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Nitrofuran Drugs as Common Subversive Substrates of *Trypanosoma Cruzi* Lipoamide Dehydrogenase and Trypanothione Reductase

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ABSTRACT. Lipoamide dehydrogenase (LipDH), trypanothione reductase (TR), and glutathione reductase (GR) catalyze the NAD(P)H-dependent reduction of disulfide substrates. TR occurs exclusively in trypanosomatids which lack a GR. Besides their physiological reactions, the flavoenzymes catalyze the single-electron reduction of nitrofurans with the concomitant generation of superoxide anions. Here, we report on the interaction of clinically used antimicrobial nitrofurans with LipDH and TR from *Trypanosoma cruzi*, the causative agent of Chagas' disease (South American trypanosomiasis), in comparison to mammalian LipDH and GR. The compounds were studied as inhibitors and as subversive substrates of the enzymes. None of the nitrofurans inhibited LipDH, although they did interfere with the disulfide reduction of TR and GR. When the compounds were studied as substrates, *T. cruzi* LipDH showed a high rate of nitrofuran reduction and was even more efficient than its mammalian counterpart. Several derivatives were also effective subversive substrates of TR, but the respective reaction with human GR was negligible. Nifuroxazide, nifuroxime, and nifurprazine proved to be the most promising derivatives since they were redox-cycled by both *T. cruzi* LipDH and TR and had pronounced antiparasitic effects in cultures of *T. cruzi* and *Trypanosoma brucei*. The results suggest that those nitrofuran derivatives which interact with both parasite flavoenzymes should be revisited as trypanocidal drugs. BIOCHEM PHARMACOL **58**;11:1791–1799, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. Chagas' disease; lipoamide dehydrogenase; nitrofurans; trypanothione reductase; *Trypanosoma cruzi*; redox cycling

Trypanosomatids are parasitic protozoa that cause a variety of tropical diseases including African sleeping sickness (*Trypanosoma brucei gambiense*, *T. brucei rhodesiense*), Chagas' disease (South American trypanosomiasis, *T. cruzi*) and the visceral, cutaneous, and mucocutaneous manifestations of leishmaniasis (e.g. *Leishmania donovani*, *L. tropica*, *L. braziliensis*). Most drugs used in the treatment of these diseases have serious side effects [1, 2]. The limited available treatment and the spread of drug resistance emphasize the need for new therapeutic agents. One approach is to take advantage of the metabolic differences between parasite and host and to develop new drugs on the basis of specific enzyme inhibitors. Another approach is to reevaluate drugs for their trypanocidal effect and their interaction with parasite target enzymes. Among these enzymes, thiol-generating oxidoreductases are of special interest. Trypanosomes and leishmania possess a unique thiol metabolism. In contrast to other eukaryotes, trypanosomatids use spermidine conjugates such as trypanothione [N^1, N^8-bis(glutathionyl) spermidine, $(T(SH)_2)$§ to maintain a reducing intracellular milieu [3]. In these protozoa, the otherwise nearly ubiquitous glutathione reductase (GR, EC 1.6.4.2; GSSG + NADPH + H^+ → 2 GSH + $NADP^+$) is replaced by trypanothione reductase (TR, EC 1.6.4.8) which keeps trypanothione in the dithiol form (TS_2 + NADPH + H^+ → $T(SH)_2$ + $NADP^+$) [4, 5]. Interestingly, *Euglena gracilis* has very recently been shown as the first protozoan to contain both reductase systems [6].

The physiological function of lipoamide dehydrogenase (LipDH, EC 1.8.1.4; dihydrolipoamide + NAD^+ → lipoamide + NADH + H^+) is the dehydrogenation of protein-bound dihydrolipoamide as part of the mitochondrial pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes. In addition, LipDH, which is identical to the earlier described diaphorase [7], catalyzes the reduction of naphthoquinone and nitrofuran derivatives such as mena-

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§ *Abbreviations:* GR, glutathione reductase; HIFBS, heat-inactivated foetal bovine serum; LipDH, lipoamide dehydrogenase; MDCK, Madin-Darby canine kidney; SOD, superoxide dismutase; TR, trypanothione reductase; and TS_2, trypanothione disulfide.

dione and nifurtimox [8, 9]. The one-electron reduced intermediates formed in this reaction can then undergo redox cycling with the concomitant generation of superoxide anion radicals and/or hydrogen peroxide [10].

Trypanothione reductase, glutathione reductase, and lipoamide dehydrogenase are closely related flavoenzymes [11]. The catalytic mechanisms and 3-dimensional structures of the three FAD-cystine oxidoreductases have been thoroughly studied [for reviews see Refs. 11 and 12]. TR and LipDH have been isolated from *T. cruzi* [5, 13] and the genes have been cloned and overexpressed [14, 15]. Both enzymes are attractive target molecules for the development of new antiparasitic drugs [12, 16, 17]. The unique TR is the key enzyme of the parasite thiol metabolism and LipDH may be involved in the trypanocidal effect of nifurtimox (Lampit®), one of two drugs used to treat acute Chagas' disease [18]. The mode of action of nifurtimox has been proposed to be due to the generation of reactive oxygen species [9, 19, 20]; this is in agreement with the known sensitivity of *T. cruzi* towards oxidative stress [12, 21]. Recently, it has been shown that nifurtimox and benznidazole, the other drug used for Chagas' disease, lower the intracellular thiol level of the parasite. Reduction of the nitroaromatic compounds, for instance to the nitroso derivatives, with the subsequent conjugation to thiols has been proposed as a mechanism [22].

In recent years, different classes of compounds have been revealed as potent inhibitors of trypanothione reductase [22–27, for reviews see Refs. 12, 16], among them several nitrofurans. In addition, TR can catalyze the one-electron reduction of naphthoquinone and nitrofuran derivatives with the subsequent production of toxic radicals as is the case for LipDH. This means that both inhibition of TR and the conversion of this antioxidant enzyme into a prooxidant enzyme by so-called subversive substrates (turncoat inhibitors) are promising strategies in the design of new drugs [24, 26, 27].

Here, we report on the interaction of clinically introduced nitrofuran drugs with *T. cruzi* LipDH and TR in comparison to mammalian LipDH and GR. The compounds were selected due to their potency as antimicrobial agents. The main purpose of this study was to identify derivatives which can act simultaneously as subversive substrates of *T. cruzi* LipDH and TR but do not interact with human GR.

MATERIALS AND METHODS

Materials

DL-α-Lipoamide, menadione (2-methyl-1,4-naphthoquinone), nifuroxazide, and nifuroxime were purchased from Sigma. Pig heart LipDH was obtained from Boehringer Mannheim, TS_2 from Bachem, and cytochrome *c* from Calbiochem. SOD was from Serva, nitrofurazone from Aldrich, and nifurtimox from Bayer Leverkusen. The other nitroaromatic compounds were provided by the following companies: nifurfoline (Laboratorios Dr. S. A. Esteve, Spain), nifurprazine (Boehringer Mannheim), nifurtoinol (Zambon), nifurzide (Lipha Arzneimittel), and nimorazole (Pharmacia-Upjohn). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) was a gift from Dr. E. Davioud, Institut Pasteur de Lille. Chinifur was made available to us by Dr. N. Cenas, Lithuanian Academy of Sciences, Vilnius. Pentamidine isethionate was purchased from Rhone-Poulenc-Rorer, sodium stibogluconate ($NaSb^v$) from Glaxo Wellcome. All reagents were of the highest available purity.

The RPMI-1640 medium was obtained from Sigma Aldrich, the starch being from Merck. Dulbecco's modified Eagle's medium was from Life Technologies and HIFBS from Harlan Sera Lab. CD1 mice and female golden hamsters (Wright's strain) were purchased from Charles River Ltd. 16-Well Lab-Tek™ tissue culture slides were obtained from Nunc and Microtest III™ tissue culture plates from Becton Dickinson.

DL-α-Dihydrolipoamide was prepared by reduction of α-lipoamide with $NaBH_4$ as described [15, 28]. *T. cruzi* LipDH was purified from recombinant *Escherichia coli* cells according to Schöneck *et al.* [15]. The protein was stored at −20° in 50 mM potassium phosphate, 1 mM EDTA, pH 7.0 (LipDH assay buffer) containing 50% glycerol, which resulted in a higher stability of the enzyme than storage as a 90% $(NH_4)_2SO_4$ pellet. Recombinant *T. cruzi* TR was prepared as described [14, 29]. Recombinant human GR [30] was a kind gift from Dr. R. H. Schirmer, Biochemie-Zentrum Heidelberg. All enzymes were dialyzed against the respective assay buffer prior to use in the kinetic studies. Stock solutions (4.5 mM) of the nitrofuran and quinone derivatives were freshly prepared. If a compound was not sufficiently soluble in buffer, but only in ethanol or DMSO, control assays containing the same amount of solvent were performed.

Parasites

Trypanosoma cruzi (strain MHOM/BR/OO/Y) trypomastigotes were derived from MDCK fibroblasts in Dulbecco's modified Eagle's medium with 10% HIFBS at 37° in an atmosphere containing 5% CO_2–air mixture. *Trypanosoma brucei brucei* (strain S427) bloodstream form trypomastigotes were maintained in HMI-18 medium [31] with 20% HIFBS at 37° in a 5% CO_2–air mixture. *Leishmania donovani* (strain MHOM/ET/67/L82) was maintained routinely in special pathogen-free (SPF) female golden hamsters (Wright's strain) by serial passage every 6 to 8 weeks.

Lipoamide Dehydrogenase Assay

LipDH dehydrogenase activity was determined at 25° in a Hitachi 150-20 spectrophotometer. The standard assay (1 mL total volume) contained 1 mM NAD^+ and 1.4 mM dihydrolipoamide in LipDH assay buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.0). The reaction was started by adding the enzyme, and the absorbance decrease at 340 nm was followed. The reverse reaction was measured in the

presence of 200 μM NADH, 300 μM NAD^+ and 1 mM lipoamide.

Trypanothione Reductase Assay

TR activity was measured at 25° in a total volume of 1 mL in the presence of 100 μM NADPH and 5–10 mU enzyme in TR assay buffer (40 mM HEPES, 1 mM EDTA, pH 7.5). The reaction was started by adding 100 μM TS_2 and NADPH consumption was followed at 340 nm. V_{max} was calculated using a K_m value of 18 μM for TS_2 [24].

Glutathione Reductase Assay

Human GR was assayed at 25° in a total volume of 1 mL containing 100 μM NADPH and 1 mM glutathione disulfide in GR assay buffer (20.5 mM KH_2PO_4, 26.5 mM K_2HPO_4, 200 mM KCl, 1 mM EDTA, pH 6.9) according to published procedures [30, 32].

Inhibitor Studies

Inhibition of LipDH by the nitrofuran and naphthoquinone derivatives (100 μM) was studied at 100 μM NAD^+ and 110 μM dihydrolipoamide in the forward reaction, and in the presence of 50 μM NADH, 50 μM NAD^+, and 800 μM lipoamide in the reverse reaction. The effect of the drugs on the physiological catalysis of TR and GR was measured by adding 100 μM of the compound to the respective standard assay (see above).

Oxidase/Nitroreductase Assay

The oxidase/nitroreductase activities of LipDH, TR, and GR, respectively, were measured spectrophotometrically at 25°. In the case of LipDH, the assay mixture (assays were performed in microcuvettes with a total volume of 90 μL in a Beckmann DU-65 spectrophotometer) contained 100 μM nitrofuran and 100 μM NADH in LipDH assay buffer, pH 7.5. After monitoring the baseline for 1 min, the reaction was started by adding the enzyme and the consumption of NADH was followed at 340 nm.

In a second assay system, reduction of the nitrofurans was coupled to cytochrome *c* reduction. The assay additionally contained 25 μM cytochrome *c* (Fe^{3+}) and the absorption increase at 550 nm [cytochrome *c* (Fe^{2+})] was measured. An ε value of 18.9 mM^{-1} cm^{-1} was used [33], which represents the difference in absorption between reduced and oxidized cytochrome *c* at 550 nm. In order to distinguish between $O_2^{-\cdot}$-mediated and direct reduction of cytochrome *c*, the reaction was followed in the presence and absence of 6 μg of SOD.

TR- and GR-oxidase/nitroreductase activities were determined by following the consumption of NADPH as described for LipDH. The assay mixtures (1 mL total volume) contained 100 μM nitrofuran and 100 μM NADPH in TR assay buffer and GR assay buffer, respectively. The reaction was started by adding the enzyme, and the absorbance decrease at 340 nm was monitored.

In Vitro *Assays in Cell Cultures*

L. DONOVANI AND *T. CRUZI*. Peritoneal macrophages were harvested from female CD1 mice by peritoneal lavage 24 hr after starch-induced recruitment. After two washing steps in medium, the exudate cells were dispensed into 16-well Lab-Tek™ tissue culture slides at 4×10^4/well in a volume of 200 μL of RPMI-1640 medium and 10% HIFBS and maintained at 37° in a 5% CO_2/air mixture. After 24 hr, macrophages were infected at a ratio of 10:1 (4×10^5 parasites/well) with *L. donovani* amastigotes freshly isolated from hamster spleen or at a ratio of 5:1 (2×10^5/well) with *T. cruzi* trypomastigotes derived from the MDCK fibroblast overlay. Infected macrophages were then maintained in the presence of drug in a threefold dilution series, with quadruplicate cultures at each concentration, for 5 days for *L. donovani* cultures and 3 days for *T. cruzi* cultures.

After these periods of drug exposure, slides were methanol-fixed and Giemsa-stained and drug activity determined by counting the percentage of macrophages cleared of amastigotes in treated cultures in comparison to untreated cultures [34]. ED_{50} values were determined by linear regression analysis. Sodium stibogluconate ($NaSb^v$) and nifurtimox were used as the respective control drugs.

T. BRUCEI. The assays were performed in medium as described above [31]. Compounds were tested in triplicate in a threefold dilution series from a top concentration of 30 μM. Parasites were diluted to 2×10^5/mL and added in equal volumes to the test compounds in 96-well, flat-bottom Microtest III™ tissue culture plates. Appropriate controls with pentamidine isethionate as the positive were set up in parallel. Plates were maintained for three days at 37° in a 5% CO_2/air atmosphere. Compound activity was determined by the use of a tetrazolium salt colorimetric assay [35] on day three. ED_{50} values were determined by linear regression analysis.

RESULTS

Reduction of Nitrofuran Drugs by T. cruzi *Lipoamide Dehydrogenase*

Nine nitrofuran derivatives (Fig. 1) with antimicrobial activity were studied as subversive substrates of *T. cruzi* LipDH (Table 1). The reaction was followed in two assay systems by monitoring the oxidation of NADH at 340 nm (reaction I in the Scheme) and by coupling the reaction to the reduction of cytochrome *c* and measuring the absorbance increase at 550 nm. The latter assay system allowed the detection of the single-electron reduction of a compound and the concomitantly generated superoxide radicals (reactions II and III in the Scheme). Inhibition of cytochrome *c* reduction by SOD showed the generation of $O_2^{-\cdot}$ radicals in the overall reaction.

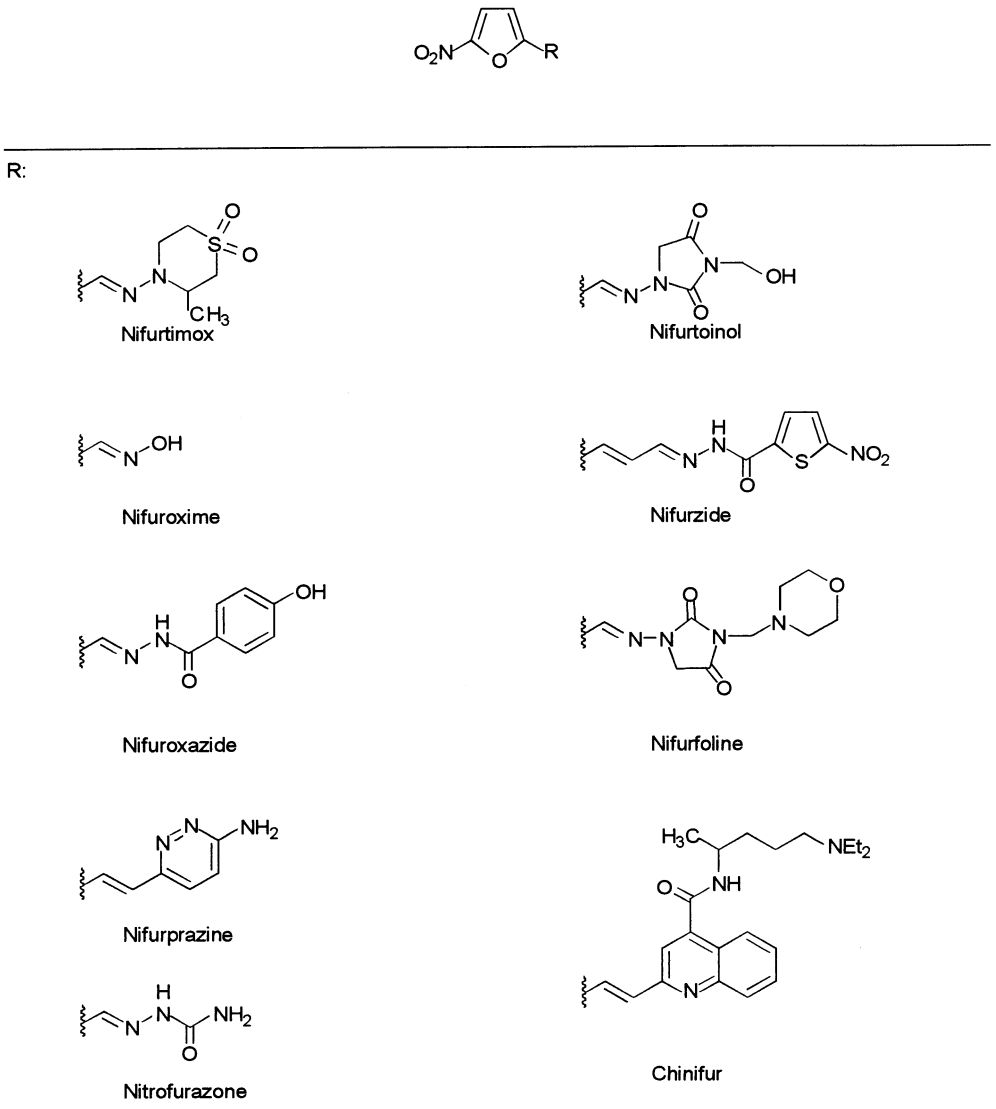

FIG. 1. Nitrofuran drugs studied as inhibitors and subversive substrates of *T. cruzi* LipDH and TR.

As summarized in Table 1, nifuroxazide, nifuroxime, nitrofurazone, and nifurprazine were readily reduced by LipDH. When compared to the intrinsic oxidase activity of LipDH, e.g. the oxidation of NADH in the absence of any substrate, these nitrofurans caused an up to 4-fold increase in the rate of NADH oxidation. In contrast, under identical conditions, the nitroimidazole nimorazole was not reduced at all (Table 1). In the cytochrome *c* assay, nifuroxazide, nifuroxime, and nifurprazine were again the most active derivatives and SOD strongly inhibited the reaction. These data clearly show that superoxide radicals are generated in the LipDH-catalyzed reduction of the nitrofurans.

In order to exclude the possibility that cytochrome *c* acts as a direct substrate of LipDH—as it does with trypanothione reductase [5]—NADH consumption was measured in the absence and presence of 25 μM cytochrome *c*. The reaction was followed at 340 nm since reduced and oxidized cytochrome *c* are isosbestic at this wavelength. The oxidase activity of LipDH did not increase in the presence of cytochrome *c*, indicating that it is not a direct substrate. This finding is in accordance with the fact that cytochrome *c* reduction by LipDH (see legend of Table 1) was completely inhibited by SOD.

When comparing the rates of NADH oxidation and cytochrome *c* reduction, a ratio of 1:2 would be expected (Scheme). In the absence of any nitro-compound, the rate of cytochrome *c* reduction at pH 7.5 was only 25% of the theoretical value. A possible explanation is that in the latter reaction only production of superoxide anions is registered, whereas the oxygen-mediated NADH consumption could also be due to formation of hydrogen peroxide. The comparably low cytochrome *c* reduction rates observed with some nitrofurans may be due to several factors. The cytochrome *c* concentration was 25 μM, which is probably

TABLE 1. Reduction of nitrofuran and naphthoquinone derivatives by *T. cruzi* lipoamide dehydrogenase

Compound [100 μm]	NADH oxidation	Cytochrome *c* reduction [U/mg]	+SOD
Nitrofuran			
Nifuroxazide	2.16 ± 0.37	4.26 ± 0.20	1.70 ± 0.03
Nifuroxime	1.73 ± 0.11	3.72 ± 0.18	1.23 ± 0.32
Nitrofurazone	1.30 ± 0.20	1.14 ± 0.06	0.40 ± 0.05
Nifurprazine	1.16 ± 0.09	2.13 ± 0.10	1.06 ± 0.04
Nifurfoline	0.73 ± 0.04	0.58 ± 0.01	≤ 0.02
Nifurzide	0.70 ± 0.11	1.52 ± 0.11	0.55 ± 0.03
Nifurtimox	0.70 ± 0.04	0.56 ± 0.06	0.10 ± 0.02
Nifurtoinol	0.62 ± 0.02	0.67 ± 0.03	≤ 0.06
Chinifur	0.61 ± 0.03	0.57 ± 0.02	0.14 ± 0.02
Nitrofuran	0.60 ± 0.02	0.44 ± 0.02	≤ 0.06
Nitroimidazole			
Nimorazole	0.57 ± 0.02	0.27 ± 0.02	0
Naphthoquinone			
Menadione	4.32 ± 0.81	10.64 ± 0.61*	2.87 ± 0.42
Plumbagin	7.34 ± 0.64	19.15 ± 0.95*	4.79 ± 0.82

Experimental conditions were as described in Materials and Methods. The activity was measured by following the consumption of NADH and by coupling the reaction to the reduction of cytochrome *c*. Inhibition of the latter reaction by SOD indicates the generation of superoxide anions. The values are the means ± SD of two to six independent measurements. The kinetics were measured with a LipDH preparation (60 U/mg) which had lost some activity during storage. Oxidation of NADH (reduction of cytochrome *c*) in the absence of any reducible substrate occurred at a rate of about 0.54 (0.27) U/mg. This activity was completely inhibited by SOD. Freshly prepared recombinant *T. cruzi* LipDH has a specific activity of about 150 U/mg.

*The observed rate of cytochrome *c* reduction of more than twice the rate of NADH oxidation is probably due to the spontaneous comproportionation of quinones and hydroquinones to the semiquinones at high concentrations.

not saturating. In addition, the activities given in Table 1 refer to a fixed nitrofuran concentration of 100 μM. Nifuroxazide, nifuroxime, nifurprazine, and nifurzide yielded a 1:2 stoichiometry under these conditions, which means that reduction of these nitrofurans results in the quantitative formation of superoxide anion radicals.

Comparison of T. cruzi *LipDH with the Mammalian Enzyme*

In order to reveal probable differences between the parasite and the host enzyme which might be exploited for the development of specific inhibitors, the interaction of *T. cruzi* LipDH with two nitrofurans as well as menadione was compared to that of the porcine enzyme. The intrinsic oxidase activity of *T. cruzi* LipDH measured at varying concentrations of NADH in the absence of any reducible

$$\text{(I)}\quad \text{NAD(P)H} + 2\text{X} \longrightarrow \text{NAD(P)}^+ + 2\text{X}^{-\cdot} + \text{H}^+$$

$$\text{(II)}\quad 2\text{X}^{-\cdot} + 2\,\text{O}_2 \longrightarrow 2\,\text{O}_2^{-\cdot} + 2\text{X}$$

$$\text{(III)}\quad 2\,\text{O}_2^{-\cdot} + 2\,\text{Cyt}\,c^{3+} \longrightarrow 2\,\text{O}_2 + 2\,\text{Cyt}\,c^{2+}$$

SCHEME 1. X = a compound which undergoes single-electron reduction by LipDH or another flavoenzyme, e.g. a nitrofuran or naphthoquinone derivative.

substrate was about 30% higher than that of the mammalian enzyme (Fig. 2). For *T. cruzi* LipDH, this activity amounted to nearly 1% of the physiological lipoamide dehydrogenase activity. Reduction of nifuroxime, nifuroxazide, and menadione by the parasite enzyme was at least two times faster than by the mammalian enzyme (Fig. 2).

Oxidase/Nitroreductase Activity of T. cruzi *TR and Human GR*

Flavoprotein oxidoreductases catalyze the (slow) oxidation of NAD(P)H by molecular oxygen in the absence of their physiological substrate. When comparing *T. cruzi* LipDH, TR, and human GR, this intrinsic oxidase activity was highest in the case of LipDH (see previous section) and lowest with GR. The ratio between the oxidase and the physiological activity was about 1:150, 1:3000, and 1:18000, respectively, for the three enzymes.

Since nifuroxazide, nifuroxime, and nifurprazine proved to be the best subversive substrates of *T. cruzi* LipDH, their interaction with *T. cruzi* TR and human GR was studied (Table 2). In the case of TR the most effective derivative was chinifur, which increased the oxidase activity 40-fold at a concentration of 100 μM [27]. The other nitrofuran derivatives also increased the oxidase activity of TR severalfold. In contrast, the oxidase activity of human GR in the presence of the nitrofurans was negligible (Table 2).

Naphthoquinones are another class of compounds that can be reduced by the flavoenzymes, leading to two- and single-electron reduced products [36, 37]. Menadione, a well-known substrate of LipDH from baker's yeast [7, 38] and an effective inhibitor of GR [39, 40], as well as plumbagin, a methyl-naphthoquinone with antileishmanial activity [41], were included in the studies. The naphthoquinones were readily reduced by *T. cruzi* LipDH and TR, but plumbagin was reduced by human GR as well (Table 2). In contrast, as described above, the nitrofurans caused only a minute increase in the oxidase activity of GR (Table 2 and [42]) and thus showed some specificity for the parasite enzymes.

Inhibition of TR and GR by Nitrofuran and Naphthoquinone Derivatives

Besides their ability to act as redox-cycling substrates, nitroaromatic compounds can interfere with the physiological activities of TR and GR [25–27, 42]. At 100 μM, none of the compounds studied here inhibited *T. cruzi* LipDH either in the forward or the reverse reactions, in accordance with studies on pig heart LipDH which showed that oxidation of dihydrolipoamide by NAD^+ was not affected by several nitrofurans [9]. The nitrofurans act as inhibitors towards *T. cruzi* TR. At fixed concentrations of 100 μM nitrofuran and 100 μM TS_2, the degree of inhibition varied between 15 and 97%. The most potent derivative was chinifur, which had previously been shown to inhibit *T. congolense* TR with a K_i of 4.5 μM [27]. In the case of *T. cruzi* TR, an IC_{50} value of about 1 μM at 100 μM TS_2 and

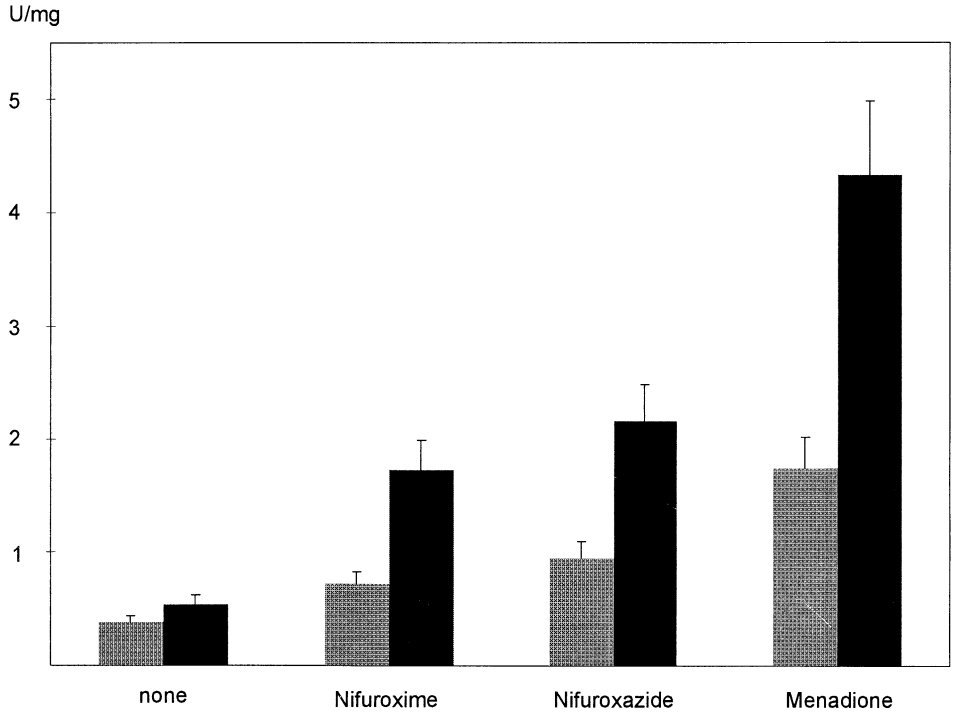

FIG. 2. Reduction of nitrofuran and naphthoquinone drugs by *T. cruzi* (dark-gray columns) and mammalian LipDH (light-gray columns). The activity was measured at 340 nm by following the consumption of NADH (100 μM) in the absence and presence of 100 μM of the respective compound. The columns are the means of at least three independent measurements, the error bars representing the deviation from the mean.

100 μM NADPH was determined. GR was inhibited with an IC_{50} value of 15 μM at 1 mM GSSG and 100 μM NADPH. At concentrations of 100 μM, nifurprazine and nifuroxazide caused a 72 and 85% inhibition of TR activity, respectively. All nitrofuran derivatives also interfered with human GR. At a saturating concentration of GSSG, 100 μM nitrofuran caused between 27 and 90% inhibition (Table 2).

The naphthoquinones inhibited *T. cruzi* TR and human GR quite effectively. Plumbagin, e.g., showed IC_{50} values of about 4 μM and 2 μM for TR and GR, respectively (data not shown). However, a preference for one of the two enzymes could not be detected.

Antiparasitic Effects of Nitrofuran Drugs in Cell Cultures

Nifuroxazide, nifuroxime, and nifurprazine, which proved to be the most effective nitrofurans in the enzyme kinetic studies, were tested for their ability to inhibit the growth of *T. cruzi*, *T. brucei*, and *L. donovani* cultures (Table 3). At 10 μM, all three nitrofurans inhibited parasite growth com-

TABLE 2. Interaction of *T. cruzi* LipDH, *T. cruzi* TR, and human GR with nitrofuran and naphthoquinone derivatives

	T. cruzi LipDH		*T. cruzi* TR		Human GR	
Compound [100 μM]	NADH oxidation [U/mg]	Inhibition of $Lip(SH)_2$ dehydrogenation [%]	NADPH oxidation [U/mg]	Inhibition of TS_2 reduction [%]	NADPH oxidation [U/mg]	Inhibition of GSSG reduction [%]
Nifuroxazide	2.16 ± 0.37	none	0.23 ± 0.03	85	0.026 ± 0.001	75
Nifuroxime	1.73 ± 0.11	none	0.42 ± 0.01	50	0.016 ± 0.001	79
Nifurprazine	1.16 ± 0.09	none	0.60 ± 0.04	72	0.014 ± 0.001	72
Nifurtimox	0.70 ± 0.04	none	0.21 ± 0.01	37	0.011 ± 0.001	62
Chinifur	0.61 ± 0.03	none	1.20 ± 0.05	97	0.013 ± 0.001	90
Nitrofuran	0.60 ± 0.02	none	0.06 ± 0.01	15	0.011 ± 0.001	27
Menadione	4.32 ± 0.81	ND	0.57 ± 0.11	85	0.043 ± 0.004	75
Plumbagin	7.34 ± 0.64	ND	1.32 ± 0.18	80	0.235 ± 0.021	72

Experimental conditions were as described in Materials and Methods. NAD(P)H oxidation was followed at 340 nm. Inhibition of LipDH was studied in the forward reaction at 100 μM NAD^+ and 110 μM dihydrolipoamide as well as in the reverse reaction. Neither reaction was inhibited. ND, not determined; inhibition could not be studied because of the rapid spontaneous reaction between dihydrolipoamide and the quinones. The assays were performed with LipDH which had a specific activity of 60 U/mg. The intrinsic NAD(P)H oxidase rate in the absence of any reducible substrate was about 0.54, 0.03, and 0.01 U/mg for *T. cruzi* LipDH, *T. cruzi* TR, and human GR, respectively.

TABLE 3. Antiprotozoal activity of nitrofuran drugs in cell cultures

Compound [μM]		Inhibition [%]					
		30	10	3	1	0.3	ED_{50}
Nifuroxazide	*T. cruzi*	t/100	100	97.8	77.0		<1
	T. brucei	100	100	40.5	0		4.2
	L. donovani	t/100	t/100	0.7	0		—
Nifurprazine	*T. cruzi*	t/100	t/100	91.2	38.2	0	2.0
	T. brucei	100	100	100	36.4	0	1.4
	L. donovani	t/100	t/100	t/+	0		—
Nifuroxime	*T. cruzi*	t/100	t/+	t/+	0		
	T. brucei	100	100	0			
	L. donovani	t/100	t/100	t/100	18		
Nifurtimox	*T. cruzi*						3.0
Pentamidine	*T. brucei*						0.02
Sodium stibo-gluconate	*L. donovani*						5.6 μg Sb^v/mL

The antiparasitic effects of the drugs were tested against the extracellular free parasites (*T. brucei*) and the intracellular parasites (*T. cruzi* and *L. donovani*), respectively as described [31, 34, 35]. t/100, the compound gives 100% clearance but is toxic to the host cells. t/+, % clearance cannot be determined because the host cells are also damaged. ED_{50}, effective doses resulting in 50% clearance.

pletely, but were also toxic to the mammalian host cells. Nifuroxime was found to damage the host cells to a greater extent than the parasites. Nifuroxazide and nifurprazine yielded ED_{50} values < 5 μM and were not as toxic towards mammalian cells. Nifurprazine (3 μM) induced a 100% growth inhibition of *T. brucei*. Nifuroxazide showed an ED_{50} value of less than 1 μM against *T. cruzi* and no toxicity towards the host cell at concentrations <10 μM (Table 3). In comparison, the ED_{50} value of nifurtimox for *T. cruzi*, the standard drug for Chagas' disease, was 3 μM.

DISCUSSION

LipDH, TR, and GR catalyze the pyridine nucleotide-dependent oxidoreduction of their respective disulfide/dithiol substrate [11]. The flavoenzymes have been shown to promote a slow non-specific oxidation of NAD(P)H by molecular oxygen, leading to reduced oxygen species. This side reaction amounts to nearly 1 and 0.03% of the physiological activity with *T. cruzi* LipDH and TR, respectively, but is practically negligible in the case of GR. Nitrofuran derivatives are able to strongly increase this oxidase activity [9, 18, 24, 26, 27, 42]. As potential antiparasitic drugs, this type of ligand may be favorable when compared to purely competitive enzyme inhibitors. Recent genetic studies on TR revealed that the enzyme must be inhibited by at least 90% in order to cause phenotypic changes such as growth arrest and an increased sensitivity towards oxidative stress [43]. Therefore, only reversible inhibitors with K_i values in the nanomolar range might become useful as potential drugs. In comparison, subversive substrates exert several synergistic effects that may be highly detrimental to the parasite: NADPH and O_2 are wasted, the thiol/disulfide ratio is lowered, and the production of free radicals can trigger multiple chain reactions [12].

Different nitrofuran derivatives have been studied with respect to their interaction with mammalian LipDH [9, 18], parasite TR [24, 26], and human or yeast GR [24, 42]. In the study presented here, the mode of action of nitrofuran drugs on *T. cruzi* LipDH and TR has been directly compared for the first time with that on mammalian LipDH and GR. The aim of this work was to identify derivatives which can trigger redox cycling in both parasite flavoenzymes.

None of the compounds inhibited *T. cruzi* LipDH, but they did strongly suppress the physiological catalysis of TR and GR (Table 2). As subversive substrates, the nitrofurans induced the highest activity in LipDH, and the parasite enzyme showed a 2- to 3-fold higher activity than the porcine protein. Sreider *et al.* [9] reported that nitrofurans with unsaturated five- or six-membered nitrogen heterocycles are more effective substrates of porcine LipDH than those bearing other groups. In our studies on the *T. cruzi* enzyme, nifuroxazide, nifuroxime, and nifurprazine caused the highest rate of superoxide generation (Table 1). Structure–activity relationships cannot be deduced from this finding, since the compounds carry quite diverse substituents (Fig. 1); nifurprazine has a nitrogen heterocyclic substituent, whereas nifuroxime has only an oxime side chain, and nifuroxazide does not possess a heterocyclic substituent.

In *T. cruzi* TR, chinifur—which is a specific inhibitor and subversive substrate of *T. congolense* TR [27]—also induced the highest oxidase activity (Table 2). In contrast, the compound caused only a very poor increase in the *T. cruzi* LipDH oxidase activity. Obviously, LipDH binds the basic ligand only weakly, an observation also made with positively charged naphthoquinones (data not shown).

Nifuroxazide, nifuroxime, and nifurprazine also increased the oxidase activity of *T. cruzi* TR. In contrast, they were barely reduced by human GR. This is in agreement with studies on the yeast enzyme which showed that nifuroxime and chinifur increased the oxidase activity of the enzyme by only 3×10^{-3} sec^{-1} [42]. As pointed out by Sreider *et al.*

[9], a significant difference between LipDH and GR is that the latter enzyme is inhibited by 5-nitrofuran derivatives but fails to catalyze redox cycling. The studies presented here indicate that TR obviously occupies a position intermediate between LipDH and GR, since the enzyme is inhibited by 5-nitrofurans and catalyzes their reduction.

The development of nitroaromatic compounds as new antiparasitic drugs may be hampered by their possible mutagenicity [2]. On the other hand, the re-evaluation of nitrofuran drugs as antitrypanosomal agents appears justified as long as trivalent arsenicals are still used for the treatment of African sleeping sickness. The nitrofurans studied here have been selected for several reasons. All compounds, except for chinifur, were licenced antimicrobial drugs. Nifurtimox is one of two drugs used in the treatment of acute Chagas' disease, and nitrofurazone can be employed in late-stage African trypanosomiasis refractory to melarsoprol, although its high toxicity makes it unsuitable for regular use.

Nifuroxazide, nifuroxime, and nifurprazine—the most effective subversive substrates of *T. cruzi* LipDH and TR—also showed antiparasitic activity in cultures of *T. brucei* and *T. cruzi*. Nifuroxazide and nifurprazine had ED_{50} values against *T. cruzi* of <1 μM and 2.0 μM, respectively, which is lower than that of the standard drug nifurtimox (3.0 μM). The molecular mode of action of nitrofurans in the parasites is not yet fully understood. In cultures of *T. cruzi* epimastigotes, nifurtimox induced an increased respiratory rate and the release of H_2O_2 from the cells [19]. In the mitochondrial extracts of the parasite, the drug stimulated superoxide production when supplemented by NADH and to a lesser extent by NADPH, which might indicate that more than one enzyme is involved. Henderson *et al.* fully attributed the antitrypanosomal effect of nitrofuran and naphthoquinone derivatives to their interaction with TR [26]. As shown here, the concerted action of the cytosolic and mitochondrial [44] TR and the mitochondrial LipDH [15] might indeed be involved in the trypanocidal activity of nifurtimox and other nitrofurans.

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