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Biochemistry

"Subversive" substrates for the enzyme trypanothione disulfide reductase: Alternative approach to chemotherapy of Chagas disease

(*Trypanosoma cruzi*/leishmaniasis/naphthoquinone/nitrofuran)

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ABSTRACT **The trypanosomatid flavoprotein disulfide reductase, trypanothione reductase, is shown to catalyze one-electron reduction of suitably substituted naphthoquinone and nitrofuran derivatives. A number of such compounds have been chemically synthesized, and a structure–activity relationship has been established; the enzyme is most active with compounds that contain basic functional groups in side-chain residues. The reduced products are readily reoxidized by molecular oxygen and thus undergo classical enzyme-catalyzed redox cycling. In addition to their ability to act as substrates for trypanothione reductase, the compounds are also shown to effectively inhibit enzymatic reduction of the enzyme's physiological substrate, trypanothione disulfide. Under aerobic conditions, trypanothione reductase is not inactivated by these redox-cycling substrates, whereas under anaerobic conditions the nitrofuran compounds cause irreversible inactivation of the enzyme. When tested for biological activity against *Trypanosoma cruzi* trypomastigotes, many of the test compounds were trypanocidal, and this activity correlated with their relative ability to act as substrates for trypanothione reductase. The activity of the enzyme with these redox-cycling derivatives constitutes a subversion of its normal antioxidant role within the cell. For this reason these compounds may be termed "subversive" substrates for trypanothione reductase.**

There is great need for new and less toxic treatments for human tropical diseases by parasitic trypanosomes [African sleeping sickness and Chagas disease (South American trypanosomiasis)] and leishmanias (oriental sore and kala-azar). In the case of Chagas disease, which is caused by *Trypanosoma cruzi*, no generally effective chemotherapy currently exists for the millions of infected people (1).

Comparative studies on the metabolism of trypanosomatids and their mammalian hosts have pointed to a number of biochemical differences that might be exploited as targets for chemotherapy. We have been investigating the biochemical basis for the well-documented (2, 3) sensitivity of parasitic protozoa towards reagents that promote free radical damage in cells. This work has led to the discovery that trypanosomatids possess highly unusual antioxidant defense mechanisms (4) based upon the glutathione-spermidine conjugate N^1,N^8-bis(glutathionyl)spermidine, which has been given the trivial name trypanothione (5).

In most aerobic organisms, the tripeptide glutathione and the glutathione reductase/glutathione peroxidase enzyme couple have key roles in the antioxidant defense process (6). In contrast, all species of trypanosomatids examined to date lack classical glutathione reductase and glutathione-dependent peroxidase activities (7). Trypanosomatids possess instead an unusual NADPH-dependent flavoprotein disulfide reductase (trypanothione reductase) (8), which maintains trypanothione in the dithiol form [$Try(SH)_2$] within the cell. In addition, trypanosomatids also possess trypanothione-dependent peroxidase activity (9, 10). Given that the antioxidant defenses of trypanosomatids are based upon trypanothione, inhibition of trypanothione reductase or subversion of its antioxidant role within the cell represents an attractive target for the design of drugs to treat trypanosomatid infections.

Trypanothione disulfide reductase has been purified from *Crithidia fasciculata* and *T. cruzi* (8, 11) and found to have similar physical and chemical properties to human glutathione reductase [NAD(P)H:oxidized-glutathione oxidoreductase, EC 1.6.4.2]. In fact, trypanothione and glutathione reductases appear to reduce their respective physiological disulfide substrates by the same catalytic mechanism. The parasite and human enzymes do, however, differ in their respective substrate specificities; glutathione disulfide is not a substrate or inhibitor of trypanothione reductase, and, conversely, trypanothione disulfide [$Try(S)_2$] is not a substrate for glutathione reductase. This mutually exclusive substrate specificity is of key importance in any approach toward the selective inhibition of trypanothione reductase. In a study on the substrate specificity of trypanothione reductase (12), the enzyme reduced various analogues of $Try(S)_2$ in which the spermidine moiety had been replaced by an aliphatic side chain that contained at least one amine function. The activity of the enzyme with these analogues most closely reflected the relative ability of the compounds to bind in the active site and suggested that trypanothione reductase might possess a binding site for the spermidine moiety of $Try(S)_2$. This work led us to propose that suitably substituted analogues of $Try(S)_2$ might take advantage of this aspect of the enzyme–substrate interaction to access the catalytic center of trypanothione reductase.

Having identified trypanothione reductase as a potential target for chemotherapeutic intervention, we developed a strategy in which compounds are designed, which effectively subvert the physiological function of this enzyme. These compounds take advantage of the ability of trypanothione reductase to catalyze reduction of substances that can undergo redox-cycling processes to produce toxic metabolites of oxygen that can kill *T. cruzi* trypomastigotes.

In this communication we report the preparation of several model compounds that appear to be trypanocidal by their ability to act as redox-cycling or "subversive" substrates for trypanothione reductase.

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Abbreviations: $Try(SH)_2$ and $Try(S)_2$, the dithiol and disulfide forms of trypanothione, respectively; HSVSM, human saphenous vein smooth muscle.

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MATERIALS AND METHODS

Materials. All reagents and chemicals were of the highest grade commercially available. $Try(S)_2$ was chemically synthesized (5), and trypanothione disulfide reductase was purified from *C. fasciculata* as described previously (8). Nifurtimox and nitrofurazone were obtained from Bayer (Leverkusen, F.R.G.) and Aldrich, respectively. Other compounds (Fig. 1) were chemically synthesized. Details of chemical synthesis (too lengthy for inclusion) are available from the authors on request.

Enzyme Assays and Kinetic Analysis. Trypanothione disulfide reductase activity was assayed spectrophotometrically by monitoring substrate-dependent oxidation of NADPH at 340 nm. Alternatively, enzyme activity was monitored by coupling radical formation to cytochrome *c* reduction and measuring absorbance changes (ϵ_{550} = 21 mM^{-1}). Absorbance changes were monitored on a Varian Cary 219 spectrophotometer with a thermostated cuvette chamber. Enzyme concentration was measured spectrophotometrically using the extinction coefficient ϵ_{464} = 11.3 mM^{-1} (8). Kinetic runs were done at 27°C in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (Hepes) buffer (pH 7.8) containing 0.1 mM EDTA and 0.25 mM NADPH. Anaerobic measurements were performed in rubber-stoppered cuvettes that were flushed repeatedly with helium. All buffers and solutions used in the assays were treated in this manner. Additions to the cuvette were made with a microsyringe piercing the septum.

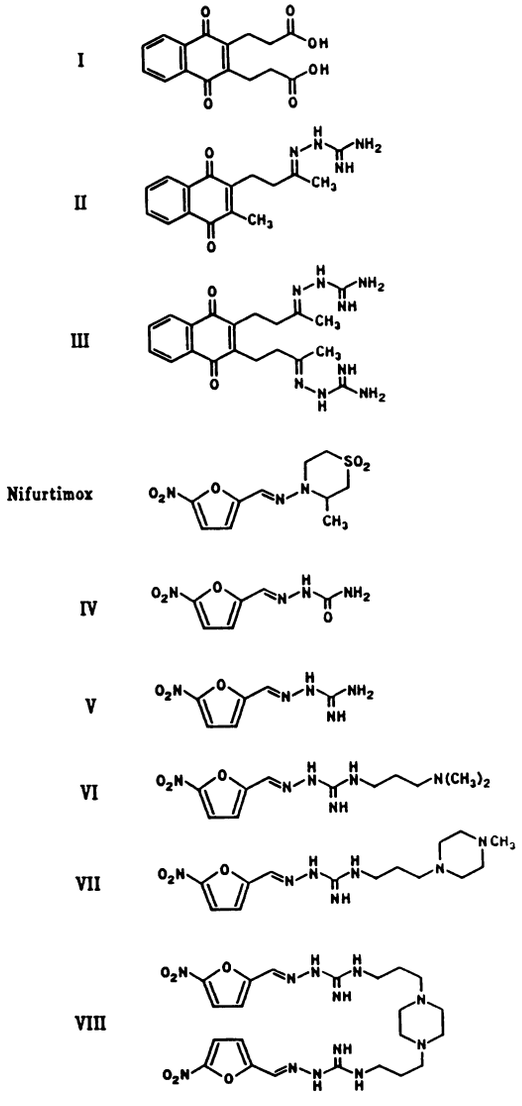

FIG. 1. Structures of naphthoquinone (compounds I–III) and nitrofuran (compounds IV–VIII) derivatives.

Interaction of Trypanothione Reductase with Nitrofuran VIII Under Aerobic and Anaerobic Conditions. Assays were done in 0.1 M Hepes (pH 7.8) containing 1 mM EDTA, 0.15 mM NADPH, trypanothione reductase at 500 pmol/ml, and 10 μM nitrofuran VIII. Reactions were started by addition of enzyme (80 μl to a 2-ml assay). Sequential spectra (200–800 nm) were collected at 20-sec intervals using a Hewlett–Packard HP8450A UV/Vis spectrophotometer. At the end of the experiment, the assay mixtures were dialyzed, and trypanothione reductase was assayed using 250 μM $Try(S)_2$.

Parasite Cell Culture. Human saphenous vein smooth muscle (HSVSM) cells were isolated from outgrowths of explants of unused portions of veins harvested for coronary artery bypass surgery as described (13). The cells were maintained in 24-well tissue culture plates (Costar, Cambridge, MA) in a solution of Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose, 25 mM Hepes, and 10% fetal bovine serum (HyClone, Logan, UT). *T. cruzi* strain Y trypomastigotes were propagated as described (14), except that HSVSM cells were used in place of bovine aortic smooth muscle cells.

Trypanocidal Assays. Test compounds were dissolved in 0.3 ml of dimethyl sulfoxide and brought to volume by dilution with serum-free DMEM. Solutions were sterilized by filtration through 0.22-μm filter units. Trypomastigotes (10^7 ml^{-1}) were harvested and washed twice in serum-free DMEM. Parasites were added to solutions of the test compounds to a final concentration of 10^6 ml^{-1} and incubated for 3 hr at 37°C in a 5% CO_2 atmosphere. After incubation with the test compound, 0.1 ml of the parasite suspension (10^5 organisms) was diluted with 0.4 ml of culture medium and added to the HSVSM cells. After 16 hr the HSVSM cells were washed twice with serum-free DMEM to remove trypomastigotes that were not internalized, and fresh culture medium was added to the wells. Infections were followed for 4–7 days by light microscopy.

RESULTS

Trypanothione Reductase-Catalyzed Reduction of Naphthoquinones and Nitrofurans. We postulated that trypanothione reductase might reduce functional groups other than disulfides, having found precedent for such a process in the reported ability of erythrocyte glutathione reductase to catalyze reduction of trinitrobenzenesulfonate (15). To test this idea, we synthesized three naphthoquinones possessing, respectively, two side-chain carboxylic acid residues, one side-chain guanidine residue, and two side-chain guanidine residues (Fig. 1, compounds I–III). In each case these side-chain residues are not conjugated to the aromatic portion of the molecule and should not influence the reduction potential of the compound. When tested as substrates for trypanothione reductase from *C. fasciculata* (Fig. 2), oxidation of NADPH was readily measured with the two basic naphthoquinones (compounds II and III), but the dicarboxylic acid (compound I) was much less active. In this fully aerobic system the naphthoquinones were found to oxidize several molar equivalents of NADPH, which suggested that redox cycling was occurring. Thus, the naphthoquinone substrates must undergo enzyme-catalyzed reduction and subsequent reoxidation by O_2 with concomitant production of superoxide. This was confirmed by monitoring oxygen consumption directly (which was approximately stoichiometric with NADPH consumption) and by monitoring cytochrome *c* reduction by superoxide in the coupled assay system described below.

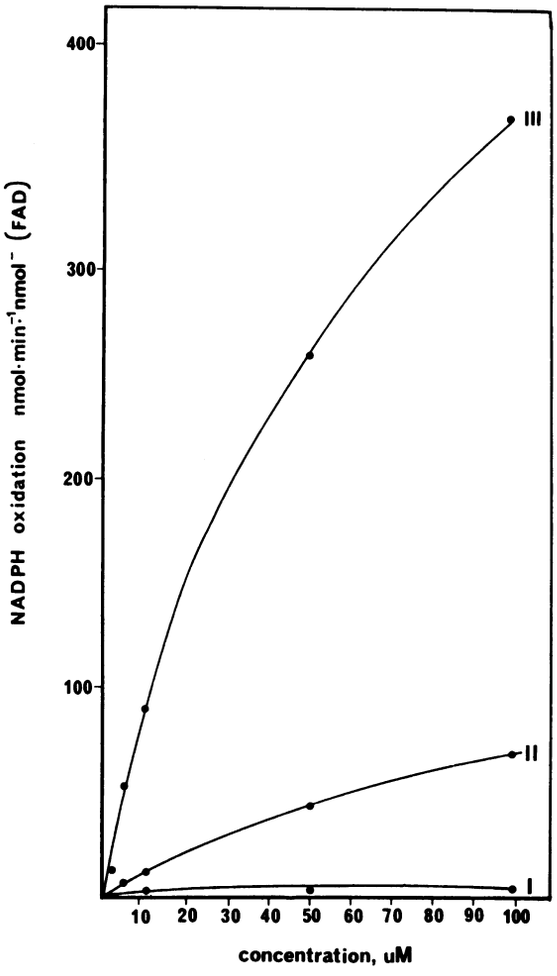

FIG. 2. Enzymatic reduction of naphthoquinone derivatives (Fig. 1, compounds I–III) as a function of substrate concentration.

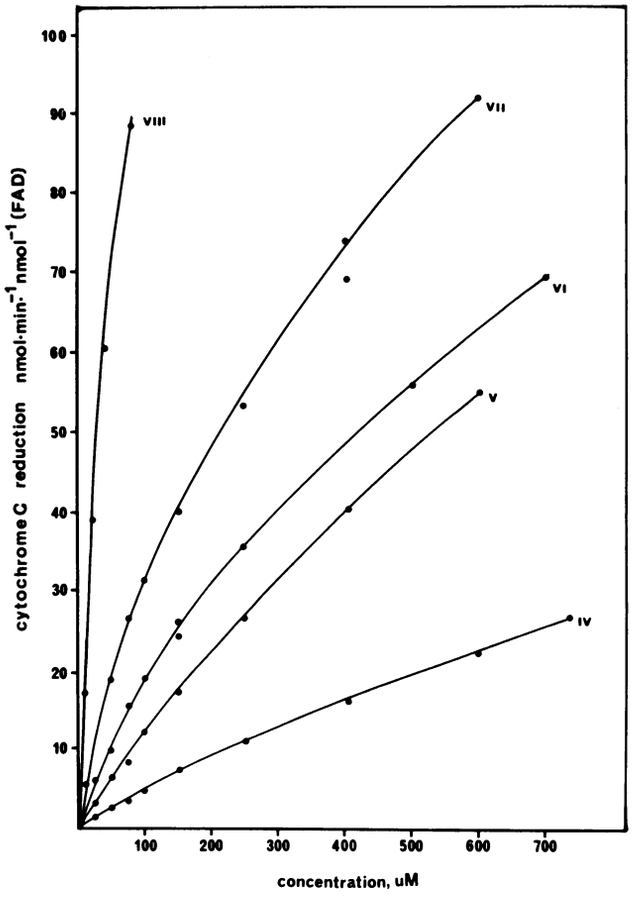

FIG. 3. Enzymatic reduction of nitrofuran derivatives (Fig. 1, compounds IV–VIII) as a function of substrate concentration. Relative rates were measured under aerobic conditions by coupling superoxide production to cytochrome *c* reduction.

The same structure–activity relationship between the three naphthoquinones extended to the relative ability of the compounds to inhibit trypanothione reductase-catalyzed $Try(S)_2$ reduction. The naphthoquinones containing one- and two-guanidine residues (compounds II and III) both inhibited $Try(S)_2$ reduction (concentrations for 50% inhibition being 1.5 μM and 0.25 μM, respectively). In contrast, the dicarboxylic acid (compound I) was a poor inhibitor of $Try(S)_2$ reduction over a much higher (millimolar) concentration range.

The ability of naphthoquinones to undergo such a redox-cycling process is reflected by the low one-electron reduction potential of these derivatives ($E_7^1 = -0.2$ V) (16). Like naphthoquinones, 5-nitrofuran derivatives also have relatively low one-electron reduction potentials ($E_7^1 = -0.25$ V) (16). We therefore tested the ability of trypanothione reductase to reduce two nitrofuran derivatives, nifurtimox and nitrofurazone (Fig. 1, nifurtimox and compound IV), which have been used to treat trypanosomatid infections. In each case, trypanothione reductase-catalyzed reduction of the nitrofuran substrates could be detected by monitoring NADPH oxidation. Redox cycling was evident by coupling superoxide production to cytochrome *c* reduction (Fig. 3). To determine whether the addition of a basic (spermidine-like) side-chain residue to the nitrofuran moiety could influence the rate of the enzyme-catalyzed reduction, we synthesized the series of nitrofuran derivatives shown in Fig. 1 (compounds V–VIII). When tested as substrates for trypanothione reductase (Fig. 3), the nitrofuran derivatives that contain basic side-chain residues were considerably more reactive than nitrofurazone (or nifurtimox; data not shown). When the nitrofuran derivatives were tested as substrates with human erythrocyte or yeast glutathione reductase, none of the compounds was active.

Aerobic and Anaerobic Inhibition of Trypanothione Reductase-Catalyzed Reduction of $Try(S)_2$ by Nitrofuran Derivatives. As for the guanidine-containing naphthoquinones, the nitrofuran derivatives also inhibited trypanothione reductase-catalyzed $Try(S)_2$ reduction (Fig. 4). The effect of the nitrofuran derivatives on this process correlated exactly with the relative ability of the compounds to undergo enzyme-catalyzed reduction—the most active substrates being also the best inhibitors of $Try(S)_2$ reduction. Inhibition of trypanothione reductase-catalyzed $Try(S)_2$ reduction was reversed after removal of the nitrofuran by dialysis.

The experiments described above were done in the presence of oxygen. Under anaerobic conditions interaction of the nitrofuran derivatives with trypanothione reductase took quite a different course. Trypanothione reductase was incubated with the bisnitrofuran derivative (Fig. 1, compound VIII) in the presence or absence of O_2 (Fig. 5). Under aerobic conditions, NADPH oxidation was linear with time. Under anaerobic conditions, the rate of NADPH oxidation was nonlinear after an initial period and did not increase upon subsequent addition of O_2. At end of the incubation ($t = 1000$ sec) assay mixtures were dialyzed extensively to remove nitrofuran and then assayed for $Try(S)_2$ reductase activity. The enzyme that had been incubated without O_2 lost 93% $Try(S)_2$ reductase activity relative to the aerobic control and was no longer active with the nitrofuran derivatives.

Effect of Naphthoquinones and Nitrofuran Derivatives and the Ability of *T. cruzi* Trypomastigotes to Infect HSVSM Cells.

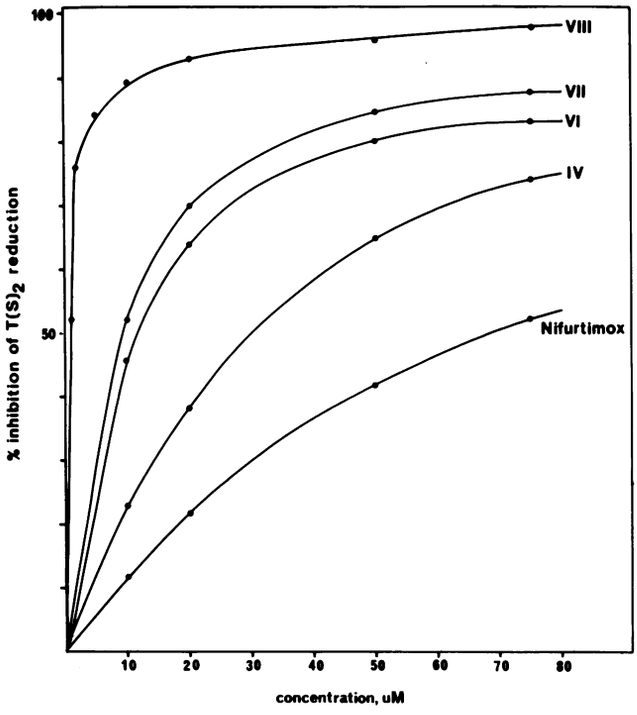

FIG. 4. Inhibition of $Try(S)_2$ [$T(S)_2$] reduction by nitrofuran derivatives (Fig. 1, compounds IV–VIII) under aerobic conditions. $Try(S)_2$ concentration was 250 μM; enzyme, 0.3 unit/ml; NADPH, 150 μM.

Pretreatment of cultured *T. cruzi* trypomastigotes with the reactive naphthoquinone and nitrofuran trypanothione reductase substrates markedly decreased the capacity of the parasite to infect HSVSM cells (Table 1). All test compounds were completely trypanocidal at 100 μM (data not shown). In the nitrofuran series all test compounds were more effective than nifurtimox at 10 μM. This concentration was taken as a starting point for the activity–concentration titration because effective serum concentration of the clinically used nifurtimox is reported in the 20 μM range. Even at 1 μM, at which point nifurtimox is completely ineffective, several test compounds significantly decreased invasion. Of the two naphthoquinones, the bisguanidine derivative was extremely trypanocidal, and this effect was evident even at submicromolar concentrations. In contrast, the naphthoquinone that contained just one guanidine residue was less effective than nifurtimox.

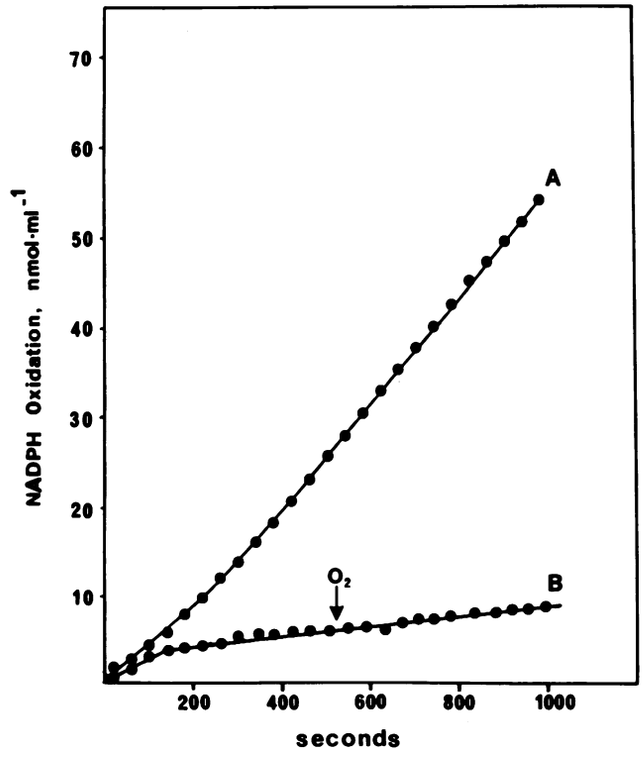

FIG. 5. Incubation of nitrofuran VIII with trypanothione reductase under aerobic (curve A) and anaerobic (curve B) conditions.

Table 1. Effect of pretreatment of *T. cruzi* trypomastigotes with substituted naphthoquinones and nitrofurans on their capacity to infect HSVSM cells

Compound	Infected cells, %			
	10 μM	5 μM	1 μM	0.5 μM
Nifurtimox	4	10	100	100
II	8	21	100	100
III	0	0	0	14
V	0	0	45	100
VII	0	0	35	64
VIII	0	0	0	100

Structures are the same as in Fig. 1. Trypomastigotes were incubated with test compounds. Results are expressed as a percentage of cells infected by pretreated trypomastigotes relative to untreated controls. At 100 μM, all test compounds were completely effective; at 0.1 μM none of the compounds was effective.

From this preliminary screen, a naphthoquinone (compound III) and two nitrofurans (compounds VII and VIII) were identified as being particularly trypanocidal. We therefore examined the effect of these compounds on an ongoing infection. HSVSM cells were infected with trypomastigotes, and after 16 hr all parasites that did not infect were removed by washing. Cells were then incubated for 48 hr after which time test compounds were added to the complete media and incubated for 16 hr; after 16 hr the treated culture medium was replaced with drug-free fresh medium and cell culture was continued for a further 24 hr. Levels of parasitemia in the treated and untreated cultures were measured (Table 2). Relative to the untreated control and the nifurtimox-treated cultures, significant trypanocidal activity was found with compound VII.

DISCUSSION

All pathogenic species of trypanosomes and *Leishmania* that have been examined possess trypanothione and the enzyme trypanothione disulfide reductase, which maintains a high intracellular concentration of this compound in the reduced (dithiol) form (17). As the enzyme trypanothione reductase appears to be ubiquitously distributed in trypanosomatids and has no direct mammalian counterpart, it represents an extremely attractive target for chemotherapy. The mammalian equivalent of trypanothione reductase is the enzyme glutathione disulfide reductase. Previous attempts to inhibit

Table 2. Effect of substituted naphthoquinone III and nitrofurans VII and VIII on an ongoing infection

Compound	Concentration, μM	Trypomastigotes per ml*
None	—	1.85×10^7
III	10	1.35×10^7
	1	4.10×10^7
VII	10	$<2.00 \times 10^4$
	1	1.80×10^7
VIII	10	2.85×10^7
	1	1.85×10^7

Structures of compounds are shown in Fig. 1.
*Number of trypomastigotes per ml counted swimming above the HSVSM cell monolayer 5 days after initial infection.

enzymes of this class (18) have been based upon alkylating or acylating agents, but such approaches have, so far, met with little practical success. For this reason we have taken a different approach to the problem.

In this study we show that trypanothione reductase can reduce compounds that, in turn, readily undergo autoxidation with concomitant production of superoxide. This finding enables us to propose a strategy for the design of trypanocidal drugs. The ability of trypanothione reductase to catalyze reduction of compounds that spontaneously reoxidize, producing superoxide, constitutes a reversal or subversion of the enzyme's normal physiological role. Thus, trypanothione reductase, normally an integral part of the trypanosomatid antioxidant defense, is induced by the compounds described here to become a source of oxidant stress. These compounds might therefore be described as subversive substrates for trypanothione reductase; in the presence of O_2 they do not inactivate the enzyme but (*i*) cause the production of free radicals, (*ii*) inhibit $Try(S)_2$ reduction, and (*iii*) cause futile consumption of NADPH. Such a combination of potentially cytotoxic events might be highly detrimental within the environment of the trypanosome. Although the present compounds were designed to examine the catalytic properties of trypanothione reductase, we were anxious to test the therapeutic potential of this approach. We therefore tested the trypanocidal activity of the compounds with *T. cruzi* trypomastigotes. The results obtained from these experiments are significant in the following sense: several compounds were found to be trypanocidal, but, more importantly, this biological activity correlated well with the ability of the compounds to act as substrates for trypanothione reductase. When the compounds were tested with crude cell extracts of *C. fasciculata, Trypanosoma brucei,* or *T. cruzi*, rates of NADPH-dependent superoxide production were only slightly higher than those generated by an equivalent amount of pure *C. fasciculata* trypanothione reductase (the differences were less by a factor of 2). Taken together these results strongly suggest that the trypanocidal activity of the compounds is specifically due to their reduction by cellular *T. cruzi* trypanothione reductase.

The compounds described here are much less efficient substrates for trypanothione reductase than trypanothione disulfide itself. However, the relative rates of reduction of these compounds correlate with the structural features designed to confer binding to the enzyme's active site. Therefore, it should be possible to design and synthesize more active substrates for the enzyme. This process will be aided by better understanding of the architecture of the enzyme's active site. Further work is also necessary to understand the mechanism of the reduction, emphasized by the finding that nitrofuran derivatives irreversibly inhibit the enzyme in O_2 absence. This inhibition may be due to enzyme-catalyzed reduction of the nitro function beyond the radical anion state. Theoretically, nitrofurans can accept up to six electrons and undergo complete reduction to the corresponding amine (19). Many potential intermediates in such a process might attach covalently to the enzyme, which would cause irreversible inhibition. In oxygen such products might not accumulate to the same extent.

In conclusion, among parasitic diseases, *T. cruzi* infection remains particularly difficult to treat by chemotherapy. At present the only drugs that are clinically available to treat Chagas disease are nitroaromatic derivatives, but the well-documented host toxicity associated with such compounds has severely limited their use. While it has been apparent for some years that the antiparasitic action of nitroaromatic drugs involves reduction of the nitro function by parasite enzymes, attempts to improve the therapeutic index of these compounds have proceeded slowly on an ad hoc basis because the parasite enzymes responsible for nitroreduction are unidentified. In the present study we have established that a specific trypanosomatid enzyme, trypanothione disulfide reductase, can catalyze redox cycling of suitably substituted compounds. These findings may lead to the development of a separate class of trypanocidal drugs with activity against the different pathogenic species that cause trypanosomiasis and leishmaniasis.

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1. Marr, J. J. & Docampo, R. (1986) *Rev. Infect. Dis.* **8,** 884–903.
2. Docampo, R. & Moreno, S. N. J. (1984) in *Free Radicals in Biology,* ed. Pryor, W. A. (Academic, London), Vol. 6, pp. 243–288.
3. Meshnick, S. R., Blobstein, S. H., Grady, R. W. & Cerami, A. (1978) *J. Exp. Med.* **148,** 569–579.
4. Fairlamb, A. H., Blackburn, P., Ulrich, P., Chait, B. T. & Cerami, A. (1985) *Science* **277,** 1485–1487.
5. Henderson, G. B., Ulrich, P., Fairlamb, A. H. & Cerami, A. (1986) *J. Chem. Soc., Chem. Commun.,* 593–594.
6. Meister, A. & Anderson, M. E. (1983) *Annu. Rev. Biochem.* **52,** 711–760.
7. Fairlamb, A. H. & Cerami, A. (1985) *Mol. Biochem. Parasitol.* **14,** 187–198.
8. Shames, S. L., Fairlamb, A. H., Cerami, A. & Walsh, C. T. (1986) *Biochemistry,* **25,** 3519–3526.
9. Penketh, P. G. & Klein, R. A. (1986) *Mol. Biochem. Parasitol.* **20,** 111–121.
10. Henderson, G. B., Fairlamb, A. H. & Cerami, A. (1987) *Mol. Biochem. Parasitol.* **24,** 39–45.
11. Krauth-Siegel, R. L., Enders, B., Henderson, G. B., Fairlamb, A. H. & Schirmer, R. H. (1987) *Eur. J. Biochem.* **164,** 123–128.
12. Henderson, G. B., Fairlamb, A. H., Ulrich, P. & Cerami, A. (1987) *Biochemistry* **26,** 3023–3027.
13. Warner, S. J. C., Aoyer, K. R. & Libby, P. (1987) *J. Exp. Med.* **165,** 1316–1331.
14. Libby, P., Alroy, J. & Pereira, M. E. A. (1986) *J. Clin. Invest.* **77,** 127–135.
15. Carlberg, I. & Mannervik, B. (1986) *J. Biol. Chem.* **261,** 1629–1635.
16. Adams, G. E., Clarke, E. D., Jocobs, R. S., Stratford, I. J., Wallace, R. G., Wardman, P. & Watts, M. E. (1976) *Biochem. Biophys. Res. Commun.* **72,** 824–829.
17. Fairlamb, A. H. & Henderson, G. B. (1987) in *Host-Parasite Cellular and Molecular Interactions in Protozoal Infections,* NATO Advanced Study Institutes Series, eds. Chang, K.-P. & Snary, D. (Springer, Berlin), pp. 29–40.
18. Schirmer, R. H., Lederbogen, F., Krauthy-Siegel, R. L. & Eisenbrand, G. (1984) in *Flavins and Flavoproteins,* eds. Bray, R. C., Engel, P. C. & Meyhew, S. G. (de Gruyter, Berlin), pp. 847–859.
19. Mason, R. P. & Josephy, P. D. (1985) in *Toxicity of Nitroaromatic Compounds,* ed. Rickert, D. (Hemisphere, New York), pp. 121–140.