Cross-Resistance to Nitro Drugs and Implications for Treatment of Human African Trypanosomiasis

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The success of nifurtimox-eflornithine combination therapy (NECT) for the treatment of human African trypanosomiasis (HAT) has renewed interest in the potential of nitro drugs as chemotherapeutics. In order to study the implications of the more widespread use of nitro drugs against these parasites, we examined the in vivo and in vitro resistance potentials of nifurtimox and fexinidazole and its metabolites. Following selection in vitro by exposure to increasing concentrations of nifurtimox, Trypanosoma brucei brucei nifurtimox-resistant clones designated NfxR1 and NfxR2 were generated. Both cell lines were found to be 8-fold less sensitive to nifurtimox than parental cells and demonstrated cross-resistance to a number of other nitro drugs, most notably the clinical trial candidate fexinidazole (~27-fold more resistant than parental cells). Studies of mice confirmed that the generation of nifurtimox resistance in these parasites did not compromise virulence, and NfxR1 remained resistant to both nifurtimox and fexinidazole in vivo. In the case of fexinidazole, drug metabolism and pharmacokinetic studies indicate that the parent drug is rapidly metabolized to the sulfoxide and sulfone form of this compound. These metabolites retained trypanocidal activity but were less effective in nifurtimox-resistant lines. Significantly, trypanosomes selected for resistance to fexinidazole were 10-fold more resistant to nifurtimox than parental cells. This reciprocal cross-resistance has important implications for the therapeutic use of nifurtimox in a clinical setting and highlights a potential danger in the use of fexinidazole as a monotherapy.

The protozoan parasites Trypanosoma brucei gambiense and T. b. rhodesiense are the causative agents of human African trypanosomiasis (HAT), commonly known as sleeping sickness. This epidemic disease was largely under control in the 1960s but has reemerged as a major threat due to limited financial resources for the maintenance of control programs as well as population displacement due to political conflict and famine (23). Currently, the World Health Organization estimates that about 400,000 people are infected with HAT, resulting in an annual death toll of around 50,000 (42), although this may be an overestimate (36). In the absence of an effective vaccine, treatment is dependent solely upon a small repertoire of drugs which suffer from a number of problems, including severe toxic side effects (12) and acquired drug resistance (2). In particular, treatment of the second stage of the disease, where parasites invade the central nervous system, has proven to be especially problematic. Two drugs are available: the arslenal melarsoprol and the ornithine decarboxylase inhibitor eflornithine (difluoromethylornithine [DFMO]). Melarsoprol is extremely toxic, with death in 3 to 6% of cases and treatment failures as high as 30% in certain areas. Although better tolerated than melarsoprol, eflornithine is not effective against T. b. rhodesiense infections, which account for 10% of all cases of sleeping sickness. The treatment regimen for T. b. gambiense is prolonged, requiring a total of 56 slow infusions over a 14-day period. As a result, melarsoprol often remains the treatment of choice in regions where health care provision is limited.

One important development in the treatment of T. b. gambiense infections is the introduction of nifurtimox-eflornithine combination therapy (NECT) (32). Nifurtimox, a drug used in the treatment of Chagas’ disease, has previously been given on compassionate grounds for treatment of melarsoprol-refractory HAT. However, its efficacy as a monotherapy is low, and prolonged treatment is limited by severe toxicity (4, 5). Patients given NECT, consisting of oral nifurtimox over 10 days with DFMO infusions for 7 days, were found to fair just as well as those given the DFMO monotherapy, with cure rates of around 97%. The reduced frequency and duration of DFMO infusions in NECT is seen as highly advantageous in terms of cost, logistics, and human resources in areas of poverty, leading to its inclusion on the Model Lists of Essential Medicines of the World Health Organization.

The success of NECT has come at a time of renewed interest in nitroheterocyclic compounds for the treatment of infectious disease. In particular, PA-824 is being assessed for the treatment of tuberculosis (3, 15, 29), while nitazoxanide is currently in phase II clinical trials for the treatment of hepatitis C (15). The increased awareness of the antimicrobial potential of these compounds has also led to the renaissance of fexinidazole (Hoe 239) (41) as a potential chemotherapeutic for late-stage HAT. In 1983, Jennings and Urquhart reported that this...
compound, given in combination with suramin, effectively cured chronic *T. brucei* infections in mice (20). Some 26 years later, fexinidazole entered phase II clinical trials as a monotherapy for use against both early- and late-stage African sleeping sickness (8; report available from http://www.dndi.org/).

It is well established that naturally occurring strains of *Trypanosoma cruzi* can be inherently resistant to nifurtimox (28), but it is not known whether a similar situation might occur in the African trypanosome. However, clinical isolates of *T. b. gambiense* from Sudan and West and Central Africa show a 10-fold range of sensitivities to nifurtimox (25, 26). It was also recently demonstrated that laboratory-generated nifurtimox-resistant *T. brucei* cell lines become resistant to another nitro drug, benznidazole (39). To determine whether this may also be the case for *T. brucei*, we have undertaken similar studies. Here, we examine the *in vivo* and *in vitro* resistance potentials of nifurtimox and fexinidazole and its metabolites in blood-stream trypanosomes. The potential for cross-resistance to other nitro compounds is also addressed.

**MATERIALS AND METHODS**

**Cell lines and culture conditions.** *Trypanosoma brucei* bloodstream-form "single-maker" S427 (TTRP0L.TETR.NEO) and nifurtimox-resistant cell lines were cultured at 37°C in HMI9-T medium (16) supplemented with 2.5 μg ml⁻¹ G418 to maintain expression of T7 RNA polymerase and the tetracycline repressor protein (17). Cultures were initiated with 1 × 10⁶ cells ml⁻¹ and subcultured when cell densities approached 1 × 10⁹ to 2 × 10⁹ cells ml⁻¹. In order to directly compare the effects of nifurtimox and other compounds on the growth of these cell lines, the 50% effective concentration (EC₅₀) of each compound was determined as previously described (21). Experimental data were corrected for background cell density and are expressed as percentages of values for nontreated cells before being fitted to the following two-parameter equation using GraFit software:

\[
y = \frac{100}{1 + \left(\frac{[I]}{EC_{50}}\right)^{-m}}
\]

In this equation, [I] represents the inhibitor concentration and m is the slope factor. Experiments were repeated at least three times, and data are presented as the weighted means plus the weighted standard deviations.

**Generation of nifurtimox- and fexinidazole-resistant *T. brucei* cell lines.** A nifurtimox-resistant *T. brucei* cell line was generated by subculturing bloodstream trypanosomes in the continuous presence of nifurtimox (a kind gift from Bayer Argentina). Parasites were exposed to stepwise-increased concentrations of drug, starting at a sublethal concentration of 1.5 μM, until they were routinely growing in 50 μM nifurtimox. After a total of 140 days in culture, resistant parasites were cloned in the absence of nifurtimox by limiting dilution, and the two clones (NfxR1 and NfxR2) demonstrating the highest levels of resistance to nifurtimox were selected for further study. A similar approach was used to generate a fexinidazole-resistant *T. brucei* cell line.

**Nitro drug resistance *in vivo*.** Groups of five mice (NMRI) were infected with either wild-type (WT) bloodstream *T. brucei* or NfxR1 parasites by a single intraperitoneal injection of 10⁶ cells in 0.2 ml of HMI9-T medium. Twenty-four hours following infection, WT- and NfxR1-infected mice were either dosed with nifurtimox (100 mg kg⁻¹ of body weight) via an intraperitoneal injection or left untreated. The dosing of nifurtimox-treated mice was repeated in an identical manner for a further 3 days. Animals were inspected daily for clinical signs of infection, and wet smears of tail blood were examined microscopically as appropriate. Parasitemia was determined using a Neubauer hemocytometer, as previously described (21). Experimental data were corrected for background cell density, and are expressed as percentages of values for nontreated cells before being fitted to the following two-parameter equation using GraFit software:

\[
y = \frac{100}{1 + \left(\frac{[I]}{EC_{50}}\right)^{-m}}
\]

**Results**

*Generation of a nifurtimox-resistant *T. brucei* cell line.** To establish the ease with which resistance to nifurtimox could be generated *in vitro*, bloodstream trypanosomes (strain 427) were cultured in the continuous presence of nifurtimox. Parasites were exposed to stepwise-increased concentrations of drug, starting at a sublethal concentration of 1.5 μM, until they were continuously growing in 50 μM nifurtimox (Fig. 1A). In the presence of drug, the doubling time of nifurtimox-resistant parasites was 12.8 h, compared to 7.3 h for WT cells in the absence of drug. After a total of 140 days in culture, resistant parasites were cloned in the absence of nifurtimox by limiting dilution. The relative sensitivities of these cloned cell lines to nifurtimox were established and compared to that of WT parasites. Cloned lines NfxR1 and NfxR2 were found to be 8-fold less sensitive to nifurtimox than parental WT cells, with EC₅₀ values of 20.1 ± 0.9 and 20.3 ± 1.3 μM, respectively (Fig. 1B and Table 1). Nifurtimox resistance in NfxR1 and NfxR2 was
to be stable over 30 passages (60 days) in culture in the absence of drug (data not shown).

Generation of nifurtimox resistance in these cells did not greatly alter their sensitivity to drugs from other chemical classes, with the diamidine pentamidine and the fluorinated amino acid eflornithine having similar potencies against NfxR and parental wild-type cells (Fig. 1B and Table 1). However, NfxR1 and NfxR2 did demonstrate cross-resistance to a number of other nitro compounds, including nitrofurazone, previously used for the treatment of late-stage sleeping sickness (1). The anticancer nitrobenzene prodrug CB1954 (22), identified in a phenotypic screen in our laboratory as one of the most potent growth inhibitors of WT parasites, also showed marked cross-resistance in the NfxR cell lines. Most strikingly, the highest levels of resistance were observed against the nitromidazole fexinidazole and its putative metabolites (sulfoxide and sulfone) (41). NfxR1 and NfxR2 were found to be 27- and 29-fold less sensitive to fexinidazole than wild-type parasites, respectively, and about 15-fold less sensitive to its sulfoxide and sulfone metabolites. As previously demonstrated in T. cruzi, nifurtimox resistance in bloodstream T. brucei also resulted in a similar level of resistance to benznidazole (5-fold higher than that of wild-type parasites). Notably, NfxR cells were not cross-resistant to the antitubercular compound PA-824, which is considered to have a mode of action different from those of other nitro drugs (27, 35). Metronidazole, a drug used to treat microaerophilic bacterial and protozoal infections, had no effect on the growth of either wild-type or NfxR parasites at the highest concentration tested (500 μM).

Nifurtimox resistance in vivo. To confirm the biological relevance of nifurtimox resistance in NfxR clones, groups of mice were inoculated with either WT or NfxR1 parasites. Previous studies had established that the WT strain has a 50% effective dose (ED$_{50}$) of 36.7 mg kg$^{-1}$ when administered once daily for 4 days ($m = 3.7$; ED$_{99}$, 128 mg kg$^{-1}$ [recalculated from reference 13]). Twenty-four hours following infection, WT- and NfxR1-infected mice were either dosed with nifurtimox (100 mg kg$^{-1}$) via an intraperitoneal injection or left untreated. The dosing of nifurtimox-treated mice was repeated in an identical manner for a further 3 days. Infections were monitored over a 30-day period, and survival curves of each infection are shown in Fig. 2. Mice infected with WT cells succumbed to infection between days 5 and 7, and this was also the case for mice infected with NfxR1, confirming that nifurtimox resistance does not compromise virulence in these bloodstream trypanosomes. Nifurtimox treatment ensured that WT-infected mice remained free from parasitemia for the entire 30 days of the study. However, one mouse from this group died due to drug toxicity issues previously seen following intraperitoneal administration of nifurtimox. Treatment of NfxR1-infected mice did little to impede the growth of these parasites, and lethal levels of parasitemia were reached within 6 days. Collectively, these results confirm that NfxR1 parasites retain their nifurtimox-resistant phenotype in vivo.

To determine if nifurtimox-resistant parasites were cross-resistant to the clinical trial candidate fexinidazole in vivo, WT- and NfxR1-infected mice were treated orally with up to 200 mg kg$^{-1}$ fexinidazole once a day for 4 days. In general, fexinidazole seemed to be well tolerated in mice, and there were no signs of the toxicity issues previously seen following intraperitoneal administration of nifurtimox. Treatment of WT-infected mice with fexinidazole at 50 mg kg$^{-1}$ affected a cure in 100% of these animals, with an established ED$_{50}$ value of 21 ± 0.3 mg kg$^{-1}$ ($m = 7.9$) (Fig. 3). In keeping with our in vitro studies, NfxR1 parasites in vivo were found to have a relative resistance to fexinidazole that was 8.5-fold higher than that of

FIG. 1. Nifurtimox resistance in vitro. (A) Schematic representation of the generation of a nifurtimox-resistant cell line in T. brucei. Each passage of cells in culture (open circle) and loss of a culture due to drug toxicity (skull and crossbones) are indicated. (B) EC$_{50}$s were determined for nifurtimox (circles) and pentamidine (squares) against WT (open symbols) and NfxR1 (closed symbols) cells, respectively. The curves are the nonlinear fits of data using a two-parameter EC$_{50}$ equation provided by GraFit (see Materials and Methods). EC$_{50}$s of 0.95 ± 0.02 and 2.6 ± 0.1 nM were determined for pentamidine against WT and NfxR1 cells, respectively, while values of 2.4 ± 0.1 and 20.1 ± 0.9 μM were determined for nifurtimox. Data are the weighted means ± standard deviations of at least triplicate measurements.
<table>
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<tr>
<th>Chemical class</th>
<th>No.</th>
<th>Name</th>
<th>Structure</th>
<th>EC₅₀ (µM) for wild type</th>
<th>NfxR1 EC₅₀ (µM)</th>
<th>RF</th>
<th>NfxR2 EC₅₀ (µM)</th>
<th>RF</th>
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<td>Nitrofurans</td>
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<td>Nifurtimox</td>
<td></td>
<td>2.4 ± 0.1 (2.5)</td>
<td>20.1 ± 0.9 (2.0)</td>
<td>8</td>
<td>20.3 ± 1.3 (1.8)</td>
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<td></td>
<td>2</td>
<td>Nitrofurazone</td>
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<td>0.31 ± 0.01 (4.5)</td>
<td>3.7 ± 0.3 (2.3)</td>
<td>12</td>
<td>3.7 ± 0.1 (2.2)</td>
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<td>3</td>
<td>Fexinidazole</td>
<td></td>
<td>1.0 ± 0.02 (1.6)</td>
<td>26.6 ± 2.2 (1.2)</td>
<td>27</td>
<td>29.3 ± 3.0 (1.1)</td>
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<td>4</td>
<td>Fexinidazole sulfoxide</td>
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<td>21.0 ± 1.0 (1.3)</td>
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<td>Fexinidazole sulfone</td>
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<td>0.62 ± 0.03 (1.6)</td>
<td>9.6 ± 0.4 (1.4)</td>
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<td>9.5 ± 0.3 (1.4)</td>
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<td>Benznidazole</td>
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<td>154 ± 20 (1.1)</td>
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<td>155 ± 17 (1.2)</td>
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<td></td>
<td>7</td>
<td>PA-824</td>
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<td>37.8 ± 3.2 (2.6)</td>
<td>30.5 ± 2.9 (2.4)</td>
<td>1</td>
<td>30.8 ± 2.2 (2.7)</td>
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<td>8</td>
<td>Metronidazole</td>
<td></td>
<td>&gt;500 (ND)</td>
<td>&gt;500 (ND)</td>
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<td>&gt;500 (ND)</td>
<td>ND</td>
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<tr>
<td>Nitrobenzene</td>
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<td>CB1954</td>
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<td>0.54 ± 0.04 (1.1)</td>
<td>8.0 ± 0.5 (1.3)</td>
<td>15</td>
<td>10.2 ± 0.7 (1.2)</td>
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<td>Fluorinated amino acid</td>
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<td>Eflornithine</td>
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<td>16.4 ± 0.6 (2.3)</td>
<td>11.9 ± 0.7 (1.6)</td>
<td>1</td>
<td>11.5 ± 0.6 (1.6)</td>
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<tr>
<td>Diamidine</td>
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<td>Pentamidine</td>
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<td>(0.95 ± 0.02) × 10⁻³</td>
<td>(2.6 ± 0.1) × 10⁻³</td>
<td>3</td>
<td>(1.7 ± 0.07) × 10⁻³</td>
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</table>

* The results are the weighted means and standard deviations from at least three independent experiments. The values in parentheses are Hill slopes, i.e., the weighted means from at least three independent experiments. The standard deviation was less than 20% for all values. Resistance factors (RF) were determined as the ratio of the EC₅₀ of the resistant cells to that of the wild-type cells. ND, not determined.

b P < 0.001 by Student’s t test compared to wild-type cells.
WT parasites (ED_{50} \approx 171 \text{ mg kg}^{-1}). At the highest dose of the drug (200 mg kg^{-1}), only 2 out of 5 mice were aparasitemic following treatment. The other three mice showed relapsing peaks of parasitemia after treatment, with one apparently being self-cured, since no further signs of infection were observed from days 18 to 60, when the experiment was terminated.

**Pharmacokinetic properties of fexinidazole.** In early studies regarding the potential of fexinidazole as a treatment for HAT, Winkelmann and Raether (41) proposed that the sulfur atom in this compound is almost certainly metabolized in vivo to sulfoxide and/or sulfone groups (see the structures in Table 1). Analysis of blood samples from mice orally dosed with fexinidazole (200 mg kg^{-1}) confirmed that this was indeed the case (Fig. 4). Fifteen minutes after oral dosing, levels of fexinidazole in the blood were found to peak at 961 ng ml^{-1}, before declining steadily over time. The sulfoxide metabolite accumulated to considerably higher levels in the blood than the parent compound, reaching 40,000 ng ml^{-1} 2 h following dosing. Fexinidazole sulfone, the final metabolite to appear in the blood, continued to accumulate throughout the study, reaching a concentration of 55,000 ng ml^{-1}.

Comparison of the maximum achievable total blood levels of fexinidazole with the previously established in vitro EC_{99} (4,900 ng ml^{-1}) suggests that the unmetabolized form of this drug is unlikely to be the major trypanocidal agent in vivo. In contrast, total blood levels of both the sulfoxide and sulfone metabolites comfortably exceeded their respective EC_{99} levels for several hours following dosing. Additional studies established that the fraction unbound (fu) of fexinidazole and its sulfoxide and sulfone metabolites in mouse plasma is high (fu, 0.147, 0.858, and 0.728, respectively), indicating that the free-drug metabolite levels are also well in excess of the parasite EC_{99}. Collectively, these findings suggest that fexinidazole acts as a prodrug, which is oxidized in vivo to these more therapeutically relevant species.

**Fexinidazole-resistant T. brucei lines.** To determine whether fexinidazole-resistant cell lines would show reciprocal cross-resistance to nifurtimox, fexinidazole-resistant parasites were generated in a manner identical to that previously described for nifurtimox. The results of a representative drug sensitivity experiment are shown for cloned line FxR1 in Fig. 5. Overall, the cloned lines (FxR1 and FxR2) were found to be 10-fold less sensitive to fexinidazole than parental WT cells, with weighted mean EC_{50} (and Hill slope) values from 4 independent exper-
DISCUSSION

The resistance potential of nitro drugs such as nifurtimox and fexinidazole has yet to be adequately studied in the African trypanosomes. With ongoing phase II clinical trials of fexinidazole as a treatment for HAT and the inclusion of NECT on the Model Lists of Essential Medicines of the World Health Organization, the need for such studies has become more pressing. T. b. brucei is a suitable model for T. b. gambiense, since it is more amenable to culture in vitro and in vivo and displays similar sensitivities to nifurtimox, eflornithine, and pentamidine (25, 26). In the current study, we have confirmed that resistance to nifurtimox can be generated relatively easily in bloodstream trypanosomes in vitro and that these nifurtimox-resistant cell lines are cross-resistant to a number of other nitro drugs. Most significantly, NfxR parasites are highly cross-resistant to fexinidazole and its active metabolites (13- to 29-fold more resistant than WT parasites). Conversely, our fexinidazole-resistant lines of T. brucei are cross-resistant to nifurtimox (10-fold more resistant than WT parasites). Should this occur in a clinical setting, widespread resistance to nifurtimox would preclude the use of fexinidazole and vice versa. In addition, based on pharmacological data from human subjects (31), it can be calculated that, with an oral-dose schedule of 15 mg kg⁻¹ of nifurtimox every 8 h for 10 days for HAT patients (32), the total serum concentration will reach approximately 3 μM. Thus, if this concentration is reflected in the central nervous system, then this might account for the partial efficacy of nifurtimox as a monotherapy (4, 5) since the mean EC₉₀ in vitro for clinical isolates of T. b. gambiense ranges from 0.26 to 2.6 μM (25, 26). This 10-fold range of inherent susceptibility to nifurtimox underlines the need for future clinical trials with fexinidazole to include testing of T. b. gambiense field isolates for their intrinsic sensitivity to this drug.

It is not known whether the success of NECT in the treatment of the Gambian form of sleeping sickness is due to the additive or synergistic toxic effects of nifurtimox and eflornithine. However, eflornithine treatment lowers the intracellular concentrations of the unique antioxidant trypanothione in African trypanosomes (14) and nifurtimox may increase oxidant stress as discussed below.

Nitroheterocyclic compounds, such as nifurtimox, are believed to function as prodrugs requiring enzyme-mediated reduction by nitroreductases (NTRs) to generate cytotoxic species that cause damage to DNA, lipids, and proteins. Depending on the organism and the nature of the nitro drug, reduction can occur via sequential one-electron or two-electron processes (6, 9, 10). Whether the lower potency of nifurtimox are related to a different mode of action in T. cruzi, whereas the nitroimidazole benznidazole does not (see the reviews in references 9 and 10). In one-electron processes, the first intermediate formed is the nitroso intermediate, or, following a second round of reduction and lower resistance factor of benznidazole compared with nifurtimox (39). Wilkinson and colleagues reported that modulation of NTR levels within these parasites directly affected their sensitivity to nitro compounds, with reduced levels of the enzyme leading to nitro drug resistance. These findings not only confirm a crucial role for NTR in drug activation in the trypanosomes but also suggest deple-
tion of this enzyme as one possible mechanism of nitro drug resistance. Reliance on a single enzyme for prodrug activation could leave these drugs particularly vulnerable to the emergence of drug resistance, a hypothesis that seems to be supported by the ease with which nitro drug resistance was generated in NfxR and FxR cell lines. NTR activity is too low to be measured in cell lysates, and our attempts to generate a specific antisera to estimate NTR protein levels have proved unsuccessful. However, we have eliminated the possibility that point mutations in T. brucei NTR are responsible for resistance. Furthermore, deletion of one copy of the NTR gene in this diploid parasite increases resistance to nitfurmitox only by a factor of 2- to 3-fold (39; A. Y. Sokolova et al., unpublished data). Characterization of the molecular basis of nitro drug resistance in these cell lines will be the focus of our subsequent studies.

Notably, many of the structurally diverse nitroheterocyclic compounds tested in this study were found to have similar potencies in inhibiting cell growth and display similar resistance factors to nitfurmitox (Table 1, compounds 1 to 5 and 9), suggesting similar mechanisms of cell killing. The most striking exceptions were the antituberculosis drug PA-824, which showed no cross-resistance, and metronidazole, which was in-}

VOL. 54, 2010 NITRO DRUG RESISTANCE IN TRYPANOSOMA BRUCEI 2899

REFERENCES


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Ibba, South Sudan, an area of high melarsoprol treatment failure rate. Acta Trop. 104:84–90.


