPH and TR in screening buffer was added, and the reaction was started by 8 μ l of trypanothione disulfide in screening buffer. The final assay volume was 20 μ l containing 280 μ M NADPH, 0.3 nM TR, 150 μ M trypanothione disulfide, and 2% DMSO. The Z' factor was calculated from the controls in columns 1 (0% inhibition, with DMSO) and 24 (100% inhibition, without TR), respectively. The assay was conducted as described above for the IC_{50} determinations with nine data points (one read every 10 min).

Cuvette-based Peroxidase Assays—In a total volume of 200 μ l, a premixture of NADPH, TR, T(SH)₂, Tpx, and Px in screening buffer was mixed with the compounds, resulting in final concentrations of 140 μ M NADPH, 220 nM TR, 100 μ M T(SH)₂, 10 μ M Tpx, 90 nM Px, and 40, 20, or 10 μ M inhibitor or 2% DMSO. The reaction was started either directly or after 40 min of preincubation by adding 100 μ M H₂O₂, and the absorption decrease at 340 nm was followed at 25 °C. To evaluate if a compound specifically targeted the peroxidase, Px was replaced by 150 nM Prx. To analyze if Tpx was the target protein, premixtures containing all components except either Tpx or Px were incubated with the compounds. After 40 min, Tpx or Px was added immediately before starting the assay with H₂O₂.

ESI-MS Analysis of *T. brucei* Tpx and Human Trx—10 μ M Tpx (His₆-tagged or tag-free) was treated with 100 μ M T(SH)₂ for 10 min at 25 °C in screening buffer without Tween 20. Reduced human Trx was obtained by incubating 10 μ M protein with 100 μ M DTT. In a final volume of 100 μ l, the reduced and oxidized protein species were incubated with 40 μ M of compounds **1–6** for 40 min at 25 °C. The final concentration of DMSO was 2%. The samples were either directly subjected to ESI-MS or stored at –20 °C. The spectra presented in Fig. 3 and supplemental Fig. S1, *a–h*, were obtained from the Core Facility for Mass Spectrometry and Proteomics, Zentrum für Molekularbiologie der Universität Heidelberg, Heidelberg, Germany. The total protein masses were determined by ESI-MS on an API-QSTAR™ Pulsar instrument (Applied Biosystems) with an HPLC (Agilent) on-line-coupled to the ESI-QTOF instrument. The reaction mixtures were diluted to 1 ml with 0.1% TCA, and 100 μ l were loaded onto a 50 Poros R1 trapping column. After 1.5 min of washing with 0.1% TFA (0.4 ml/min), the protein was eluted into the electrospray ion source with 80% acetonitrile, 0.1% TFA at 10 μ l/min as described by Rist *et al.* (32). The spectra depicted in supplemental Figs. S1, *i–p*, and S2 were recorded at the Proteomics Core Facility, EMBL Heidelberg. The protein samples were desalted using C18 ZipTips (Millipore). After wetting in 50% (v/v) acetonitrile in water, equilibrating in 0.1% TFA, binding, and washing (0.1% TFA), the samples were eluted in 10 μ l of 50% acetonitrile containing 1% (v/v) formic acid. The purified protein samples were

injected into a Q TOF II mass spectrometer (Micromass) using static nanospray borosilicate needles (PicoTip). Both instruments were calibrated with apomyoglobin (Sigma), and their mass accuracy was better than 100 ppm.

Reaction of Tpx with MAL-PEG—In 40 μ l of screening buffer without Tween 20, 125 μ M wild type His₆-Tpx, C40S-Tpx-His₆, or C43S-Tpx-His₆ was treated for 40 min at 25 °C with 500 μ M of compounds **1–6** in the presence of 3.1 mM DTT. 10 μ l of MAL-PEG (50 mM in screening buffer without Tween) and 50 μ l of screening buffer were added. After 1 h at 37 °C, 25 μ l of ice-cold TCA (100% (w/v) in 20 mM HCl) was added, and the mixture was kept for 10 min at 4 °C. After centrifugation, the pellet was washed three times with 100 μ l of ice-cold acetone, and the denatured protein was resuspended in screening buffer. 20 μ g of protein was subjected to SDS-PAGE on a 15% gel followed by Coomassie staining.

Identification of Tpx as Intracellular Target— 1×10^7 trypanosomes (1×10^6 cells/ml) were incubated with different concentrations of the compounds for 0 or 4 h at 37 °C, harvested, washed twice with 6 ml of PBS, resuspended in 15 μ l of screening buffer in the presence or absence of 5 mM TCEP, and lysed by three cycles of freezing-thawing followed by 30 min of incubation at 37 °C. 5 μ l of 50 mM MAL-PEG was added, and the reaction mixture was incubated for another 30 min. Lysates corresponding to 5×10^6 cells were subjected to SDS-PAGE as described above, followed by Western blot analysis with the polyclonal rabbit antiserum against *T. brucei* Tpx (1:2000) (19). Horseradish peroxidase-conjugated anti-rabbit IgGs served as secondary antibody (1:20000; Santa Cruz Biotechnology). Bands were visualized by chemiluminescence using the Super Signal west pico kit (Pierce).

RESULTS

Assay Principle, Miniaturization, and Optimization—Tryparedoxin peroxidase activity is measured in a coupled assay following NADPH consumption (18, 33) (see Scheme 1). The cuvette-based assay was adapted for the HTS approach of 78,637 compounds. Although *T. brucei* Px has higher activity with hydrogen peroxide (3, 33), *t*-BOOH was used as substrate because of its much slower spontaneous reduction by T(SH)₂ (34). Under the conditions chosen, the reaction rate was linear for at least 2 h and dependent on the Px activity. The slope of each graph was obtained by linear regression, and percentage of inhibition was calculated as outlined under “Experimental Procedures” and shown as an example for compound **6** (Fig. 1*a*). The fit of the curve to the data was calculated. For all compounds that were further characterized (see below), the R^2 value was ≥ 0.92 . With Z' -factors of ≥ 0.68 , the assay performance and robustness proved to be very good (Fig. 1*b*). In comparison with a single end point determination, this kinetic data acquisition not only gives a more robust assay signal but also minimizes the probability of false-positive results, for instance in the case of compounds that absorb at 340 nm (35, 36).

HTS Results and Intracellular Activity—The HTS approach yielded 36 compounds that inhibited the peroxidase system by $\geq 20\%$ at a concentration of 40 μ M. This relatively low overall hit rate is comparable with HTSs reported for other parasite

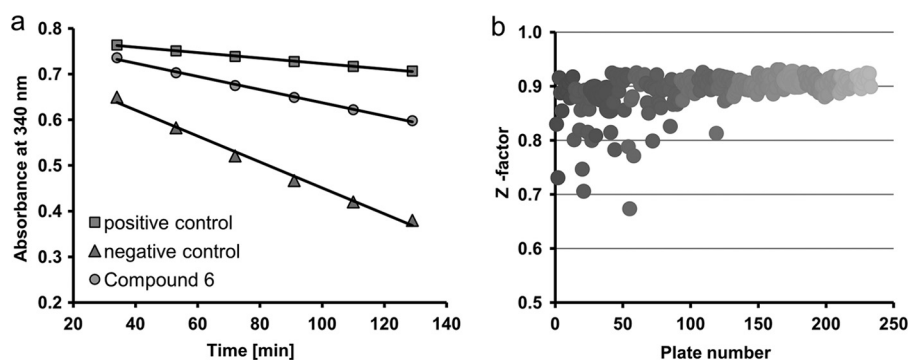


FIGURE 1. **Assay performance and Z'-factors of the coupled peroxidase assay.** *a*, peroxidase activity was measured by following NADPH consumption at 340 nm with six reads every 19 min. A reaction mixture lacking Px represents 100% inhibition (positive control), whereas one containing Px but no inhibitor corresponds to 0% inhibition (negative control). The percentage of inhibition was calculated from the respective slopes as exemplified here for compound **6** (gray circles), which resulted in an inhibition of 65%. *b*, the full screening approach comprised 78,637 compounds distributed in 246 plates (384-well plates). The Z'-factor was calculated as described under "Experimental Procedures."

TABLE 1
In vitro and cellular activity of compounds 1–12

Compound	<i>In vitro</i> inhibition ^a		EC ₅₀ ^b			Selectivity index ^c
	HTS	IC ₅₀	<i>T. brucei</i>		HeLa	
			24 h	72 h	72 h	
	%	μM	μM	μM	μM	
1	102	16.9 ± 2.6	0.7 ± 0.03	0.6 ± 0.1	>50	>83
2	103	4.8 ± 0.04	0.4 ± 0.1	0.8 ± 0.2	14.0 ± 4.5	18
3	100	13.7 ± 0.1	1.1 ± 0.04	4.2 ± 0.3	>50	>12
4	31	29.5 ± 0.2	3.1 ± 0.7	2.3 ± 0.1	22.1 ± 3.2	10
5	99	16.0 ± 0.1	1.3 ± 0.2	3.1 ± 0.5	28.4 ± 3.2	9
6	65	7.2 ± 0.2	0.4 ± 0.1	1.5 ± 0.9	10.0 ± 2.0	7
7	64	30 ± 0.3	0.2 ± 0.05	3.6 ± 0.6	16.3 ± 4.9	5
8	76	22 ± 0.1	0.5 ± 0.1	4.3 ± 1.1	12.2 ± 4.1	3
9	88	31 ± 0.8	0.5 ± 0.1	3.2 ± 0.1	8.0 ± 3.1	3
10	107	3.5 ± 0.02	3.1 ± 0.7	3.6 ± 0.6	8.0 ± 3.2	2
11	102	3.9 ± 0.06	5.1 ± 1.2	7.9 ± 1.7	12.3 ± 5.3	2
12	80	13.9 ± 2.6	2.2 ± 0.1	ND	8.3 ± 0.3	ND

^a Percentage of inhibition of the peroxidase system in the initial HTS with 40 μM compound and IC₅₀ value are shown, and both were measured in 384-well plate format.

^b Bloodstream *T. brucei* and HeLa cells were cultured in the presence of different concentrations of the compounds. The parasites were counted (*T. brucei*, 24 h), and the ATP level of living *T. brucei* and HeLa cells was determined using ATPLite (72 h). The EC₅₀ values are the inhibitor concentrations that resulted in 50% living cells compared with those grown in the presence of 0.5% DMSO.

^c Ratio of the EC₅₀ value for HeLa cells and *T. brucei* (72 h). The values are the means ± S.D. of three independent assays. ND means not determined, because of lack of material.

enzyme systems (6, 35). Subsequently, the library was virtually re-screened for derivatives with common core structures or similar side chains. The aim was to identify putative false-negatives and structure activity relationships. A total of 39 compounds (31 primary hits and 8 analogs) was reordered from the suppliers. Five chemicals were no longer available. For 25 of the 31 candidates, the inhibitory potency was confirmed by IC₅₀ determinations yielding values between 3.5 and 66 μM . 12 Compounds with IC₅₀ values \leq 31 μM were further analyzed (Table 1). They grouped in chemicals with common core structure or with an identical reactive substituent (Fig. 2). None of the reordered analogs **1a**, **1b**, **3a**, **3b**, and **7a** showed activity (supplemental Table S1). In the case of **1a**, **3a**, and **3b**, this can be explained by the absence of the reactive leaving group. The analogs **9a–d** revealed some inhibition but were less active when compared with the parent **9**. The compounds **9** and **9a–c** have in common the possession of a chloroacetamido substituent suggesting a similar mode of action. The core structure of **9d** resembles those in **9** and **9b**, but the compound carries a chloromethyl group as do compounds **1** and **5–8**. In summary, except for compound **3**, all compounds that significantly interfered with the peroxidase system possess a chloromethyl, chlo-

roacetamido, or chloroacetyl group. The data also showed that, although necessary, the chloroalkyl substituents are not sufficient for the inhibitory potency indicating some specificity based on the core structures. The finding that none of the reordered analogs showed a pronounced effect confirmed the results of the HTS.

The 12 compounds were then studied for their activity toward bloodstream *T. brucei*. The parasites were treated with different inhibitor concentrations, and after 24 h living cells were counted. In a second approach, a luciferase-based viability assay was used. *T. brucei* and HeLa cells (31) were incubated with the compounds for 72 h, and the cellular ATP level was measured (37). The antitrypanosomal effect of the compounds became apparent after 24 h and remained practically constant for 72 h. The mammalian HeLa cells revealed generally higher EC₅₀ values, which resulted in selectivity indices of up to >83 (Table 1). For most compounds, the EC₅₀ values were an order of magnitude lower than the IC₅₀ values obtained in the *in vitro* peroxidase assay. As outlined below, this does not contradict the peroxidase system to be targeted but is probably due to the fact that these compounds cause a time-dependent inactivation of their target protein.

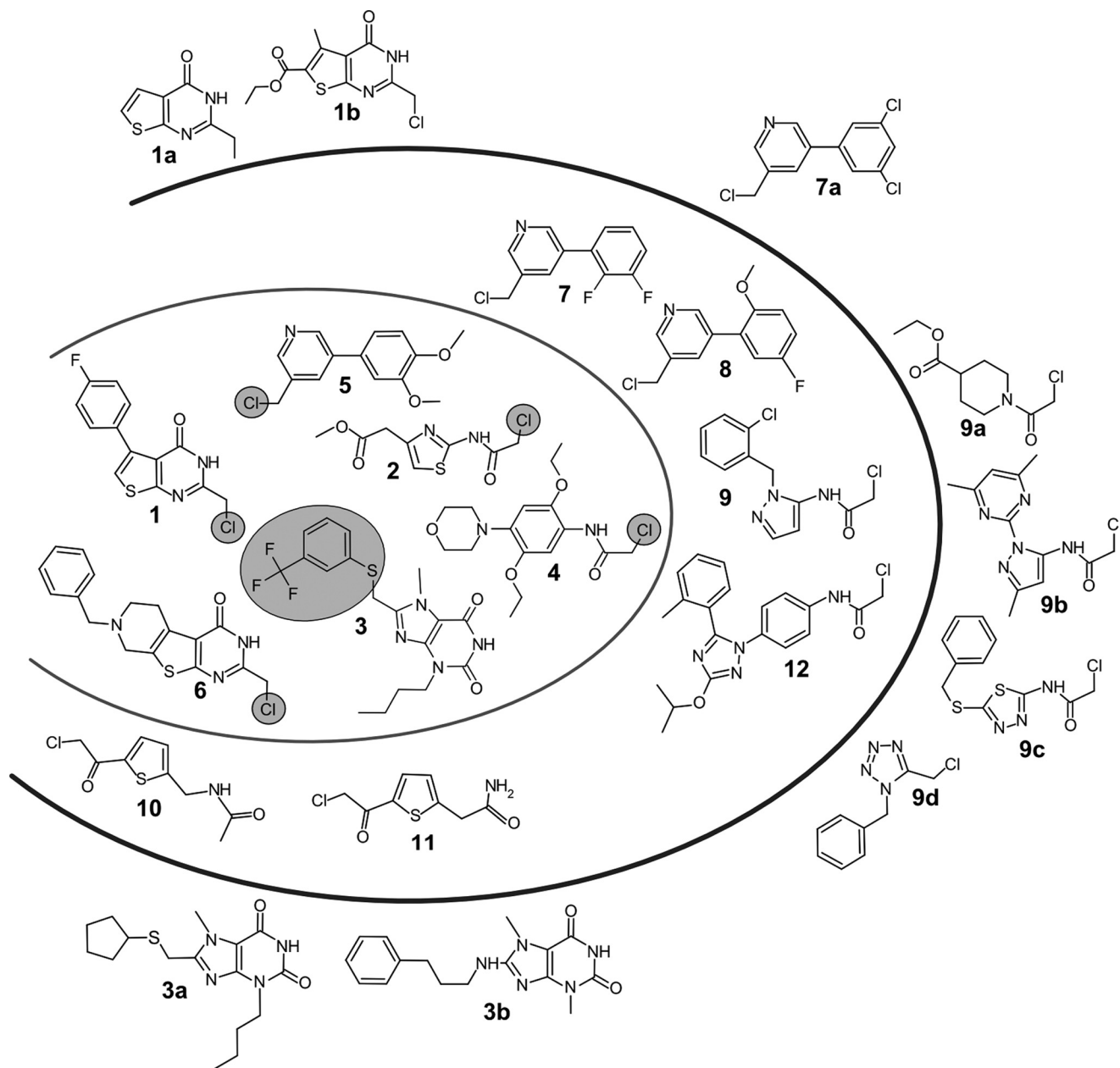


FIGURE 2. **Compounds active against the peroxidase cascade in the HTS and analogs identified by virtual screening.** The 12 compounds within the outer oval revealed IC_{50} -values of $\leq 31 \mu\text{M}$. Compounds **1–6** (inner oval) displayed higher toxicity against *T. brucei* compared with HeLa cells (Table 1). The leaving group upon inactivation of Tpx is circled. The thienopyrimidine-4-ones **1**, **1a**, **1b**, and **6**, the purine 2,6-diones **3**, **3a**, and **3b**, the chloromethyl-phenylpyridines **5**, **7**, **7a**, and **8**, as well as the chloroacetyl-thiophenes **10** and **11** each have a common core structure. Compounds **2**, **4**, **9**, **9b**, **9c**, and **12** carry a 2-chloroacetamido substituent. None of the analogues (marked by a–d) revealed pronounced inhibitory potency.

Target Identification—To identify the distinct protein affected within the peroxidase cascade, the compounds were first subjected to a TR assay. Most of them yielded IC_{50} values of $\geq 200 \mu\text{M}$ (Table 2). Only the chloromethyl-phenylpyrimidines **5**, **7**, and **8** revealed IC_{50} values of 123, 29, and $2.3 \mu\text{M}$, respectively. Subsequent cuvette-based assays and spectroscopic and mass analyses showed for **7** and **8**, but not **5**, a time-dependent inhibition of TR suggesting modification of the active site cysteine(s).³ This is not due to a generally higher reactivity of these derivatives because in the complete peroxidase system compounds **7** and **8** were less efficient than **5** (Table 1). Detailed

analyses are required to reveal putative specific interactions with TR, especially because the halogen and methoxy substituents occupy different positions at the phenyl ring. Taken together, TR can be ruled out as target molecule of compound **5**, whereas in the case of compounds **7** and **8**, inhibition of the enzyme may contribute to the effect of the compounds toward the peroxidase cascade.

The remaining 10 compounds were further characterized in cuvette-based peroxidase assays. In the first series, the inhibitor was added to a reaction mixture composed of NADPH, TR, Tpx, T(SH)₂, and Px (complete), and the assay was immediately started with H₂O₂. The percentage of inhibition was calculated from the reactions without compound (0% inhibition) and

³ A. Dietl and R. L. Krauth-Siegel, unpublished data.

without Px (100% inhibition). At 10 μM , compounds **6**, **10**, and **11** revealed $\geq 71\%$ inhibition (Table 3). For all other compounds, inhibition was significantly weaker when compared with the HTS results (Table 1). In the case of compounds **1** and **2**, this may partially be due to the lower inhibitor concentration used (10 μM instead of 40 μM in the HTS). However, the main difference between both systems was that in the cuvette-based assay, the activity was determined from the absorption decrease directly after adding the inhibitor, whereas in the HTS, the activity was calculated from the data recorded 34 min after starting the reaction. In the next series of kinetics, the reactants were thus preincubated for 40 min before the reaction was started by H_2O_2 . As expected, this procedure resulted in significantly stronger inhibition. With compound **2**, the time-dependent inactivation already became visible in assays that were started directly (see legend of Table 3). All assays in the plate format (HTS, IC_{50} determination, and TR inhibition) were conducted under identical conditions to allow comparison of the time-dependent reactions.

The time-dependent mode of inhibition indicated a covalent modification of the target protein. In the next series of kinetics, the Px was replaced by the Prx-type enzyme. The degree of inhibition was comparable with that in the corresponding Px

assay suggesting that none of the peroxidases was the main target. Finally, the compounds were studied as inhibitors of Tpx. Reaction mixtures that lacked either Px (control) or Tpx were preincubated with the inhibitors for 40 min. Immediately before starting the reaction with H_2O_2 , the missing protein was added. With the exception of compound **2** (see below), preincubation of the reaction mixtures lacking Px resulted in 78–100% inhibition as it was observed for the complete mixtures and strongly suggested Tpx as target protein. In contrast, the preincubation of reaction mixtures that lacked Tpx resulted in minimal inactivation. Only compound **6** caused 70% inhibition under these conditions. This can be explained by the high reactivity of the compound toward Tpx, which already resulted in 97% inactivation without any preincubation and does not reflect inhibition of Px. T(SH)_2 is not an efficient direct reductant of the peroxidase (33). Thus, in the absence of Tpx, Px should be mainly in the inactive form, characterized by an intramolecular disulfide (38). In the case of compound **2**, only 21% inhibition was observed when the preincubation mixture lacked Px in comparison with 85% for the complete system, which indicates that both Tpx and Px react with this compound.

Characterization of Tpx after Treatment with Compounds 1–6—10 μM recombinant Tpx was treated with 40 μM compound **2** for 40 min in the presence and absence of 100 μM T(SH)_2 and subjected to ESI-MS analysis as described under “Experimental Procedures.” Oxidized Tpx, independent of the presence of the compound, as well as untreated reduced Tpx revealed the mass of the unmodified protein (Fig. 3). In contrast, when Tpx was incubated with compound **2** in the presence of T(SH)_2 , a new peak with a 214-Da higher mass appeared in accordance with covalent modification of Tpx under elimination of HCl. Table 4 and the supplemental Fig. S1 provide the respective analyses for compounds **1–6** using His-tagged Tpx. As shown by HPLC MS/MS, all compounds showed a purity of 99%, except for compound **6** with a content of 84% (supplemental Fig. S3). Treatment of reduced, but not oxidized, Tpx resulted in a mass increase that corresponded to the mass of the inhibitor minus HCl. An exception was compound **3**. Here, the

TABLE 2
Effect of 1–12 toward TR

TR activity was measured in 384-well plates as described under “Experimental Procedures.” The IC_{50} values are the means \pm S.D. from three experiments.

Compound	IC_{50}
	μM
1	>200
2	>200
3	>200
4	>200
5	123 \pm 16
6	>200
7	29 \pm 4
8	2.3 \pm 0.3
9	>200
10	146 \pm 22
11	>200
12	>200

TABLE 3
Identification of the target protein(s)

Peroxidase activity was measured as described under “Experimental Procedures.” The reaction was started either directly or after 40 min preincubation of the reaction mixture (complete, without either Px or Tpx) by adding the missing protein component immediately followed by H_2O_2 . Values are the means \pm S.D. of three assays. ND means not determined.

Compound	Inhibition					
	No Preincubation, complete		Preincubation, complete		Preincubation-Px	Preincubation-Tpx
	Px	Prx	Px	Prx	Px	Px
	%					
1	29 \pm 4 ^a	40 \pm 3 ^b	84 \pm 1 ^a	ND	87 \pm 1 ^a	13 \pm 2 ^a
2	3 \pm 1 ^{a,d}	9 \pm 0.2 ^{c,d}	85 \pm 4 ^a	ND	21 \pm 4 ^a	3 \pm 1 ^a
3	29 \pm 1 ^c	ND	102 \pm 2 ^c	102 \pm 2 ^c	100 \pm 1 ^c	21 \pm 2 ^c
4	3 \pm 1 ^c	ND	78 \pm 3 ^c	75 \pm 2 ^c	90 \pm 1 ^c	11 \pm 3 ^c
5	10 \pm 2 ^c	ND	93 \pm 1 ^c	ND	100 \pm 1 ^c	13 \pm 1 ^c
6	97 \pm 1 ^a	88 \pm 2 ^a	105 \pm 0.3 ^a	ND	99 \pm 2 ^a	70 \pm 2 ^a
9	2 \pm 0.4 ^c	ND	75 \pm 1 ^c	85 \pm 2 ^c	78 \pm 1 ^c	10 \pm 3 ^c
10	84 \pm 3 ^a	74 \pm 3 ^a	99 \pm 2 ^a	ND	96 \pm 1 ^a	6 \pm 4 ^a
11	71 \pm 1 ^a	64 \pm 1 ^a	102 \pm 1 ^a	ND	101 \pm 2 ^a	12 \pm 2 ^a
12	19 \pm 3 ^c	ND	101 \pm 2 ^c	102 \pm 1 ^c	99 \pm 1 ^c	17 \pm 2 ^c

^a The assay contained 10 μM of the respective inhibitor.

^b The assay contained 20 μM of the respective inhibitor.

^c The assay contained 40 μM of the respective inhibitor.

^d The values refer to the activity calculated for the 1st min. In the presence of compound **2**, NADPH oxidation declined rapidly during the course of the assay. This resulted after 3 min in 32% and 45% inactivation in the Px and Prx assay, respectively.

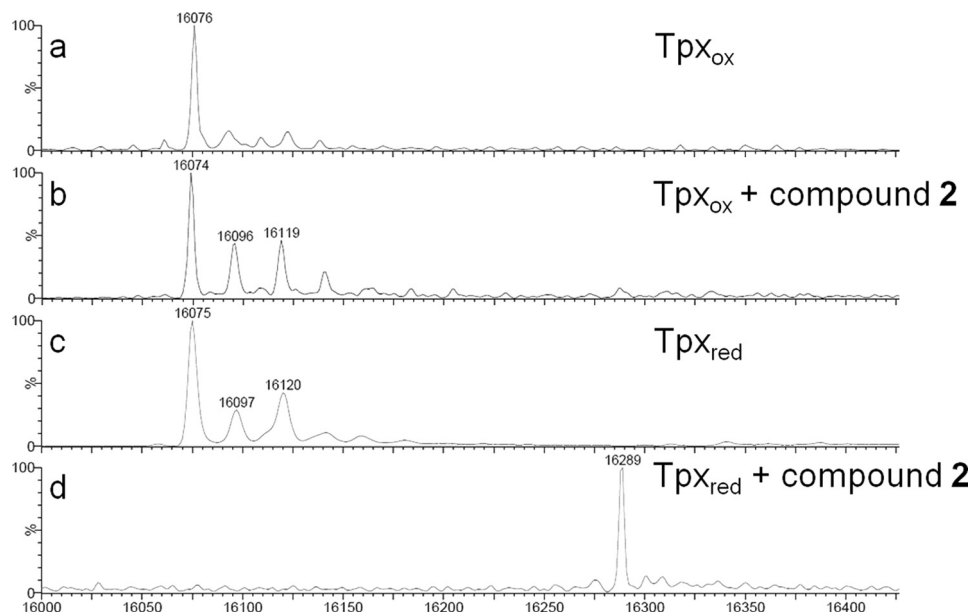


FIGURE 3. **Deconvoluted ESI-MS spectra of Tpx after reaction with compound 2.** Tpx was incubated with compound 2 in the absence (Tpx_{ox}) or presence of $T(SH)_2$ (Tpx_{red}) and subjected to ESI-MS analysis. *a*, oxidized Tpx. The peak at 16,076 Da corresponds to the calculated mass (16,074.2 Da) of the full-length tag-free protein with an artificial N-terminal GAG tripeptide. *b*, oxidized Tpx incubated with compound 2. No mass shift was observed. *c*, reduced Tpx. *d*, reduced Tpx incubated with compound 2 showed a new peak at 16,289 Da. The mass increase by 214 Da corresponds to Tpx modified by compound 2 after elimination of HCl (expected mass difference 213 Da). The peaks at 16,096 (16,097) Da and 16,119 (16,120) Da with 23 and 45 Da higher masses probably represent mono- and di-sodium adducts of Tpx. The accuracy of the measurement was ± 2 Da.

TABLE 4
ESI-MS data of His₆-Tpx after reaction with compounds 1–6

Compound	Molecular mass Da	Observed masses ^a Da	Mass increase Da	Mass of the leaving group Da
		17921.5, 18100.2		
1	294.7	18181.4, 18359.4	259.9, 259.2	34.8, 35.5
2	248.7	18135.0, 18313.3	213.5, 213.1	35.2, 35.6
3	412.4	18158.2, 18336.5	236.7, 236.3	175.7, 176.1
4	342.0	18229.8, 18408.0	308.3, 307.8	33.7, 34.2
5	263.7	18149.7, 18328.0	228.2, 227.8	35.5, 35.9
6	345.8	18232.5, 18410.5	311.0, 310.3	34.8, 35.5

^a The calculated mass of reduced His₆-Tpx without and with the N-terminal Met is 17,923.2 and 18,054.3 Da, respectively. The analysis revealed two masses, one for the protein lacking the N-terminal Met and another one that corresponded to the full-length protein with an additional mass of 48 ± 1 Da. The latter mass did not appear when tag-free Tpx was used (see Fig. 3) and probably represents an oxidation product. Both peaks showed an identical mass increase when Tpx was reacted with the respective compound. The spectra are depicted in the supplemental Fig. S1.

mass of the leaving group corresponded to the trifluoromethyl benzenethiol moiety of the compound (Fig. 2). Remarkably, under the conditions chosen, Tpx became completely modified by these compounds despite the presence of a 10-fold molar excess of $T(SH)_2$. The highly reactive small dithiol did not interfere with the reaction.

In Vitro, Compounds 1–6 Inactivate also Human Thioredoxin—Tpx is a distant relative of thioredoxins. Both proteins have in common that under physiological conditions the first cysteine of their active site motif is a solvent-exposed reactive thiol(ate). As expected, reduced but not oxidized recombinant human Trx also became covalently modified by compounds 1–6 (supplemental Fig. S2 and Table S2). Interestingly, the reaction of compounds 5 and 6 with Trx did not result in the complete modification of the protein, as observed with Tpx, which may indicate some selectivity or higher reactivity of the parasite protein. This is supported by the finding that compounds 1–6 were more cytotoxic toward the parasites compared with mammalian cells.

Compounds 1–6 Modify Cys-40 of Tpx—During catalysis, Tpx cycles between the oxidized state with Cys-40 and Cys-43

forming an intramolecular disulfide and the reduced dithiol state (10, 11). A MAL-PEG-based gel shift assay was established that should result in a mass increase of 5 kDa per cysteine reacting. Although the apparent mass shifts observed on the SDS gel were much larger, mono- and bis-modified Tpx species were clearly distinguishable. In the absence of DTT, Tpx did not react with MAL-PEG, independent of a prior treatment with the compounds, and the unmodified protein was the most prominent band (Fig. 4, *a* and *b*). In the presence of the reducing agent but no inhibitor, both cysteines reacted with MAL-PEG, and the double-modified protein was formed. When reduced Tpx was reacted with compounds 1–6 followed by MAL-PEG, the mono-modified protein was observed. Treatment of 125 μ M Tpx with 500 μ M inhibitor in the presence of 3.1 mM DTT resulted in almost 100% modification of one cysteine residue. Thus, as described above for $T(SH)_2$, the presence of excess DTT did not interfere with Tpx inactivation.

To elucidate which cysteine residue of the WCPC active site motif was targeted, C40S-Tpx and C43S-Tpx mutants were studied (Fig. 4c). Incubation of C40S-Tpx with MAL-PEG resulted in the mono-modified protein independent of a pre-

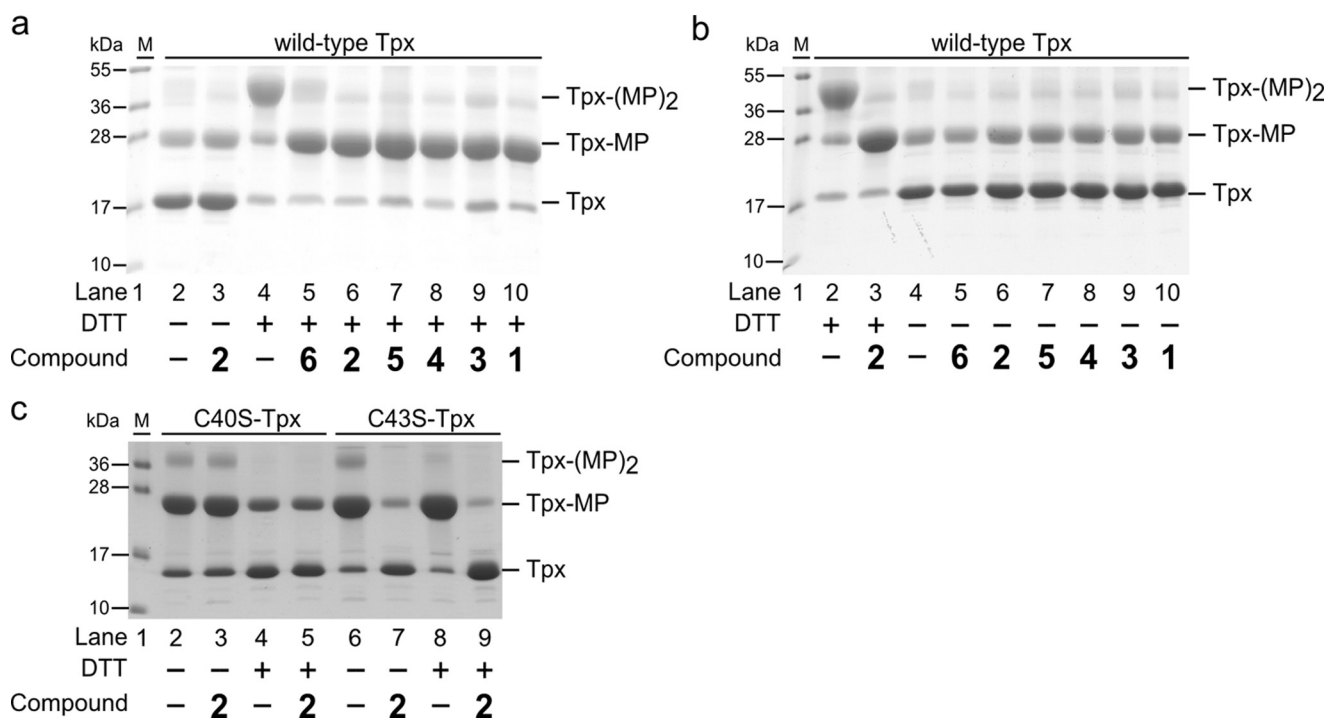


FIGURE 4. SDS-PAGE of Tpx inactivated by compounds 1–6. His-tagged Tpx was treated with the compounds in the presence or absence of DTT, and remaining free cysteines were modified by MAL-PEG (~5 kDa) as described under "Experimental Procedures." *a*, in the absence of DTT, Tpx did not react with MAL-PEG independent of prior incubation without (lane 2) or with compound 2 (lane 3). In the presence of DTT, but no inhibitor, both cysteines of Tpx reacted with MAL-PEG (Tpx-(MP)₂; lane 4). In contrast, if reduced Tpx was incubated with the compounds prior to MAL-PEG treatment, the mono-modified protein was the main product (Tpx-MP; lanes 5–10). *b*, reduced Tpx, untreated (lane 2) or treated with 2 (lane 3) (corresponding to lanes 4 and 6 in *a*), served as controls. Oxidized Tpx either untreated (lane 4) or pretreated with compounds 1–6 (lanes 5–10) revealed the unmodified protein as main species. *c*, reaction of Tpx mutants with compound 2. In the absence of DTT, reaction of C40S-Tpx with MAL-PEG resulted in the mono-modified protein as main species independent of a prior treatment with compound 2 (lanes 2 and 3). In contrast, in the case of C43S-Tpx, treatment with compound 2 prevented the subsequent reaction with MAL-PEG (lanes 6 and 7). For further details see the text. In lane 1 the protein marker was loaded. Depicted are representative gels of at least three independent analyses.

treatment with compound 2. Thus, Cys-43 did not react with the compound but remained accessible to MAL-PEG modification. In both reactions, with or without inhibitor treatment, the presence of DTT resulted in a weaker modification of C40S-Tpx by MAL-PEG compared with the reaction in the absence of the reducing agent. This is probably due to the low reactivity of Cys-43 (12, 39), which allows DTT to compete for the reagent. In the absence of compound 2, C43S-Tpx reacted with MAL-PEG yielding the mono-modified protein as was the case with C40S-Tpx. However, pretreatment of C43S-Tpx with compound 2 prevented the subsequent modification by MAL-PEG. Here, the MAL-PEG reaction was not affected by DTT in accordance with the high reactivity of Cys-40. From the three-dimensional structure of *C. fasciculata* Tpx, it is known that Cys-40 is solvent-exposed, whereas Cys-43 is more buried (40, 41). Cys-40 is the catalytic thiolate that interacts with T(SH)₂ as well as Prx or Px, its different substrates in the peroxidase cascade (see Scheme 1) (38–42). Taken together, compounds 1–6, disclosed by the HTS approach against the peroxidase cascade, target Tpx by covalently modifying Cys-40.

Modification of Tpx in the Intact Parasite—Bloodstream *T. brucei* were treated with 0.6 μM compound 1, 0.8 μM compound 2, 3.1 μM compound 5, or 3.6 μM compound 7, concentrations that corresponded to the respective EC₅₀ value at 72 h (Table 1). After 4 h, the densities of the treated cultures were 80–100% of cells grown in the presence of DMSO, clearly proving viability of the parasites at this time point. Western blot

analyses of total cell lysates from parasites that were treated with DMSO or with compounds 1, 2, 5, and 7, respectively, but immediately washed (0 h) followed by MAL-PEG treatment revealed the unmodified protein (Fig. 5*a*). Thus, in the cell lysates Tpx was present in oxidized form. When the parasites were cultured in the presence of the compounds for 4 h and then treated with MAL-PEG, mono-modified Tpx was the main species. Some remaining oxidized protein as seen here for compounds 5 and 7 was not observed in other series of experiments (see also Fig. 5*b*). In the case of compound 1, even without incubation (0 h), a small amount of Tpx appeared in mono-modified form probably because of the high reactivity or very rapid cellular uptake of the compound.

Finally, the cells were incubated with the compounds as described above but reacted with MAL-PEG in the presence of TCEP. When the parasites were treated with either DMSO or with the compounds but then immediately washed, both cysteines of Tpx were susceptible to modification by MAL-PEG (Fig. 5*b*) confirming the successful removal of the inhibitors before cell lysis. In contrast, when the parasites were cultured for 4 h in the presence of compounds 1, 2, 5, or 7, the main product was again the mono-modified protein. These results clearly established that compounds 1, 2, 5, and 7 entered the cell and covalently modified Tpx. In addition, the finding that the protein reacted with the compounds in the intact parasites revealed that intracellular Tpx is present in the accessible reduced form. We cannot rule out that other proteins might be

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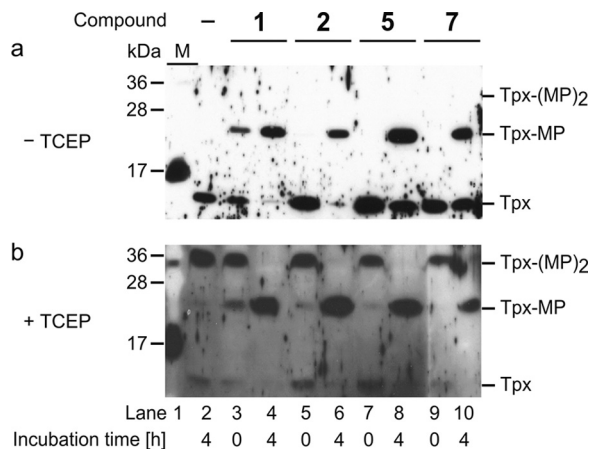


FIGURE 5. Western blot analysis of bloodstream *T. brucei* cultured in the presence of compounds 1, 2, 5, or 7. *a*, cell lysates treated with MAL-PEG in the absence of reducing agent. In cells incubated with DMSO (4 h) or with compounds 2, 5, and 7 for 0 h, Tpx did not show any modification by MAL-PEG (Tpx). Treatment of the parasites with the compounds for 4 h, or in the case of compound 1 to a minor degree immediately, resulted in a mono-modification of Tpx by MAL-PEG (Tpx-MP). *b*, cell lysates treated with MAL-PEG in the presence of TCEP. In parasites cultured with DMSO for 4 h or the inhibitors for 0 h, MAL-PEG reacted with both cysteines of Tpx (Tpx-(MP)₂). In contrast, if the parasites had been incubated with the compounds for 4 h, and the cell lysate was then reacted with MAL-PEG only Tpx-MP was obtained. Lane 1 of both gels shows the protein marker (M). Depicted are representative blots of at least three independent experiments.

affected as well. The fact that the 4-h treatment resulted in 100% inactivation of Tpx but was not accompanied by a significant growth defect might indicate that the compounds have additional off-target effects. However, the phenotype observed can also be explained by Tpx being the main target. The delayed onset of growth impairment closely resembles that of parasites subjected to RNA interference against Tpx. Depletion of the mRNA in bloodstream *T. brucei* leads within 24 h to a down-regulation of the protein to concentrations of $<1 \mu\text{M}$, but a significant growth defect becomes obvious only after 48 h (19). The depletion of Tpx is accompanied by an up-regulation of the cellular low molecular mass thiols that transiently may compensate for the lack of the dithiol protein (19). Because Tpx delivers the reducing equivalents for the synthesis of DNA precursors by ribonucleotide reductase (14), the main consequence of Tpx inactivation is probably an impaired proliferation of the parasites.

DISCUSSION

Of the nearly 80,000 chemicals studied against the peroxidase system of African trypanosomes, 32 compounds displayed activity. All 12 compounds that were mechanistically further characterized proved to be time-dependent inhibitors. As shown for compounds 1, 2, and 4–6, the compounds reacted with Cys-40 of Tpx with the elimination of HCl. The finding that Tpx was not only targeted *in vitro* but also in the intact parasite is remarkable. It clearly shows that free T(SH)₂ present in high molar excess does not interfere with the modification of the parasite oxidoreductase. The specificity for Tpx observed is further corroborated by the fact that all three proteins that form the peroxidase cascade, namely TR, Tpx, and Px, possess reactive cysteine residues.

The inhibitors identified can be classified according to their common core structures or reactive substituents. Compounds 10 and 11 are chloroacetyl-substituted thiophenes. Structurally related compounds have been described as irreversible inhibitors of human glycogen synthase kinase 3 (GSK3- β) (43). The cysteine residue that is putatively affected is conserved in the two GSK3 homologs of *T. brucei* (44). Both compounds 10 and 11 readily inhibited the peroxidase system *in vitro* but did not reveal any specificity for the parasite compared with HeLa cells in the cell-based assays. These derivatives are probably not specific toward the target protein nor the organisms and were not further analyzed. All compounds of the second group possess a chloromethyl substituent. Compounds 1, 2, and 6–8 reacted with Tpx, but only 7 and 8 displayed also activity toward TR. Thus, despite the common reactive substituent, the distinct core structures appear to play a critical role for target interaction. This is supported by the finding that 7a, in contrast to the related 7 and 8, did not reveal significant inhibition of the peroxidase system. The third group formed by compounds 2, 4, 9, and 12 had in common a chloroacetamido substituent. None of the compounds inhibited TR. Compound 2 may interact not only with Tpx but also with Px. Taken together, despite the presence of a chloroalkyl substituent, the compounds revealed distinct reactivities toward the three thiol redox proteins of the peroxidase system.

The parasite Tpx is a distant relative of the thioredoxin protein family (11, 19). Therefore, we studied the compounds also toward recombinant human Trx. The same type of inactivation was observed, namely modification of its redox-active cysteine residue. Nevertheless, some of the compounds reacted much slower with Trx compared with Tpx. Indeed, compounds that interact with Trx do not necessarily react with Tpx. The anti-tumor benzothiazole-substituted quinole PMX464 inhibits Trx (21–23). A recent study on the *T. brucei* peroxidase system revealed that the quinole reacts with T(SH)₂ as well as Px and Prx but not with Tpx or TR (20). This supports the conclusion that a selective inhibition of these structurally related proteins should be possible.

The finding that the compounds described here inactivate human Trx *in vitro* and can affect the proliferation of HeLa cells may even be exploited for anticancer drug development approaches. Trx has been shown to be up-regulated in certain malignancies and is currently studied as a target for anticancer drugs (22). In the work presented here, we chose HeLa cells as controls because this cancer cell line expresses Trx at high levels (45), which may at least partially explain the rather low selectivity indices observed. Fibroblast MRC-5 cells that are often used as control cells in antiparasitic drug testing (see for instance Ref. 20) have been reported to have undetectable Trx1 protein levels (22) and might have resulted in higher selectivity indices.

A critical issue that could be raised is that all compounds identified here are covalent inhibitors of Tpx. Drugs that covalently attach to their target are traditionally considered as unfavorable. However, as outlined in an excellent recent review (46), covalent inhibitors have proved to be successful therapies for various indications and about one-third of all approved covalent drugs are anti-infectives. Current covalent drug discovery

programs mainly try to target a noncatalytic nucleophile. However, this restriction may not be crucial when regarding antimicrobial agents. As the next step toward the development of Tpx inhibitors as putative novel antitrypanosomal drugs, work is in progress to test the most active compounds in the animal model.⁴ To our knowledge this is the first report on inhibitors of this parasite-specific dithiol/disulfide protein. Indeed, our data suggest that it should be possible to develop specific inhibitors of Tpx, a protein that occupies a central position in the trypanothione-based thiol redox metabolism of these parasites.

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⁴ M. Comini, D. Benitez, and G. Fernández (Pasteur Institut Montevideo), personal communication.

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