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Evaluating 5-Nitrofurans as Trypanocidal Agents

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The nitroheterocycle nifurtimox, as part of a nifurtimox-eflornithine combination therapy, represents one of a limited number of treatments targeting *Trypanosoma brucei*, the causative agent of human African trypanosomiasis. The mode of action of this prodrug involves an initial activation reaction catalyzed by a type I nitroreductase (NTR), an enzyme found predominantly in prokaryotes, leading to the formation of a cytotoxic unsaturated open-chain nitrile metabolite. Here, we evaluate the trypanocidal activities of a library of other 5-nitrofurans against the bloodstream form of *T. brucei* as a preliminary step in the identification of additional nitroaromatic compounds that can potentially partner with eflornithine. Biochemical screening against the purified enzyme revealed that all 5-nitrofurans were effective substrates for *T. brucei* NTR (TbNTR), with the preferred compounds having apparent k_{cat}/K_m values approximately 50-fold greater than those of nifurtimox. For several compounds, *in vitro* reduction by this nitroreductase yielded products characterized by mass spectrometry as either unsaturated or saturated open-chain nitriles. When tested against the bloodstream form of *T. brucei*, many of the derivatives displayed significant growth-inhibitory properties, with the most potent compounds generating 50% inhibitory concentrations (IC_{50}s) around 200 nM. The antiparasitic activities of the most potent agents were demonstrated to be NTR dependent, as parasites having reduced levels of the enzyme displayed resistance to the compounds, while parasites overexpressing TbNTR showed hypersensitivity. We conclude that other members of the 5-nitrofuran class of nitroheterocycles have the potential to treat human African trypanosomiasis, perhaps as an alternative partner prodrug to nifurtimox, in the next generation of eflornithine-based combinational therapies.

Human African trypanosomiasis (HAT) is caused by the protozoan parasite *Trypanosoma brucei*. This infection is endemic throughout sub-Saharan Africa, where transmission occurs predominantly through the blood-feeding habits of its insect vector, the tsetse fly. In the last 15 years, greater political stability, coupled with improved vector control and health surveillance programs, has significantly reduced the number of new cases of HAT from a high of around 450,000 in 1997 to about 30,000 in 2009 (1, 2). An important component of these plans involves the treatment of infected individuals. However, the current therapies against this disease are problematic, as they are costly, require medical supervision for their administration, have limited efficacy, and cause numerous adverse side effects (3). Additionally, parasite strains refractory to certain front-line drugs have been isolated from patients (2, 4, 5). Against this backdrop, there is an urgent requirement for new antitrypanosomal treatments.

The interest in using nitrofuran derivatives to treat trypanosomal infections began >60 years ago following reports that the antibacterial agent nitrofurazone displayed antiparasitic activity against *Trypanosoma equiperdum* and *T. brucei* (6–8). However, clinical trials using this agent were suspended, as it became apparent that it failed to totally eradicate parasitemia and it caused several adverse side effects. The finding that trypanosomal infections can potentially be treated with nitrofurazone sparked a series of screening programs to explore the parasite-killing activities of other nitrofurans, with nifurtimox showing particular promise against the causative agent of Chagas disease, *Trypanosoma cruzi* (9). Marketed under the name Lampit (Bayer HealthCare AG), nifurtimox, administered orally at 8 to 10 mg/kg/day in three doses for 30 to 120 days, has formed one of the front-line treatments against Chagas disease for >40 years (3). However, concerns about gastrointestinal (GI) tract and central nervous system (CNS) toxicity, the refractory nature of some *T. cruzi* strains, and the limited efficacy and genotoxicity of nifurtimox led to the withdrawal of its use as an anti-Chagasic chemotherapy in Brazil, Argentina, Chile, and Uruguay; this resulted in reduced demand and the cessation of production (10). Following successful trials of a nifurtimox-eflornithine combination therapy (NECT) and its subsequent addition to the WHO Essential Medicines List as a treatment against the cerebral stage of West African trypanosomiasis (11), coupled with emerging reports about its effectiveness against pediatric neuroblastoma (12), the manufacture of nifurtimox has recommenced with Bayer HealthCare AG, guaranteeing supplies until 2017.

As with many nitroheterocyclic agents, nifurtimox must undergo activation before mediating its cytotoxic effects, reactions catalyzed by nitroreductase (NTR) enzymes. Based on their cofactors, oxygen sensitivities, and product profiles, NTRs can be broadly divided into two groups (13). The ubiquitous oxygen-sensitive type II NTRs are flavin (flavin mononucleotide [FMN] or flavin adenine dinucleotide

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[FAD]) binding NAD(P)H-dependent enzymes that mediate the $1e^-$ reduction of the nitro substrate to form a nitro radical anion (reaction 1) (14). In an aerobic environment, this radical undergoes futile cycling, resulting in the production of superoxide anions and the regeneration of the parent nitro compound (reaction 2) (15):

$$RNO_2 + e^- \rightarrow RNO_2^{\bullet -} \quad (1)$$

$$RNO_2^{\bullet -} + O_2 \rightarrow RNO_2 + O_2^{\bullet -} \quad (2)$$

Initial findings showed that the addition of nifurtimox to *T. cruzi* mitochondrial fractions or rat liver microsomes stimulated the production of superoxide anions, leading to a widely accepted view that its trypanocidal mode of action involves the induction of oxidative stress (16, 17). Several trypanosomal enzymes, including cytochrome P450 reductases and trypanothione reductase, have been shown to mediate this reaction *in vitro*, appearing to confirm earlier findings (18–20); this idea gained strength following reports that trypanosomes had a limited enzymatic capacity to metabolize reactive oxygen species (21). To date, the only functional evidence for the involvement of superoxide anions in nifurtimox toxicity is indirect, coming from studies on the superoxide dismutase B1 isoform, where *T. brucei SODB1* (*TbSODB1*) null mutants and *T. cruzi SODB1* (*TcSODB1*)-overexpressing cells are more susceptible to the nitrofuran than are wild-type cells (22, 23). Genetic manipulation of *T. brucei* or *T. cruzi* engineered to express altered levels of oxygen-sensitive NTR activators—for example, cytochrome P450 reductase, trypanothione reductase, or other components of the trypanosomal oxidative defense system—generates parasites that display the same susceptibilities to nifurtimox as control cells (24, 25). Therefore, as both trypanosomal and mammalian cells can mediate futile cycling leading to superoxide anion production, the induction of oxidative stress via activation by oxygen-sensitive NTRs does not explain the selectivity of nifurtimox toward *T. brucei* or *T. cruzi*.

The other class of enzymes shown to activate nitroaromatic prodrugs are the oxygen-insensitive type I NTRs. These NAD(P)H-dependent FMN-containing proteins have a restricted distribution, as they are found predominantly in prokaryotes and were recently identified in some protozoans (13, 26, 27). They catalyze two sequential $2e^-$ reduction reactions resulting in the conversion of the nitro substrate to its hydroxylamine form via a nitroso intermediate (see reactions 3 and 4 below) (13). As this process does not involve oxygen and does not result in the production of reactive oxygen species, this activity is said to be "oxygen insensitive."

$$RNO_2 + 2e^- \rightarrow RNO \quad (3)$$

$$RNO + 2e^- \rightarrow RNHOH \quad (4)$$

Based on *in vitro* drug selection, gene manipulation, biochemical analysis, and functional genomics, it is now clear that a type I NTR expressed by trypanosomes plays a key role in nifurtimox activation within the parasite itself (25, 27–30). The hydroxylamine derivative generated from this activation event causes a redistribution of electrons within the structure of the compound, promoting cleavage of a C-O bond present on the furan ring (25). This results in the formation of an unsaturated open-chain nitrile, which, unlike the parental prodrug, displays equal cytotoxicity toward trypanosomal and mammalian cells. For some nitrofurans, the unsaturated open-chain nitrile can convert rapidly to a nontoxic saturated state (31). Although nifurtimox does undergo this change *in vitro*, this process occurs at a low rate (25). Based on the accumulated data, the basis of nifurtimox selectivity against trypanosomal pathogens appears to be directly related to the expression of type I NTRs, with the relative stability of the unsaturated open-chain nitrile contributing to nitrofuran's antiparasitic activity.

The prostaglandin F2α synthase (also known as "old yellow enzyme") from *T. cruzi* is an atypical nitroreductase, displaying features characteristic of both type I and II NTRs. In biochemical studies, this enzyme was shown to catalyze the $2e^-$ reduction of nifurtimox, but only under anaerobic conditions (32). Immunoprecipitation of this activity in parasite lysates lowered the capacity of the extract to reduce the nitrofuran, suggesting that it may have an important role in nifurtimox metabolism (32). However, functional studies on this enzyme have not yet been performed in *T. cruzi*, while for *T. brucei*, cells expressing elevated levels of prostaglandin F2α synthase displayed the same susceptibility to nifurtimox as control cells; this indicates that this enzyme does not play a key role in the *in vivo* metabolism of this prodrug (25).

Given the success of the NECT combination and the renewed interest in using nitroaromatics for the treatment of trypanosomal infections, we evaluated the growth-inhibitory properties of a nitrofuran series against the bloodstream form (BSF) of *T. brucei*. Included in this compound set are a number of agents proposed to mediate their activity through the inhibition of sterol biosynthesis (33, 34). From these screens, six compounds showed significant potency against trypanosomes and were more selective toward the parasite than nifurtimox in an activation mechanism where the oxygen-insensitive NTR plays a key role.

MATERIALS AND METHODS

Chemicals. The nitrofuran structures used are shown in Table 1. Their synthesis has been described previously (33–36).

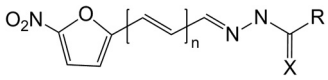
Cell culturing. *T. brucei brucei* BSF trypomastigotes (MITat 427 strain; clone 221a and a derivative [2T1] engineered to constitutively express the tetracycline repressor protein) were cultured in Hirumi's modified Iscove's 9 (HMI-9) medium with 10% tetracycline-free fetal calf serum (Autogen Bioclear) as described previously (37, 38). The 2T1 line was grown in the presence of 1 μg ml^{-1} phleomycin. Transformed 2T1 parasite lines overexpressing *T. brucei* NTR (TbNTR), prostaglandin F2α synthase (TbPGS), squalene epoxidase (TbSQE), or two cytochrome P450 reductases (TbCPR2 and TbCPR3; numbering is in accordance with that of Portal et al. [39]) were maintained in the medium, supplemented with 2.5 μg ml^{-1} hygromycin.

African green monkey kidney (Vero) cells were grown at 37°C under a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal calf serum, 20 mM HEPES (pH 7.4), 2 mM sodium glutamate, 2 mM sodium pyruvate, 2.5 U ml^{-1} penicillin, and 2.5 μg ml^{-1} streptomycin.

Plasmids. DNA fragments encoding *TbPGS* (GenBank accession no. AB034727), *TbSQE* (XP_828409), *TbCPR-2* (XM_822460), or *TbCPR-3* (XM_823737) were amplified from *T. brucei* genomic DNA and cloned into the trypanosomal expression vector pBSF-TbNTR-9e10 (27), such that the insert DNA replaced the *TbNTR* gene. Constructs were digested with AscI, and purified fragments were electroporated into *T. brucei* 2T1 BSF cells.

Drug assays. All assays were performed in a 96-well plate format. *T. brucei* BSF parasites (200 cells per well) were treated with growth medium containing different concentrations of nitrofuran. Where appropriate, protein expression was induced by adding tetracycline (1 μg ml^{-1}). After incubation at 37°C for 3 days, alamarBlue was added to each well and the plates incubated for a further 8 h (40). The fluorescence of each culture was determined using a Gemini fluorescent plate reader (Molecular Devices) at an excitation wavelength of 530 nm, emission wavelength of 585 nm, and an emission cutoff filter at 550 nm. The change in fluorescence

TABLE 1 Structures of nitrofuran compounds



Compound	n	X	R	ϵ ($M^{-1}cm^{-1}$)[a]	CLogP[b]
Thiosemicarbazones					
HC1	0	S	NH_2	12,300	0.75
HC2	0	S	$NHCH_3$	13,900	0.98
HC3	0	S	$NHCH_2CH_3$	17,090	1.34
HC4	0	S	$NHCH_2CHCH_2$	12,250	1.71
HC5	0	S	NHPh	4,770	3.00
HC15	1	S	$NHCH_3$	4,570	1.27
HC16	1	S	$NHCH_2CHCH_2$	7,940	0.33
Carbazates					
HC6	0	O	$OCHCH_2$	6,390	1.32
HC7	0	O	OCH_3	9,840	0.82
HC8	0	O	$O(CH_2)_3CH_3$	8,840	2.14
HC9	0	O	$O(CH_2)_5CH_3$	2,010	3.03
HC10	0	O	$O(CH_2)_6CH_3$	7,040	3.48
HC11	0	O	$O(CH_2)_7CH_3$	2,120	3.92
HC12	0	O	OPh	4,920	2.48
HC17	1	O	OPh		2.77
Semicarbazones					
Nitrofurazone	0	O	NH_2	12,000	−0.14
HC13	0	O	$NH(CH_2)_3CH_3$	13,490	1.41
HC14	0	O	$NHCH_2CH_2OCH_3$	15,130	0.04

[a] The molar extinct coefficients (ϵ) for HC1 to HC16 at 400 nm were determined. HC17 displayed no significant absorbance at this wavelength.
[b] CLogP values were determined using the ChemAxon CLogP calculator program.

resulting from the reduction of alamarBlue is proportional to the number of live cells. The concentration of compound that inhibited parasite growth by 50% (IC_{50}) was then established.

To assess mammalian cell cytotoxicity, Vero cells (2,000 cells per well) were treated with growth medium containing different concentrations of nitrofurans. After incubation at 37°C for 6 days, alamarBlue was added to each well and the plates incubated for a further 8 h. The cell density of each culture was determined as described above and the IC_{50} value was established.

Biochemistry. Recombinant His-tagged TbNTR was purified from *Escherichia coli* extracts as described previously (40). Enzyme activity was determined by following the changes in absorbance at 400 nm corresponding to the reduction of nitrofuran. A reaction mixture (1 ml) containing 50 mM Tris-Cl (pH 7.0), 100 μM NADH, and nitrofuran (0 to 100 μM) was incubated at room temperature for 5 min. The reaction was initiated by the addition of 20 μg trypanosomal NTR, and the initial reaction rate was measured. In the case of HC17, no significant absorbance at 400 nm was observed. For reactions involving this substrate, the change in absorbance at 340 nm was followed, corresponding to NADH oxidation (extinction coefficient value of 6,220 mM^{-1} cm^{-1}). For nifurtimox, the change in absorbance at 435 nm was followed, corresponding to the reduction of this compound (extinction coefficient value of 19,000 $mM^{-1}cm^{-1}$). For compounds HC1 to HC16, an extinction coefficient value at 400 nm was calculated and used to determine enzyme activity (Table 1). To standardize data values, enzyme activity was expressed in nmol NADH oxidized min^{-1} mg^{-1}, and it is assumed that four NADH molecules are consumed during the reduction of one nitrofuran molecule (B.S.H. and S.R.W., unpublished data). Kinetic analysis (apparent V_{max}, k_{cat}, and K_m values ± standard deviations [SD]) was performed by nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software). As the k_{cat}/K_m value is derived from two parameters, each with their own associated error, k_{cat}/K_m lower and upper error bar values were calculated by using ([mean k_{cat} − SD]/[mean K_m + SD]) and ([mean k_{cat} + SD]/[mean K_m − SD]), respectively.

Analysis of metabolites. The substrate (nitrofurazone, HC1, HC2, HC4, HC10, or HC11, all at 100 μM) was completely reduced by the activity of recombinant TbNTR (40 μg ml^{-1}). The His-tagged enzyme was removed by binding to nickel-nitrilotriacetic acid (Ni-NTA), the protein was pelleted, and the supernatant was analyzed using a Series 1100 liquid chromatograph (LC) with SL Ion Trap mass spectrometer (MS) (Agilent). Fractionation was carried out on a 5-mm HyPurity Elite C_{18} column (15 mm by 2.1 mm) (Thermo Scientific) preequilibrated with 10% acetonitrile eluting with a 10 to 30% acetonitrile gradient at a flow rate of 0.2 ml min^{-1}. Metabolites were detected using a diode array (λ at 250, 300, 340, and 450 nm) and *m/z* was determined by positive electrospray ionization. Mass spectrometry was carried out with a drying gas temperature of 325°C, drying gas flow of 10 liters min^{-1}, nebulizer gas pressure of 25 lb/in^2, and a capillary voltage of 3,500 V in full scan mode in the *m/z* range 50 to 400. Negative tandem mass spectrometry was performed in automatic mode using SmartFrag for ion fragmentation.

RESULTS

Nitrofurans are substrates for the trypanosomal NTR. Most nitroheterocyclic compounds function as prodrugs and must undergo activation before mediating cytotoxic effects. For the trypanocidal agent nifurtimox, a type I NTR has been shown to play a key role in this process (27). To evaluate whether other nitrofuran-based compounds are activated via this mechanism, a preliminary enzyme activity screen using purified recombinant His-tagged *T. brucei* NTR was performed (Fig. 1A). The previously reported NTR assay that monitored the oxidation of NADH

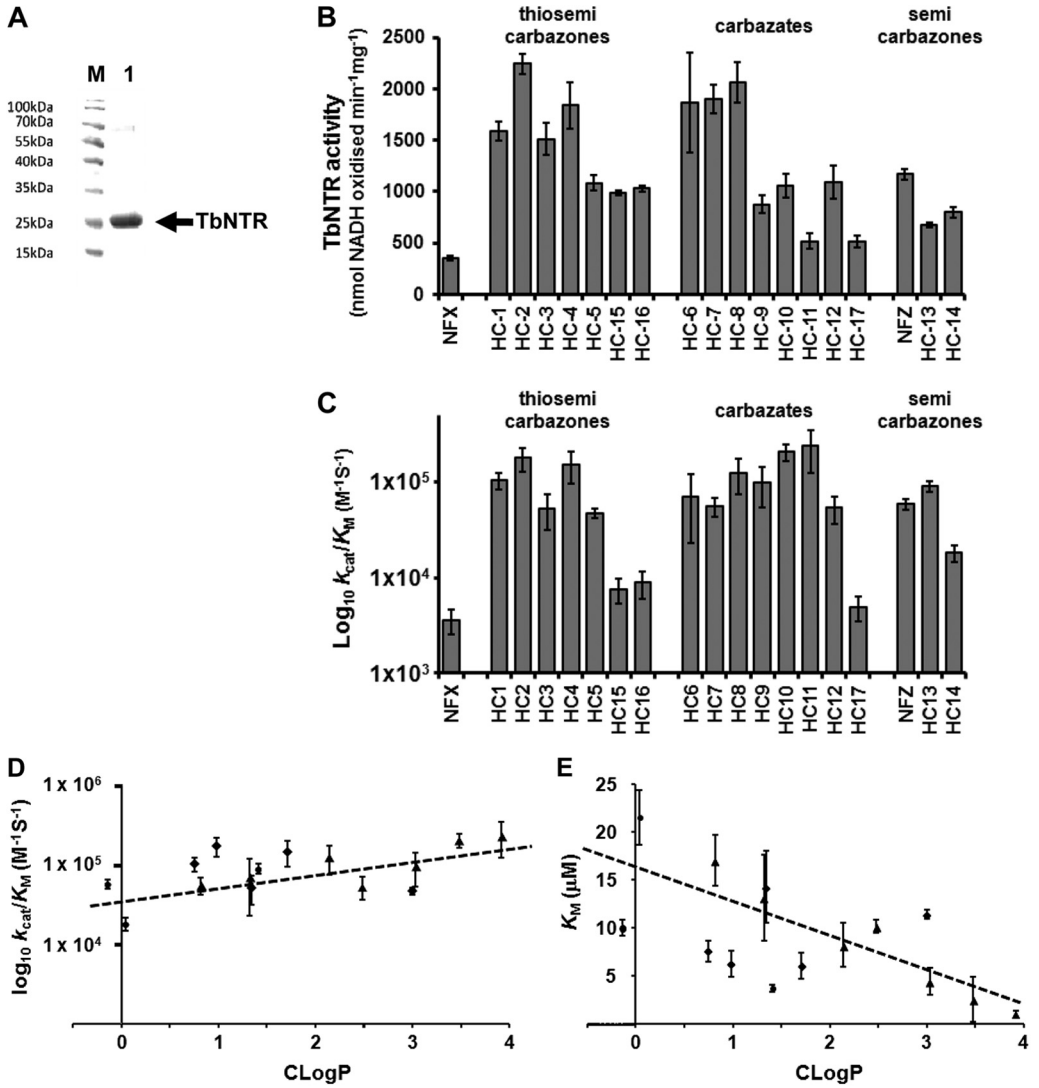


FIG 1 Analysis of TbNTR activity toward nitrofurans. (A) SDS-PAGE gel (10%) stained with Coomassie blue. Lane M, size standards; lane 1, recombinant TbNTR purified as described previously (41). (B) In the presence of NADH (100 mM), the activity of purified His-tagged TbNTR was assessed by following the reduction of the nitrofuran substrates at 400 nm (HC1 to HC16 and nitrofurazone) or 435 nm (nifurtimox [NFX]). For HC17, the oxidation of NADH at 340 nm was monitored due to the absorption properties of this compound. To standardize the data, activity was expressed in nmol NADH oxidized min^{-1} mg^{-1} (see Materials and Methods). (C) Catalytic efficiency (k_{cat}/K_m) of TbNTR toward different non-alkene-linked nitrofurans. (D) The hydrophobicity of the nitrofurans was determined (CLogP) using the ChemAxon CLogP calculator program and compared against the catalytic efficiency of TbNTR (k_{cat}/K_m) toward each thiosemicarbazone (diamonds), semicarbazone (circles), and carbazate (triangles). (E) Comparison of CLogP with apparent K_m values for thiosemicarbazone (diamonds), semicarbazone (circles), and carbazate (triangles). For each compound, the enzyme activity using several nitrofurans (0 to 100 μM) was determined, from which apparent V_{max}, k_{cat}, and K_m values ± standard deviations were generated by nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software). These kinetic values were then used to calculate k_{cat}/K_m values, with the associated error bars obtained by comparison of the k_{cat} and K_m standard deviations (see Materials and Methods).

could not be used here, as many nitrofurans displayed significant absorbance at 340 nm (data not shown). Therefore, the direct reduction of the nitroheterocycle itself was followed by monitoring the change in absorbance at 400 nm (for HC1 to HC16 and nitrofurazone) or 435 nm (for nifurtimox); the molar extinct coefficient for each compound was established (Table 1). For HC17, no significant absorbance at 435, 400, or 340 nm was observed, so for reactions involving this substrate, the change in absorbance at 340 nm was followed, corresponding to NADH oxidation.

In total, 19 compounds, including nifurtimox and nitrofurazone, were screened against TbNTR. All structures contained a common 5-nitrofuran core with different substituent groups at the 2-position on the furan ring. Based on the nature of this side chain, these compounds could be divided into thiosemicarbazones (HC1 to HC5, HC15, and HC16), semicarbazones (nitrofurazone, HC13, and HC14), and carbazates (HC6 to HC12 and HC17); nifurtimox is distinct in that the substituent contains a thiomorpholine ring linked to nitrofuran by a hydrazone moiety. For 3 derivatives (HC15 to HC17), an alkene linker group was inserted between the thiosemicarbazone/carbazate chain and furan ring. All compounds were metabolized by the trypanosomal enzyme, generating a higher activity, as judged by apparent V_{max}

values, than that of nifurtimox (Fig. 1B). A comparison of the catalytic efficiency of TbNTR toward each substrate revealed that no single agent or class of nitrofuran (thiosemicarbazone, semicarbazone, or carbazate) was preferentially metabolized by the parasite enzyme (Fig. 1C); k_{cat}/K_m values for the nitrofurans reported here were between 6.5×10^3 and 2.4×10^5 $M^{-1}s^{-1}$, compared to 3.6×10^3 $M^{-1}s^{-1}$ for nifurtimox. For nitrofuran derivatives containing an alkene linker, lower apparent V_{max} values were observed than for their nonlinkered counterparts (HC2 versus HC15, HC4 versus HC16, and HC12 versus HC17) (Fig. 1B), a difference manifested by their lower k_{cat}/K_m values (Fig. 1C); nitrofurans containing an alkene linker had a lower affinity for TbNTR than their nonlinkered counterparts (K_m for HC2 was 6.3 ± 1.4 μM versus 65.1 ± 16.2 μM for HC15; K_m TbNTR for HC4 was 6.1 ± 1.4 μM versus 57.9 ± 15.6 μM for HC16). An association was observed when analyzing the relationship between the catalytic efficiency values of TbNTR toward each non-alkene-linkered compound and the calculated hydrophobicity (CLogP) of each nitrofuran (Fig. 1D). This indicated that the more hydrophobic a substrate is, the higher its k_{cat}/K_m value. Given that the apparent V_{max} values for most hydrophobic substrates are low, the observed catalytic efficiencies are probably due to the higher affinities that such compounds have for the parasite enzyme, and this is reflected in their low apparent K_m values (Fig. 1E).

Trypanocidal activity of nitrofuran derivatives. The nitrofuran derivatives used in this study were selected on the basis of their anti-*T. cruzi* properties (33–36). To determine whether these agents also affected *T. brucei*, growth-inhibitory assays were performed (Table 2). An initial screen revealed that only 1 of the 19 compounds (HC5) did not affect the growth of BSF trypanosomes at concentrations up to 20 μM. For the remaining 18 compounds, further studies revealed that 13 had IC_{50}s of <1 μM, with 6 yielding values of <250 nM; these were the thiosemicarbazones HC1, HC2, and HC4, the carbazates HC10 and HC11, and the semicarbazone nitrofurazone. In contrast to other nitroheterocyclic compounds (41, 42), no relationship between the antiparasitic properties of nitrofurans and the enzymatic activities of TbNTR toward these substrates was observed (Table 2; Fig. 1).

To demonstrate that the nitrofurans were metabolized by TbNTR in the parasite itself, the susceptibilities of trypanosomes with altered levels of the oxidoreductase were investigated (Fig. 2). In most cases, cells expressing elevated levels of TbNTR (+tet) were between 4- and 15-fold-more sensitive to the nitrofurans than controls (−tet) (Table 3; Fig. 2A). For 1 compound, HC6, the recombinant and wild-type lines displayed equivalent susceptibilities. When the converse experiments were performed using *TbNTR* heterozygous parasites, cells expressing lower levels of the oxidoreductase were between 1.5- and 3-fold more resistant to most nitrofurans than controls (Table 4; Fig. 2B). For two compounds, HC6 and HC10, the recombinant and wild-type lines displayed equivalent susceptibilities. In the case of HC6, the alteration of TbNTR levels had no effect on the susceptibility of the parasites to this derivative, suggesting that the trypanocidal activity of this nitrofuran does not involve the type I nitroreductase. When the susceptibilities of *TbNTR* heterozygous lines and controls to HC10 were investigated, similar IC_{50}s were obtained, whereas cells overexpressing this enzyme displayed hypersensitivity. This suggests that the metabolite(s) generated from HC10 following TbNTR reduction might be extremely toxic to the parasite and that reducing the enzyme levels by half has little effect on the trypanocidal activity of this drug; of all the 5-nitrofurans screened, HC10 is the most potent agent against both BSF *T. brucei* and mammalian cells.

TABLE 2 Susceptibilities of bloodstream-form *T. brucei* and mammalian cells to nitrofurans

Compound	IC_{50} ± SD (nM)		Selectivity index[c]
	T. brucei[a]	Vero[b]	
Nifurtimox	2,780 ± 200	64,000 ± 1,000	23
Thiosemicarbazones			
HC1	180 ± 10	24,000 ± 1,000	133
HC2	200 ± 10	22,000 ± 1,000	110
HC3	500 ± 50		
HC4	240 ± 303	20,000 ± 1,000	83
HC5	>20,000		
HC15	670 ± 20		
HC16	790 ± 40		
Carbazates			
HC6	540 ± 70		
HC7	1,430 ± 90		
HC8	2,520 ± 330		
HC9	1,650 ± 60		
HC10	120 ± 20	14,000 ± 1,000	116
HC11	170 ± 11	45,000 ± 2,000	264
HC12	380 ± 40		
HC17	430 ± 60		
Semicarbazones			
Nitrofurazone	230 ± 80	36,000 ± 3,000	157
HC13	1,280 ± 60		
HC14	570 ± 30		

[a] Data shown are means ± SD from four experiments.
[b] Data shown are means ± SD from three experiments.
[c] The selectivity index (SI) was calculated as a ratio of the IC_{50} against Vero cells to the IC_{50} against the parasite.

Other parasite proteins have been postulated to interact with nitrofuran derivatives. These include prostaglandin F2α synthase and cytochrome P450 reductases, which are enzymes believed to be activators of nifurtimox, and squalene epoxidase, which is hypothesized to be directly inhibited by parent nitrofurans or metabolites derived from nitroheterocycles (32, 33, 39). To examine whether these enzymes affect or are affected by the 6 most potent compounds identified through our screens, recombinant TbPGS, TbCPR2, TbCPR3, or TbSQE was expressed in BSF parasites, as described for TbNTR (Fig. 3). In all cases, the expression of these other enzymes had no effect on parasite susceptibility (data shown in Fig. 3 relates to HC2 and nitrofurazone, but all six compounds behaved similarly); it must be noted that the expression of these enzymes was determined through Western blot analysis and not via functional activity. This indicates that in the *T. brucei* form that is able to replicate in the mammalian host, these enzymes do not play a significant role in the metabolism of the nitroheterocyclic compounds.

Cytotoxicity of nitrofuran derivatives to mammalian cells. The 6 compounds displaying the highest trypanocidal activities (IC_{50}s < 300 nM) against BSF *T. brucei* were assayed for cytotoxicity against Vero cells (Table 2). The selectivity index (calculated as a ratio of the IC_{50} against the mammalian line to the IC_{50} against the parasite) for each agent was then determined. In all cases, the 6

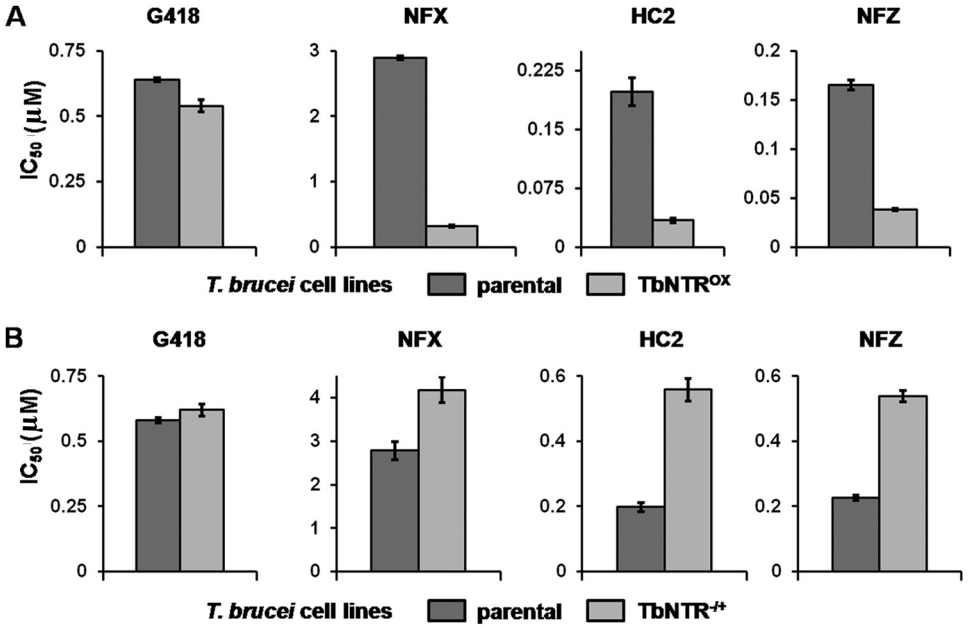


FIG 2 Susceptibility of the bloodstream form of *T. brucei* with varied levels of TbNTR to nitrofurans. Growth-inhibitory effects (IC_{50}s in μM) of HC2 and nitrofurazone (NFZ) on *T. brucei* cells overexpressing ($TbNTR_{ox}$) (A) or with lowered levels ($TbNTR^{-/+}$) (B) of TbNTR are shown. Data are means from 4 experiments ± the SD, and the differences in susceptibilities were statistically significant ($P < 0.01$), as assessed by Student's *t* test. The other 4 most potent nitrofurans (HC1, HC4, HC10, and HC11) behaved similarly to HC2 and nitrofurazone. G418 and nifurtimox were used as drug controls.

TABLE 3 Susceptibilities of bloodstream-form *T. brucei* with elevated levels of TbNTR to nitrofurans[a]

Compound	*T. brucei* IC_{50} ± SD (nM)[b]	
	−tet	+tet
Nifurtimox[c]	2,890 ± 30	315 ± 15
Thiosemicarbazones		
HC1	290 ± 20	70 ± 5
HC2	200 ± 5	35 ± 5
HC3	500 ± 40	70 ± 5
HC4	270 ± 15	35 ± 5
HC15	880 ± 60	65 ± 5
HC16	760 ± 45	75 ± 5
Carbazates		
HC6	475 ± 40	320 ± 20
HC7	1,620 ± 15	145 ± 30
HC8	2,065 ± 225	350 ± 50
HC9	2,165 ± 140	180 ± 5
HC10	100 ± 10	25 ± 5
HC11	385 ± 40	50 ± 5
HC12	2,765 ± 240	835 ± 25
HC17	1,945 ± 134	145 ± 10
Semicarbazones		
Nitrofurazone	165 ± 5	40 ± 5
HC13	1,165 ± 140	75 ± 5
HC14	625 ± 15	160 ± 5
G418[c]	640 ± 10	540 ± 20

[a] Growth-inhibitory effect as judged by the IC_{50}s (in nM) of all nitrofuran compounds on *T. brucei* control (−tet) and TbNTR-overexpressing (+tet) cells.
[b] Data shown are means from 4 experiments ± the standard deviations.
[c] G418 and nifurtimox were used as drug controls.

compounds displayed selective toxicity toward the parasite *in vitro*, having selectivity index values between 3- and 11-fold greater than that of nifurtimox.

TbNTR reduces nitrofurans to open-chain nitriles. Reduction of nitroheterocyclic compounds by type I NTRs can result in the formation of various metabolites (31, 43). To identify which products are generated following TbNTR reduction, the 6 most potent nitrofurans were enzymatically reduced and the resultant material analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. 4). For all thiosemicarbazones (HC1, HC2, and HC4), LC-MS analysis identified a single analyte whose mass spectrum was compatible with the saturated open-chain nitrile form of the parent compound. In all cases, negative tandem MS unequivocally confirmed the nature of this structure. For example, HC2 reduction leads to formation of an *m/z* fragment for $[M\text{-}H]^-$ at 197 (Fig. 4A, peak a). This then fragments to yield four ions with *m/z* values of 89, 97, 107, and 124 (Fig. 4B). These ions are the result of the eliminative cleavage of weak N-N or N-C bonds to give thioamide (*m/z* value of 89) and truncated open-chain nitrile (*m/z* values of 107 and 124) forms, with the 124 ion undergoing further fragmentation to release a nitrile moiety (*m/z* value of 97) as NCH.

In the case of the semicarbazones (nitrofurazone) and carbazonates (HC10 and HC11), TbNTR-mediated reduction results in two major products. The mass spectrum of one ion was compatible with that of the unsaturated open-chain nitrile form of the parent compound, while the second might have been generated by either a saturated open-chain nitrile or an amine. For nitrofurazone, reduction led to the formation of two ions of $[M\text{-}H]^-$ with *m/z* values of 165 and 167 (Fig. 4C, peaks b and a, respectively), with peak b corresponding to the unsaturated open-chain nitrile and peak a representing the saturated open-chain nitrile or amine.

TABLE 4 Susceptibilities of bloodstream-form *T. brucei* with reduced levels of TbNTR to nitrofurans[a]

Compound	*T. brucei* IC_{50} (nM)[b]	
	Wild-type	$TbNTR^{+/-}$
Nifurtimox[c]	2,800 ± 195	4,200 ± 290
Thiosemicarbazones		
HC1	180 ± 5	320 ± 10
HC2	200 ± 15	560 ± 35
HC3	500 ± 45	760 ± 5
HC4	240 ± 35	1,295 ± 30
HC15	665 ± 20	1,080 ± 125
HC16	790 ± 40	1,350 ± 95
Carbazates		
HC6	535 ± 70	620 ± 25
HC7	1,430 ± 90	2,100 ± 135
HC8	2,520 ± 330	4,595 ± 550
HC9	1,650 ± 65	2,930 ± 250
HC10	120 ± 15	140 ± 15
HC11	170 ± 5	310 ± 55
HC12	380 ± 40	700 ± 75
HC17	430 ± 60	765 ± 1,250
Semicarbazones		
Nitrofurazone	225 ± 10	540 ± 15
HC13	1,275 ± 55	1,720 ± 85
HC14	575 ± 25	905 ± 30
G418[c]	580 ± 10	625 ± 25

[a] Growth-inhibitory effect as judged by IC_{50}s (in nM) of all nitrofuran compounds on *T. brucei* wild-type parasites and *TbNTR* heterozygous line (designated $TbNTR^{+/-}$).
[b] Data shown are the means from 4 experiments ± the standard deviations.
[c] G418 and nifurtimox were used as drug controls.

To determine the nature of peak a, the saturated open-chain nitrile or amine ion was subjected to negative tandem MS. In all cases, the resultant fragmentation profile was consistent with the saturated open-chain nitrile. For nitrofurazone, negative tandem MS on the 167 fragment (Fig. 4C, peak a) resulted in the formation of two distinct ions with *m*/*z* values of 140 and 123. The 140 ion was generated by the loss of the nitrile, as NCH, from the saturated open-chain nitrile structure, while the 123 ion corresponds to the loss of a carboxamide group at the opposite end of the molecule (Fig. 4D).

DISCUSSION

The effectiveness of nitrofurans against *T. brucei* was demonstrated >40 years ago, although this promise did not immediately translate to clinical treatments against HAT (7, 9). However, the problems related to current therapies have stimulated a renewed interest in using nitroaromatic compounds to target *T. brucei* (41, 42, 44), with NECT being added to the WHO Essential Medicines List for the treatment of West African trypanosomiasis (11). Here, we conducted a screening program against BSF *T. brucei*, making use of a 5-nitrofuran series that was originally developed for use against Chagas disease (33–36). Most compounds exhibited higher activities toward BSF parasites than nifurtimox (Table 2), although the 6 most potent agents (those with IC_{50}s < 300 nM) were also toxic to mammalian cells. However, by virtue of their effects on trypanosome growth, these 6 all had improved selectivities against the parasite compared to nifurtimox. Understanding how these compounds mediate their antimicrobial activities may help in developing trypanocidal derivatives with reduced host toxicity.

Previous studies have shown that the type I nitroreductase-mediated activation of nifurtimox is key to how this prodrug me-

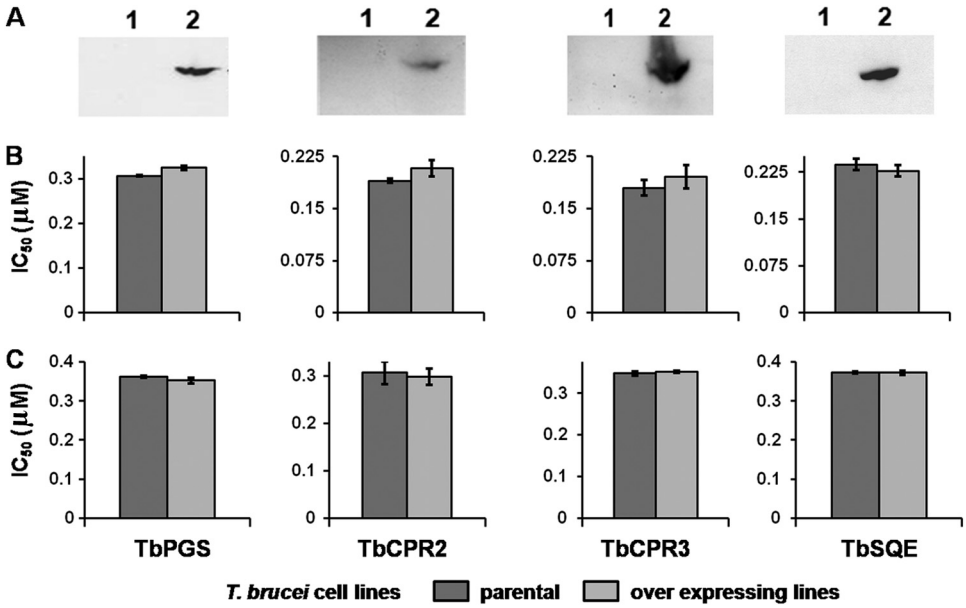


FIG 3 Susceptibility of the bloodstream form of *T. brucei* ectopically expressing other enzymes proposed to interact with nitrofurans. (A) Blot containing lysates (10 μg) from the bloodstream forms of uninduced (1) and tetracycline-induced (2) cultures probed with a 9E10 antibody (Merck Millipore). Extracts were generated from cells induced to express c-Myc-tagged TbPGS, TbCPR2, TbCPR3, or TbSQE. Protein loading was judged by Coomassie blue staining (not shown). (B and C) Growth-inhibitory effect (IC_{50}s in μM) of HC2 (B) and nitrofurazone (C) on *T. brucei* cells induced to express recombinant TbPGS, TbCPR2, TbCPR3, or TbSQE. The other 4 most potent nitrofurans (HC1, HC4, HC10, and HC11) behaved similarly to HC2 and nitrofurazone. Data shown are means from 4 experiments ± the SD.

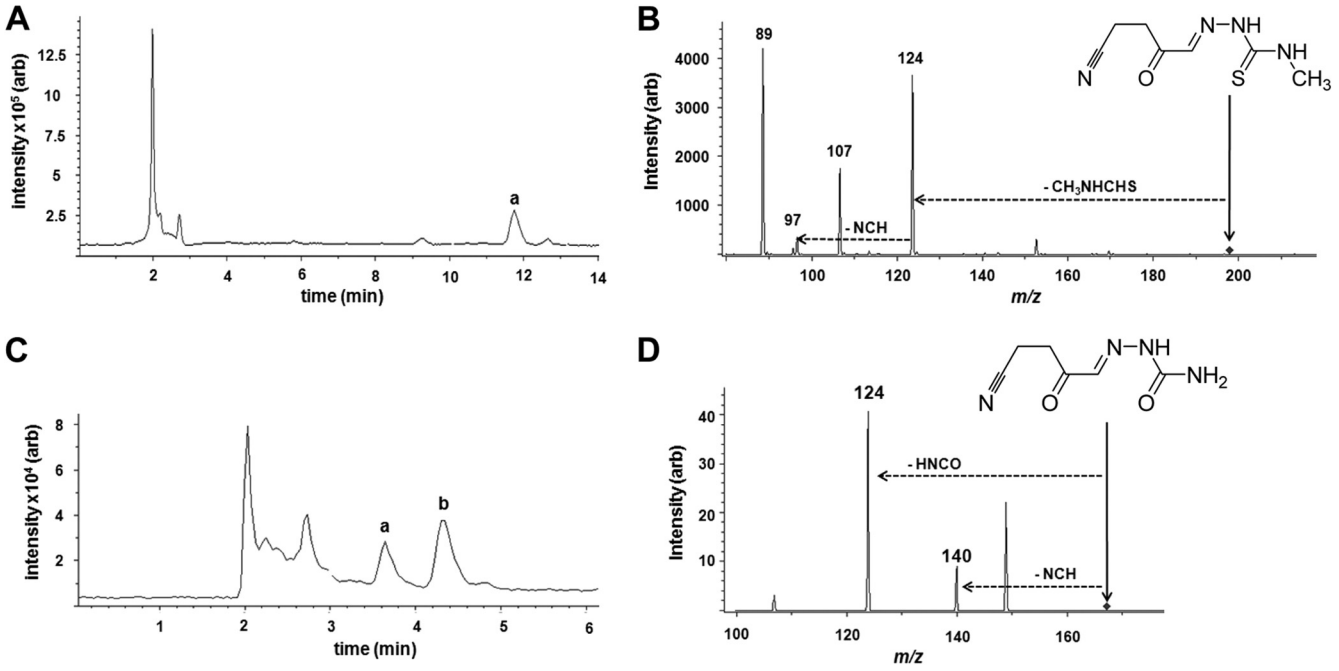


FIG 4 Characterizing the TbNTR-mediated nitrofuran reduction products. (A) Negative enhanced MS (EMS)-MS analysis of the TbNTR-mediated HC2 reduction product identifies a single analyte (peak a) whose mass spectrum contained a molecular ion for $[M-H]^-$ with *m/z* values of 197. (B) Negative electrospray ionization (ESI) MS-MS analysis of peak a (identified in panel A) generates $[M-H]^-$ ions with *m/z* values of 90, 97, 107, and 124. Analysis of these fragments indicates that the $[M-H]^-$ ion having an *m/z* value of 198 corresponds to a saturated open-chain nitrile (molecular weight of 198). (C) Negative EMS-MS analysis of the TbNTR-mediated nitrofurazone reduction products identifies two ions (peaks a and b) with *m/z* values of 167 (peak a) and 165 (peak b). (D) Negative ESI MS-MS analysis of the *m/z* 167 metabolite (identified in panel C) generates $[M-H]^-$ ions with *m/z* values of 124 and 140. Fragment analysis indicates that the $[M-H]^-$ ion having an *m/z* value of 167 corresponds to a saturated open-chain nitrile structure, while the *m/z* 165 ion corresponds to its unsaturated derivative.

diates its antiparasitic selectivity (25, 27). Using biochemical and phenotypic screens, we demonstrated that this activation mechanism extended to most of the 5-nitrofuran series examined here. *In vitro* studies revealed that all compounds were metabolized by purified TbNTR (Fig. 1B), with the enzyme having a high affinity for hydrophobic substrates (Fig. 1E), while parasite lines expressing elevated or reduced levels of TbNTR had altered susceptibilities to most of these agents (Tables 3 and 4; Fig. 2). This activation step appears to be TbNTR-specific, as phenotypic screening using parasite lines expressing elevated levels of cytochrome P450 reductase or prostaglandin F2α synthase, which are enzymes postulated to activate 5-nitrofurans (32, 39), had no effect on parasite susceptibility (Fig. 3). This is not to say that the 5-nitrofuran derivatives do not act as substrates for cytochrome P450 reductase or prostaglandin F2α synthase, merely that any interaction with these enzymes is not the primary route of parasite growth inhibition. Treatment of *T. cruzi* with certain nitrofurans leads to an intracellular accumulation of squalene, a phenomenon attributed to the inhibition of squalene epoxidase (33). Overexpression of this enzyme in BSF parasites did not affect the susceptibility of the trypanosomes to the most potent nitrofurans and indicates that these agents do not mediate their anti-*T. brucei* activities through squalene epoxidase inhibition. Why the nitrofurans appear to display distinct mechanisms of action toward these two trypanosomes species might reflect differences in the relative importance of the sterol biosynthesis pathway to these organisms; this pathway has long been regarded as a chemotherapeutic target against Chagas disease, while BSF *T. brucei* can readily scavenge these organic molecules from their environment (45).

To characterize any stable products arising from TbNTR-mediated reduction of selected 5-nitrofurans, an analysis of the metabolites was performed using liquid chromatography coupled to tandem mass spectrometry. Using the semicarbazone nitrofurazone, two major end products were isolated and identified as saturated and unsaturated open-chain nitrile derivatives (Fig. 4). During nitrofuran reduction with bacterial type I nitroreductases, the saturated form is frequently detected, whereas the unsaturated structure, although postulated as an intermediate (13), is not. Toxicity studies have shown that the saturated form does not affect cell growth, resulting in the hypothesis that the intermediates generated during its formation are responsible for the antimicrobial properties of this prodrug. In contrast, analysis of the metabolites generated following nifurtimox reduction by the *T. cruzi* or *T. brucei* type I nitroreductases identified the unsaturated open-chain nitrile as the major end product, which then undergoes further reduction to the saturated form, albeit at a very low rate (25). Analysis of the toxicity of the unsaturated form demonstrated that this affected *T. brucei* and mammalian cells at equivalent levels, unlike the parental prodrug, which shows selectivity toward the parasite. The data reported here clearly show that following the initial nitrofurazone reduction, conversion of the unsaturated to saturated form occurs more readily than for nifurtimox. Based on the above reports, it is tempting to speculate that the trypanocidal activity displayed by nitrofurazone stems from the unsaturated open-chain nitrile, at least in part. However, the effects of other, unstable intermediaries cannot be ruled out at this stage. When analyzing two structurally related carbazate derivatives (HC10 and HC11), the corresponding unsaturated and sat-

urated end products were also observed (data not shown), indicating that this reaction is common to many semicarbazone- and carbazate-based nitrofuran derivatives and is therefore unlikely to be an experimental artifact.

When these studies were extended to thiosemicarbazone derivatives (HC1, HC2, and HC4), only the saturated open-chain nitrile end metabolite was observed (Fig. 4). Presumably, the unsaturated open-chain nitrile metabolites resulting from the type I nitroreductase-mediated reactions are too unstable to isolate with this analytical technique. The obvious difference between these compounds and those described earlier is the presence of the much bulkier sulfur-containing thiosemicarbazone group in place of the oxygen-based semicarbazone group, which might influence the stability of any intermediates.

As noted, nitroheterocyclic drugs are experiencing a renaissance as treatments for HAT, with nifurtimox being deployed alongside eflornithine against the West African form and the 5-nitroimidazole fexinidazole entering phase I clinical trials (www.dndi.org). Fexinidazole activity against *T. brucei* was first discovered almost 30 years ago, yet its full potential has only recently been appreciated (44). While it has yet to be explicitly demonstrated that fexinidazole is activated by the trypanosomal type I nitroreductase, it is noteworthy that a *T. brucei* cell line resistant to nifurtimox shows cross-resistance to fexinidazole (46). Like many nitroheterocyclic drugs, fexinidazole fails the "classical" Ames test of mutagenicity, yet it is not mutagenic to type I nitroreductase-deficient bacteria or to mammalian cells (44). Whether the type I nitroreductase-mediated activation of nitroheterocyclic drugs, such as nifurtimox and fexinidazole, to their toxic metabolites is the sole cause of all short- and long-term cytotoxic effects observed remains unclear and seems unlikely, given the observation of nitroheterocyclic drug metabolites in animal tissues (47, 48). Therefore, perhaps the more subtle question to be addressed is, do nitroheterocyclic drugs have the potential to be designed with enough specificity towards the trypanosomatid type I nitroreductase, so as to present only a reasonable risk for acceptable associated side effects to human patients over both the short and longterm? Appreciating the shared mechanisms of nitroaromatic drug activation and predicting the downstream metabolites across many classes of compounds by the trypanosomal nitroreductase are fundamental aspects of overcoming this challenge.

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