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Trypanocidal Activity of Melamine-Based Nitroheterocycles

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A series of nitroheterocyclic compounds were designed with linkages to melamine or benzamidine groups that are known substrates of the P2 aminopurine and other transporters in African trypanosomes of the brucei group. Several compounds showed in vitro trypanotoxicity with 50% inhibitory concentrations in the submicromolar range. Although most compounds interacted with the P2 transporter, as judged by their ability to inhibit adenosine transport via this carrier, uptake through this route was not necessary for activity since *TbAT1*-null mutant parasites, deficient in this transporter, retained sensitivity to these drugs. One compound, a melamine-linked nitrofuran, also showed pronounced activity against parasites in mice. Studies into the mode of action of this compound indicated that neither reductive, nor oxidative, stress were related to its trypanocidal activity ruling out a genotoxic effect in *T. brucei*, distinguishing it from some other, mammalian cell toxic, trypanocidal nitroheterocycles.

There is an urgent need for the development of new drugs to treat human African trypanosomiasis, owing to poor clinical efficacy and toxic side effects of current drugs and a growing problem of resistance at a time when the disease has become resurgent (25).

In order to exert trypanocidal activity, drugs must first enter the parasites or else act on essential plasma membrane-associated targets. An unusual aminopurine transporter, termed the P2 transporter, is one of several that can carry purine nucleosides and bases into these cells (27, 37). The P2 transporter recognizes adenine and adenosine as substrates, but it can also transport melamine (triazine) derivatives and benzamidine derivatives that include several known drugs used against the African trypanosomiases (Fig. 1) (4, 11, 14).

Other transporters can also carry drugs into trypanosomes and contribute to their selectivity (15). A better understanding of these routes of uptake and the design of agents that can be delivered to trypanosomes via these portals offers one route to urgently needed trypanocidal drugs. We have previously reported approaches to selectively deliver compounds to trypanosomes using the P2 transporter (6, 23, 38). The principal of this approach was to attach P2 recognition motifs (in particular the melamine unit) to cytotoxic agents. The cytotoxic agents first selected were polyamine analogues, which are known to be cytotoxic to trypanosomes (26, 32). Several series of compounds were prepared and some of the compounds were shown to have high activity against *Trypanosoma brucei* trypomastigotes (23). However, they were too toxic in mammals for use in vivo.

Alternate cytotoxic moieties to couple to the P2 recognition motifs have now been considered. Nitroaromatic compounds are of particular interest. Nitroheterocycles have long been known to be effective against trypanosomes. Throughout the 1950s and 1960s a variety of compounds, notably furacin (nitrofurazone), were used in clinical trials (1, 19). In spite of good antitrypanosomal activity, toxicity issues prevented further development of nitrofurazone. A nitrofuran, nifurtimox, however, was licensed for use against Chagas' disease caused by *Trypanosoma cruzi* (36). Nifurtimox is also active against *T. brucei* and has been used in treating melarsoprol refractory trypanosomiasis (25, 33). The World Health Organization and Bayer are currently engaged in efforts to extend the license for nifurtimox for routine use against human African trypanosomiasis (7). A nitroimidazole, megazol, has recently received attention for its potent trypanocidal activity (9, 17), although toxicity issues (34) have stifled further development. Reports of novel trypanocidal nitroheterocycles continue to appear (8, 30), which emphasizes the fact that trypanosomes are particularly sensitive to this class of compound.

Given the potent trypanocidal activity of various nitroheterocycles, but the disadvantageous impact of host toxicity, selectively targeting nitroheterocycles to the trypanosome's interior could greatly increase their therapeutic index. We have therefore set out to produce a number of nitroheterocycles carrying either a melamine ring or a benzamidine moiety aiming to facilitate selective uptake into trypanosomes via the P2 and other transporters and circumvent host toxicity. Trypanocidal nitroheterocycles appear to be optimally active if attached to a second moiety, possibly because of steric requirements in binding to particular enzymes with nitroreductase activity. Consequently, both delivery across the plasma membrane and targeting to the active sites of particular enzymes could be bestowed by coupling nitroheterocycles to melamine or benzamidine moieties.

MATERIALS AND METHODS

Chemistry. The compounds were prepared from the 2,4-diamino-6-chlorotriazines by displacement of the chlorine by hydrazine, followed by condensation

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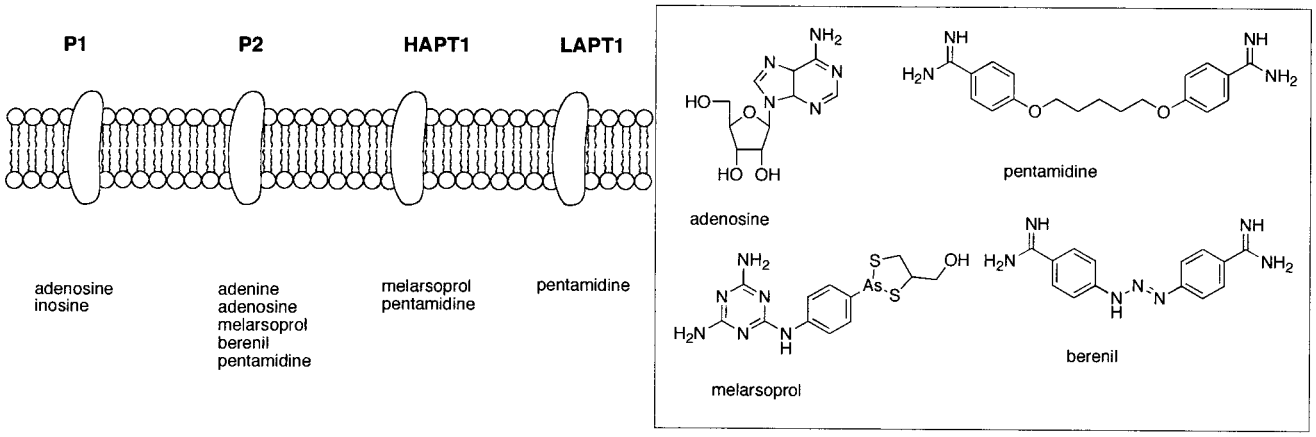


FIG. 1. Uptake of triazines and benzamidines into *T. brucei.* The left-hand side of the diagram shows the known routes of uptake of diamidines and melaminophenyl arsenicals into *T. brucei*. P2 is the product of the *TbAT1* gene and encodes an unusual transporter capable of nucleoside and nucleobase uptake. It recognizes a motif also present on diamidine- and melamine-based drugs. These are shown on the right hand side of the diagram. Pentamidine enters via two additional transporters: HAPT1 and LAPT1. The uptake routes of selected triazine- and benzamidine-derived drugs is depicted.

with the appropriate nitrofuraldehyde (Fig. 2). Synthesis was handicapped by the insolubility of the reagents and products. For control purposes, nitrofurans 4 and 5 were also screened.

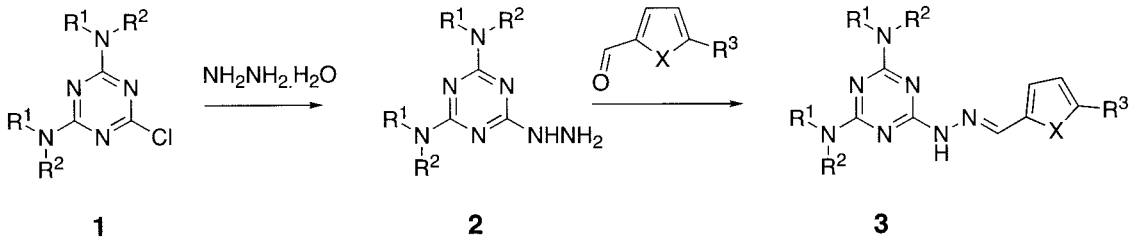
The synthesis of benzamidine derivatives of the imidazoles are shown in Fig. 3. The starting point was 4-aminobenzamide (compound 6). The amidine functionality was protected with a BOC protecting group, which allowed derivatization of the aniline position with chloroacetyl chloride. The intermediate compound 8 was then coupled with either 2-nitroimidazole or 4-nitroimidazole, which followed by deprotection gave the required products 11 and 12.

Biological assays. (i) Cultivation of parasites. Bloodstream-form *T. brucei brucei* (strain 427) (13) was cultivated in HMI-9 medium containing 20% fetal calf serum (22) at 37°C in a humidified CO_2 environment. The bloodstream-form *RAD51*$^{-/-}$ deletion mutant (29) derived from strain 427 was a gift from R. McCulloch (University of Glasgow), and these cells were cultured in HMI-9 medium with 20% fetal calf serum supplemented with puromycin (1 mg/ml) and bleomycin (2 μg/ml), which select for the respective genes giving resistance to these antibiotics targeted to the *RAD51* loci of the *T. brucei* genome.

N-Acetylcysteine (NAC) has been used frequently as an antagonist of oxidative stress in different cellular systems since it reacts readily with a number of the reduced oxygen species produced during oxidative stress (2, 12). When administered at 0.5 mM, no detrimental effect on trypanosomes was induced by this compound in in vitro assays.

For uptake analysis bloodstream-form parasites were grown to peak parasitemia (10^9 cells per ml of blood) in Wistar rats and then purified from blood by using DEAE anion-exchange chromatography (24).

(ii) P2 transporter affinity measurements. Parasites purified from blood were stored on ice in Carter's buffered saline solution (11). Transport assays used the centrifugation through oil technique, which is routinely used in analyses (5, 11, 14, 15, 23, 38). Radiolabeled adenosine (0.05 μM) uptake via P2 was measured in the presence of 1 mM inosine that blocks the P1 transporter (11). Compounds



1a, $R^1 = R^2 = H$
1b, $R^1 = H$, $R^2 = Me$
1c, $R^1 = R^2 = Me$

2a, $R^1 = R^2 = H$
2b, $R^1 = H$, $R^2 = Me$
2c, $R^1 = R^2 = Me$

3a, $R^1 = R^2 = H$, $R^3 = NO_2$, X = O
3b, $R^1 = R^2 = H$, $R^3 = NO_2$, X = S
3c, $R^1 = R^2 = H$, $R^3 = H$, X = O
3d, $R^1 = R^2 = H$, $R^3 = H$, X = S
3e, $R^1 = H$, $R^2 = Me$, $R^3 = NO_2$, X = O
3f, $R^1 = R^2 = Me$, $R^3 = NO_2$, X = O

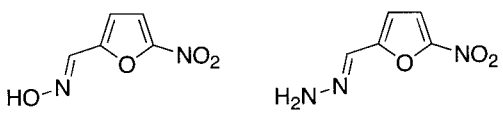


FIG. 2. Preparation of compounds from 2,4-diamino-6-chlorotriazines by displacement of the chlorine by hydrazine, followed by condensation with the appropriate nitrofuraldehyde.

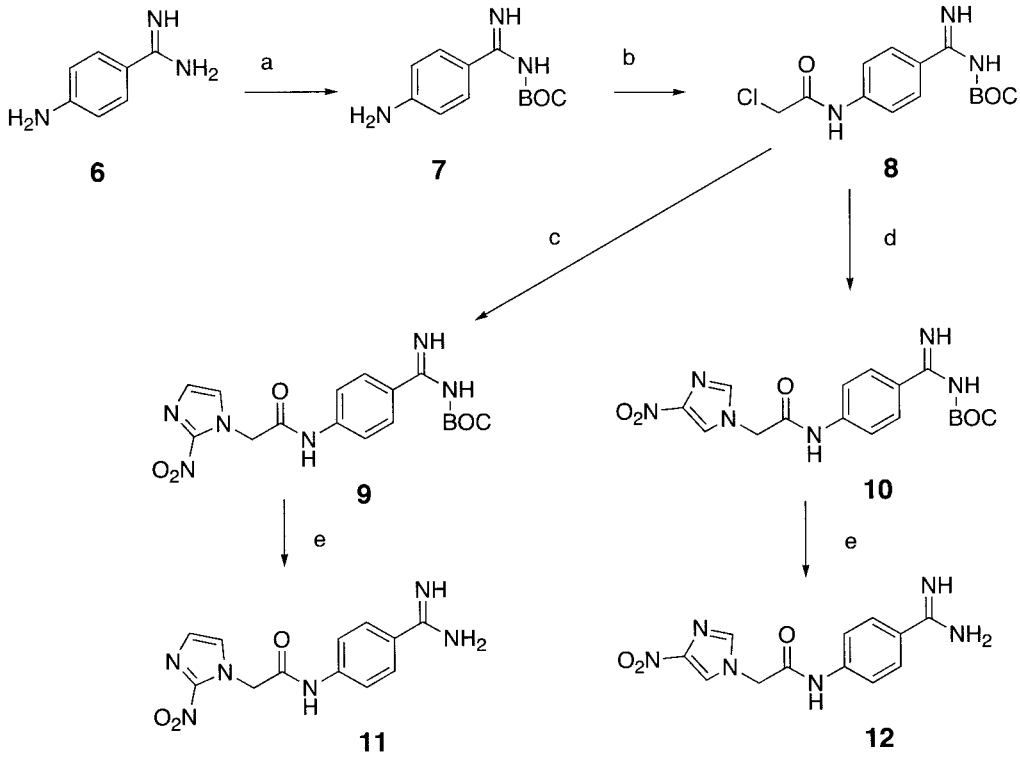


FIG. 3. Synthesis of benzamidine derivatives of the imidazoles. Lettered arrows: a, $(BOC)_2O$, NaOH, THF/H_2O; b, chloroacetyl chloride, Et_3N, MeCN; c, 2-nitroimidazole, Et_3N, MeCN; d, 4-nitroimidazole, Et_3N, MeCN; e, TFA, anisole, DCM.

were assayed for affinity for the P2 transporter by using three separate concentrations of adenosine and a range of inhibitor concentrations. The data were plotted by using the competitive inhibition algorithm of the Grafit 4.0 software (Erithacus). Plots were viewed by eye to verify inhibition type (Table 1).

(iii) In vitro activities against parasites and cytotoxicity. Activity of compounds was determined for *T. brucei rhodesiense* trypomastigotes of STIB 900. This stock was isolated in 1982 from a human patient in Tanzania. Minimum essential medium (50 μl) supplemented with 2-mercaptoethanol and 15% heat-inactivated horse serum (3) was added to each well of a 96-well microtiter plate. Serial drug dilutions were prepared covering a range from 90 to 0.123 μg/ml. Then, 50 μl of a trypanosome suspension was added to each well, and the plate incubated at 37°C under a 5% CO_2 atmosphere for 72 h. Alamar Blue (10 μl) was then added to each well, and incubation continued for a further 2 to 4 h. The plate was then read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices Corp., Sunnyvale, Calif.) by using an excitation wavelength of 536 nm and an emission wavelength of 588 nm (35). Fluorescence development was expressed as a percentage of the control, and the 50% inhibitory concentration (IC_{50}) values were determined. Cytotoxicity was assessed by using the same assay and rat skeletal myoblasts (L-6 cells).

To investigate whether transport of these compounds through the P2 trans-

TABLE 1. Activities of compounds against the P2 transporter and against various strains of *T. brucei* trypomastigotes[a]

Compound	K_i, P2 uptake (μM)	*T. brucei brucei AT1* wild type (IC_{50} [μM])	*T. brucei brucei AT1* knockout (IC_{50} [μM])	*T. brucei rhodesiense* (IC_{50} [μM])	L-6 cells (IC_{50} [μM])
2a	11.9	>200	>200	ND	ND
2b	59.3	>200	>200	ND	ND
3a	22.9	0.23	0.38	0.025	183
3b	1.9	0.85	1.52	0.24	11.8
3c	15.9	16.5	29.3	12.9	>400
3d	4.9	89	170	10.2	78.2
3e	4.6	11.9	14.8	0.25	18.8
3f	129	0.2	0.3	0.003	18.7
4	–[b]	1.1	1.2	0.68	40.4
5	404	23.5	13.7	2.3	20.0
9	13.09	111	119	ND	ND
10	3.65	>200	>200	ND	ND
11	1.58	>200	191	6.2	>300
12	2.88	>200	>200	79.8	>300
Melarsoprol	1.2	0.053	0.12	0.006	7.8
Nifurtimox	ND	5.6	ND	1.5	68

[a] *T. brucei AT1* Knockout is a mutant with a nonfunctional P2 transporter. ND, not determined.
[b] –, No inhibition.

porter is necessary for activity, compounds were assayed against the *T. brucei brucei* trypomastigotes using either the wild type or P2 knockout mutants (*TbAT1*$^{-/-}$) (28). The Alamar Blue assay (35) was also used to determine IC_{50} values against wild-type lines and the *TbAT1*$^{-/-}$ derivative. To determine whether DNA damage was associated with trypanocidal activity the Alamar Blue assay was also used to determine IC_{50} values against the *RAD51*$^{-/-}$ deletion mutant.

(iv) In vivo activities. Female NMRI mice weighing 22 to 25 g were infected with cryopreserved stabilates of *T. brucei brucei* STIB 795 (derivative of strain 427 (13) or *T. brucei rhodesiense* STIB 900. Each mouse was infected intraperitoneally with 2 to 4 $\times$ 10^4 bloodstream forms. Melarsoprol (Arsobal; Aventis) acted as a standard drug and was diluted with sterile distilled water to an appropriate concentration. Groups of four mice were treated on days 3, 4, 5, and 6 intraperitoneally with 20 mg/kg. A control group remained untreated. The parasitemia of all animals was checked on day 7 and 10 postinfection and every second day thereafter until day 60. Death of animals was recorded to calculate the mean survival time. Surviving and aparasitemic mice were considered cured at 60 days and then euthanized.

RESULTS

Activities of melamine nitroheterocycles against *T. brucei* in vitro. Table 1 shows the in vitro activities of a number of nitroheterocyclic compounds versus *T. brucei rhodesiense* and *T. brucei brucei* and a *TbAT1*$^{-/-}$ derivative that is deficient in P2 transport. It is noteworthy that all compounds are substantially more active against the *T. brucei rhodesiense* line in vitro than against *T. brucei brucei*. This observation has been made for a number of other compounds. In vitro, compound 3f (see Fig. 3) is of similar activity as melarsoprol against *T. brucei rhodesiense*, whereas in the *T. brucei* model melarsoprol is somewhat more active than any of the new compounds. However, a number of compounds (notably compounds 3a and 3f) did show pronounced in vitro activity against these parasites.

Role of the P2 transporter in activity. The compounds were designed with the intention of eliciting selective uptake into trypanosomes via the P2 transporter. Loss of the P2 transporter can be a key determinant of drug resistance. In order to determine the ability of these compounds to interact with that transporter, K_i values (which give an approximation to the affinity for the compounds) against P2-dependent adenosine uptake were determined. Most of the compounds did interact with the transporter, as judged by their ability to inhibit adenosine uptake; however, there was no correlation between the sensitivity of trypanosomes to these compounds and their apparent affinity for the transporter (Table 1). Toxic activities were also measured against a parasite line that had been rendered deficient in P2 transporter activity through knockout of the *TbAT1* gene that encodes the transporter. In no instance was an increase in sensitivity to the nitroheterocyclic compounds of >2-fold observed. This indicates that the activity of these compounds is, for the most part, not dependent upon the P2 transporter, although it is possible that other transporters may be involved in the uptake of compounds.

Compounds 2a and 2b, melamine-based binding units, were not active against trypanosomes, and they showed moderate affinity for the P2 transporter. The lack of activity of these compounds indicates that the nitrofuran group present on 3a and 3e is necessary for trypanocidal activity when coupled to a second aromatic group. In general, the benzamidine-coupled nitroimidazole compounds showed less trypanocidal activity than did the melamine-coupled nitrofurans.

TABLE 2. Activities of compounds against RAD51$^{-/-}$ mutant *T. brucei* in the presence or absence of NAC[a]

Compound(s)	Mean IC_{50} (μM) $\pm$ SD (n = 3)	
	Wild type	RAD51$^{-/-}$ mutant
Megazol	0.18 $\pm$ 0.012	0.034 $\pm$ 0.004
Megazol + NAC	0.20 $\pm$ 0.018	0.032 $\pm$ 0.001
Nifurtimox	5.6 $\pm$ 0.48	4.8 $\pm$ 0.67
Nifurtimox + NAC	14.35 $\pm$ 2.1	12.3 $\pm$ 2.2
3a	0.32 $\pm$ 0.03	0.36 $\pm$ 0.06
3a + NAC	0.34 $\pm$ 0.02	0.34 $\pm$ 0.04

[a] The effect of 0.5 mM NAC was assessed by adding this to wild-type cells only prior to the addition of drug.

In vivo activity in mice. Selected compounds were evaluated in several rodent models of trypanosome infection. Compounds 3a, 3e, and 3f were examined in rodent models infected with *T. brucei brucei* STIB 795 and *T. brucei rhodesiense* STIB 900. Neither compound 3e nor compound 3f was effective in vivo at 20 mg/kg. Compound 3a, however, cured the STIB 795 *T. brucei brucei* model mice (four of four mice all cured, as defined by no infection at 60 days) at a dose of 20 mg/kg given for 4 days (days 2 to 5). No overt signs of toxicity were observed in these mice. Having successfully treated an acute stage model, the more stringent *T. brucei rhodesiense* STIB 900 model was then tested in rodents. This model is not cured by pentamidine, suramin, or any other drugs active against early stage disease, although it does respond to melarsoprol, which is used in late-stage disease. It is thought that parasites may leave the vasculature early in a STIB 900 infection so that drugs must penetrate extravascular compartments to effect radical cure. As such, STIB 900 infection is perceived to represent a good model for determining likely outcomes of drugs in late-stage models, while providing the advantage of enabling experiments to be conducted within 2, rather than 6, months. Given intraperitoneally at 20 mg/kg for 4 days, compound 3a cured only one of the four mice as defined by their remaining infection free at 60 days. The compound did, however, promote a significant increase in life span of the mice from 8 days for untreated control animals to 35 days for the treated animals. In comparison, in the assay for the STIB 900 model, pentamidine was not curative in any of a group of four mice when given intraperitoneally at 20 mg/kg for 4 days, although the average life expectancy was extended to 43 days.

Genotoxicity is not associated with trypanocidal activity. It is important to determine whether DNA damage plays a role in activity of nitroheterocyclic trypanocides. For example, the principal stumbling block in the development of another nitroheterocycle, megazol, for use in trypanosomiasis therapy related to its propensity to induce mutations in DNA. Megazol is positive in Ames tests (20) and mutagenic in mammalian cell tests (34). Trypanosomes deficient in their own DNA repair enzymes are also hypersensitive to megazol, indicating that the genotoxic effects of this drug are also manifest in these cells (18). A *T. brucei* mutant deficient in a DNA repair enzyme (RAD51) was tested for susceptibility to megazol, to nifurtimox, and to compound 3a. The *RAD51*$^{-/-}$ line was more susceptible than wild-type to megazol but not to compound 3a or to nifurtimox (Table 2), indicating that megazol, but not nifurtimox or compound 3a, induces DNA damage in trypano-

somes. Culture of *T. brucei* in the presence of 0.5 mM NAC reduces free radical damage due to oxidative stress. Although the activity of nifurtimox was antagonized by NAC, that of compound 3a was not, indicating that induction of oxidative stress might not be responsible for the trypanocidal activity of this compound.

DISCUSSION

The chemotherapy of human African trypanosomiasis recently reached the crisis point (25). The lack of surveillance has led to a resurgence of the disease in sub-Saharan Africa (25). Treatment failures with melarsoprol, the principal drug used against late stage disease, have also increased sharply in recent years (10). New drugs are urgently needed.

Trypanosomes have long been known to be highly susceptible to a number of nitroheterocyclic compounds. The nitrofuran furacin was used in several trials in the 1950s and 1960s (1, 19), but its development halted when it became clear that it was responsible for severe toxic side effects. Another nitrofuran, nifurtimox, was registered for use against *T. cruzi* that causes Chagas' disease in Latin America (36). Nifurtimox is also active against *T. brucei*, and this compound has been used against melarsoprol refractory sleeping sickness as a monotherapy (33) or in combination with melarsoprol (25), and a license extension to allow its use for this indication is currently being sought. Nifurtimox, too, has been linked to important side effects.

Since African trypanosomes live free in the bloodstream and cerebrospinal fluid, and not intracellularly, it is possible to exert selectively toxic effects against parasites, but not against host cells, by selectively targeting compounds to the parasite's interior by the use of transporter proteins present in the parasite membrane (21). The P2 aminopurine transporter has been shown to be responsible for the uptake of the melamine-based arsenicals and diamidine classes of drugs. The reason for this relates to the substrate recognition motif of this transporter that is capable of recognizing melamine and benzamidine rings in addition to 6-aminopurine samples. We have previously reported a series of toxic polyamine analogues that possess the P2 transporter recognition motif in the form of melamine groups and shown some of these to have a high activity against *T. brucei* (6, 23, 38).

Given the susceptibility of *T. brucei* to nitroheterocycles but the difficulties in taking such compounds through clinical development due to toxicity issues, we considered the possibility of enhancing the therapeutic index of representatives of this class of molecule by linking nitrofurans and nitroimidazoles to the melamine and benzamidine P2 recognition motifs, respectively.

A number of such compounds were derived. The activity of these compounds is not dependent on uptake via the P2 transporter. First, no correlation between affinity for the P2 transporter, as measured by the ability of these molecules to inhibit uptake of adenosine via this route, and trypanocidal effect was noted. Moreover, it was demonstrated that parasites lacking the P2 transporter were not substantially less sensitive to these compounds than wild-type cells. It is not currently known what routes the compounds do take into the cell. It is plausible that other transporters could be involved in uptake or that the general lipophilic character of the agents could enable uptake through passive diffusion as is the case for the nitroimidazole megazol (5). This observation has an important corollary. It implies that loss of the P2 transporter, an event that appears to occur relatively easily (4, 11, 27), will not lead to resistance to this class of compound. However, in the absence of selective uptake, the reasons for selective activity against the trypanosome compared to the mammalian host are not certain.

Several of the compounds showed pronounced trypanocidal activity. The best compound, 3a, showed in vitro IC_{50} values against *T. brucei* of 0.23 μM and against *T. brucei rhodesiense* of 0.025 μM. In comparison, nifurtimox has an in vitro IC_{50} value of 5.6 μM against *T. brucei* and 1.5 μM against *T. brucei rhodesiense.* Compound 3a is therefore some 20-fold better in terms of in vitro efficacy than this rival nitroheterocycle that is in use clinically (Table 1). Crucially compound 3a was able to cure mice infected with *T. brucei rhodesiense* when given at 20 mg/kg for 4 days. In the difficult to cure *T. brucei rhodesiense* STIB 900 model, the same treatment schedule resulted in cure of one of four animals with a mean survival of 35 days (untreated controls had a mean survival of 8 days). The good trypanocidal activities in vitro and in rodent models seen for this class of compound suggest that further investigations into novel derivatives might yield even better trypanocidal agents, and further studies are planned.

Many nitroheterocyclic drugs are believed to exert their activity through either reductive stress or oxidative stress. In the case of reductive stress, single electron reduction of the nitro group is believed to create a highly reactive radical, derivatives of which can then form covalent bonds with numerous cellular macromolecules, including DNA. Oxidative stress is believed to arise from reduced oxygen intermediates that arise once oxygen accepts electrons from the reduced nitro group (16). We have developed assays using trypanosomes that give insight as to whether reductive or oxidative stress underlies the activity of these compounds (18). Reductive stress can be assessed by measuring the susceptibility of DNA repair-deficient trypanosomes to toxic agents. Oxidative stress can be assessed by determining the ability of NAC to antagonize an agent's activity, since this compound interacts with several reactive oxygen intermediates.

Using the RAD51 knockout line and NAC assay system, we have shown that the nitroimidazole-thiadazole megazol exerts its activity through reductive stress as *RAD51* knockout parasites, which are deficient in DNA repair and hypersensitive to this drug (18). These cells are not hypersensitive to the nitrofuran nifurtimox. The action of nifurtimox, however, is antagonized by NAC, whereas that of megazol is not. It therefore appears that megazol acts through reductive stress, whereas nifurtimox acts through oxidative stress (16, 18). Compound 3a does not have any enhanced activity against the *RAD51* knockout cells, which indicates that this compound may not exert its activity through reductive stress. Moreover, NAC fails to antagonize the activity of compound 3a. This could indicate that the agent does not act through oxidative stress. These results are important, given that the development of the nitroimidazole megazol was arrested when it became clear that the compound was genotoxic in mammalian cells, an activity also apparent in trypanosomes (18). Nifurtimox is also toxic to mammals, probably through events related to oxidative stress

(31). Compound 3a is notable in that it apparently does not act through either reductive or oxidative stress in trypanosomes.

It is noteworthy that the activity against L6 cells in vitro is 2 to 3 orders of magnitude lower than against trypanosomes. Moreover, no overt adverse effects became apparent in mice challenged with the drug up to 100 mg kg^{-1}. It is possible that the action of compound 3a occurs independently of reduction of the nitro group. In this case, the chances of the compound's passing stringent tests on mammalian cell toxicity would be greatly enhanced. Such testing has yet to be performed but, based on the data reported here, further studies into the efficacy and toxicology of this important lead compound are justified.

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