Anti-trypanosomal activity of Fexinidazole – A New Oral Nitroimidazole Drug Candidate for the Treatment of Sleeping Sickness

Marcel Kaiser²,³*, Michael A Bray¹,⁴, Monica Cal²,³, Bernadette Bourdin Trunz¹,⁴, Els Torreele¹,⁴, Reto Brun²,³

¹Drugs for Neglected Diseases initiative, 15 Chemin Louis-Dunant, CH-1202 Geneva, Switzerland; ²Parasite Chemotherapy, Swiss Tropical and Public Health Institute, Socinstrasse 57, CH-4002 Basel, Switzerland; ³University of Basel, Basel, Switzerland.

Running title: Anti-trypanosomal activity of Fexinidazole

*Corresponding author. Mailing address: Swiss Tropical and Public Health Institute, Parasite Chemotherapy, Socinstrasse 57, CH-4002 Basel, Phone: +41 612848205, Fax: +41 612848101. E-mail: marcel.kaiser@unibas.ch.

⁴Current address: Michael A. Bray, Bray Pharma Consulting, Sevogelstrasse 36A, CH-4132 Muttenz, Switzerland; Bernadette Bourdin Trunz, 54 avenue du Petit-Lancy, 1213 Petit-Lancy/Geneva, Switzerland; Els Torreele, 456 Broadway, NYC, NY 10013, USA.
Abstract

Fexinidazole is a 5-nitroimidazole drug currently in clinical development for the treatment of human sleeping sickness (human African trypanosomiasis (HAT)) caused by infection with species of the protozoan parasite *Trypanosoma brucei*. The compound and its two principal metabolites the sulfoxide and sulfone have been assessed for their ability to kill a range of *T. brucei* parasite strains *in vitro* and to cure both acute and chronic HAT disease models in the mouse. The parent molecule and both metabolites have shown trypanocidal activity *in vitro* in the 0.7 – 3.3 µM (0.2 to 0.9 µg/ml) range against all parasite strains tested. *In vivo* fexinidazole is orally effective in curing both acute and chronic disease in the mouse at doses of 100 mg/kg/day for 4 days and 200 mg/kg/day for five days respectively. Pharmacokinetic data indicate that it is likely that the sulfoxide and sulfone metabolites provide most if not all of the *in vivo* killing activity (33). Fexinidazole and its metabolites require up to 48 hours exposure in order to induce maximal trypanocidal efficacy *in vitro*. The parent drug and its metabolites show no *in vitro* cross reactivity in terms of trypanocidal activity with either themselves or other known trypanocidal drugs in use in man. The *in vitro* and *in vivo* anti-trypanosomal activity of fexinidazole and its two principal metabolites provides evidence that the compound has the potential to be an effective oral treatment for both the *T. b. gambiense* and *T. b. rhodesiense* forms of human sleeping sickness and both stages of the disease.
Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is caused by two subspecies of the protozoan parasite *Trypanosoma brucei* and is transmitted through the bite of infected tsetse flies. In west and central Africa *T. b. gambiense* is responsible for the chronic form of the disease whereas *T. b. rhodesiense* is responsible for a more acute form of the disease endemic in eastern Africa. Poor and neglected populations living in remote rural areas of sub-Saharan Africa are at risk for HAT and in 2006, it was estimated that 50 - 70,000 individuals were infected (35). In recent years the reported HAT cases have decreased to approximately 10,000 (29, 36) with over 95% of the reported cases due to *T. b. gambiense* infection.

There are four drugs currently registered for use against sleeping sickness. Pentamidine and suramin are used against the hemolymphatic stage (stage 1) of the disease whilst melarsoprol and eflornithine (DFMO) are used against stage 2 of the disease when the parasites have invaded the central nervous system (CNS). The disease is fatal if left untreated. The drugs currently in use are unsatisfactory due to cost, toxicity, poor oral bioavailability, long treatment and lack of efficacy. Melarsoprol treatment is highly toxic and up to 5% of the second stage patients treated with melarsoprol die of a reactive encephalopathy. Eflornithine treatment requires four daily intravenous infusions over fourteen days meaning that this therapy is expensive and logistically difficult in rural clinics. The only advance in the last twenty-five years has been the introduction of the eflornithine-nifurtimox combination therapy (NECT) (26). Despite the reduced
toxicity and treatment duration of NECT when compared to melarsoprol or eflornithine, the requirements for seven days of intravenous administration is still a limitation.

The aim of the present study was to characterize the anti-trypanosomal activity of the 5-nitroimidazole drug candidate fexinidazole and its two principal metabolites fexinidazole sulfoxide and fexinidazole sulfone using phenotypic in vitro and in vivo screening. Fexinidazole is targeted for the treatment of HAT, currently in phase I clinical studies and had been in preclinical development as a broad spectrum antimicrobial agent during the 1970's when the in vivo efficacy in the *T. b. brucei* strain GVR35 mouse CNS model of HAT was first demonstrated (14).

Some of the data presented here have previously been published in summary form (33).

**Materials and Methods**

**Materials**

Fexinidazole (1-methyl-2-((p-(methylthio)phenoxy)methyl)-5-nitroimidazole) manufactured under GMP conditions (Axyntis), its sulfoxide and sulfone derivatives (1-Methyl-2-(4-methylsulfonyl phenoxy)methyl)-5-nitro imidazole and 1-Methyl-2-(4-methylsulfonyl phenoxy)methyl)-5-nitro imidazole) at laboratory grade (Axyntis) and nifurtimox (Bayer) were provided by DNDi, pentamidine isethionate and diminazene aceturate were purchased from Sigma-Aldrich; melarsoprol (Aventis) was provided by the WHO. The chemical structures of the
experimental drug fexinidazole and the two metabolites fexinidazole sulfoxide
and fexinidazole sulfone have been previously published (33).

All other reagents were of standard laboratory grade and purchased from
commercial suppliers.

**Preparation of compounds**

For *in vitro* studies compounds were dissolved in 100% DMSO and finally diluted
in culture medium prior to assay. The maximum DMSO concentration in the *in
vitro* assays was 1%.

For *in vivo* studies, the compounds were dissolved in DMSO and further diluted
with distilled water to a final DMSO concentration of 10%, unless stated
otherwise. In some studies fexinidazole was prepared in an optimized
suspension medium for oral administration comprising 5% w/v Tween 80/0.5%
w/v Methocel in water which has previously been shown to maximize absorption
of the drug (33).

**Parasites and cell culture conditions**

(i) *T. b. rhodesiense*: The STIB900 strain is a derivative of the STIB704 strain
isolated from a patient in Ifakara, Tanzania, in 1982 (5). STIB900mel and
STIB900pent are melarsoprol and pentamidine resistant lines, respectively which
were generated by growing STIB900 in increasing sub-curative drug
concentrations (3).
(ii) *T. b. gambiense*: The STIB930 strain is a derivate of the TH1/78E(031) strain isolated from a patient in Côte d’Ivoire in 1978 (9). The DAL 898R strain was also isolated from a patient in Côte d’Ivoire in 1985 (5).

*T. b. gambiense* strains 40R, 45R, 130R, 349Pi and 349R were all isolated from patients in the Democratic Republic of Congo in 2003-2004 (24). The K03048 strain was isolated from a patient in South Sudan in 2003 (20).

(iii) *T. b. brucei*: The strains used include BS221, a derivative of the S427 strain isolated in Uganda in 1960 (7); AT1KO, a P2 transporter knockout of the BS221 strain (21); STIB950mdr strain which is a derivative of the CP 2469 strain isolated in 1985 from a cow in Hakaka, Soakow District, Somalia (15). The GVR35 strain was isolated from a wildebeest in the Serengeti in 1966 (primary isolate S10) (13).

*T. b. rhodesiense* and *T. b. brucei* parasites were cultured at 37°C under a humidified 5% CO₂ atmosphere in Minimum Essential Medium (MEM) with Earle’s salts, supplemented according to the protocol of Baltz et al. (2) with the following modifications: 0.2mM 2-mercaptoethanol, 1 mM Na-pyruvate, 0.5mM hypoxanthine, and 15% heat-inactivated horse serum as supplement. *T. b. gambiense* strains were grown in HMI-9 medium (11) supplemented with 15% heat-inactivated fetal bovine serum (FBS) and 5% human serum. To ensure maintenance of a log growth phase, parasites were sub cultured into fresh medium at appropriate dilutions every 2 to 3 days.

*In vitro growth inhibition assays*
The compounds were tested in a serial drug dilution assay in order to determine the IC\textsubscript{50} values (concentration of drug causing 50% growth inhibition) by using the Alamar Blue assay (27).

Serial drug dilutions were prepared in 96-well microtitre plates containing appropriate culture medium as described above for each parasite strain, and wells were inoculated with either 2,000 bloodstream forms for \textit{T. b. rhodesiense} or \textit{T. b. brucei} assay or 10,000 trypanosomes for \textit{T. b. gambiense} assay. Cultures were incubated for 70 h at 37°C under a humidified 5% CO\textsubscript{2} atmosphere. After this time ten microliters of resazurin (12.5 mg resazurin [Sigma] dissolved in 100 ml phosphate buffered saline) was added to each well. The plates were incubated for an additional 2 to 4 h for \textit{T. b. rhodesiense} and \textit{T. b. brucei} and an additional 6-8 h for \textit{T. b. gambiense} isolates. The plates were read in a Spectramax Gemini XS microplate fluorescence scanner (Molecular Devices) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC\textsubscript{50} values were calculated by linear regression (12) from the sigmoidal dose inhibition curves using SoftmaxPro software.

**In vitro dynamic assays**

\textit{T.b.rhodesiense} (STIB900) was seeded in clear 96-well V-bottom plates at a density of 10,000 parasites per well in 100 µl medium and incubated for 1, 6, and 24 h with serially diluted test compounds. One plate was prepared for each time point. At the designated time point a plate was spun at 650 rcf (relative centrifugal force) for 5 min to sediment the parasites. The supernatant was removed and 100 µL of warmed MEM media was added to each well to
resuspend the parasites. The wash process was repeated four more times. After the washing procedure the parasites were resuspended in 100 µL media and transferred into new culture plates and further incubated. After a total of 70 h incubation resazurin was added and the trypanocidal activity (IC$_{50}$ and IC$_{90}$ values) determined as described for the *in vitro* growth inhibition assays.

**In vitro combination assays**

Drug combination studies were performed as previously described (10). Initially, the IC$_{50}$ values of the test drugs alone were determined. Subsequently, drug solutions were diluted with culture medium to initial concentrations of 10 times the predetermined IC$_{50}$ value. The solutions were combined in ratios of 1:3, 1:1, and 3:1. Single and combination drug solutions were then introduced into 96-well plates and the parasites cultured as described above. The IC$_{50}$ values of the drugs alone and in combination were determined as described above. For data interpretation, the IC$_{50}$ values of the drugs in combination were expressed as fractions of the IC$_{50}$ values of the drugs alone. These data were expressed as fractional inhibitory concentrations (FIC) for drug A and drug B, respectively.

Isobolograms were constructed by plotting the FIC of drug A against that of drug B for each of the three drug ratios, with concave curves indicating synergism, straight lines indicating addition and convex curves indicating antagonism. To obtain numeric values for the interactions, results were expressed as the sum FICs (ΣFICs) of the FIC-A and FIC-B. Cutoff ranges were determined by mixing the same drug at various ratios and accounting for experimental variation. Changes in FIC values indicate the nature of the interactions as follows:
ΣFIC<0.5 is synergism; ΣFIC 0.5 to 4.0 is indifferent, ΣFIC>4 is antagonism (8,23). Mean ΣFICs were used to classify the overall nature of the interaction.

In vivo experiments

Adult female NMRI mice (Harlan Laboratories, The Netherlands) weighing between 20 and 25 g at the beginning of the study were housed under standard conditions with food pellets and water ad libitum. All protocols and procedures used in the current study were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt, Switzerland.

T. b. rhodesiense (STIB900) acute mouse model

The STIB900 acute mouse model mimics the first stage of the disease. Experiments were performed as previously described (32), with minor modifications. Female NMRI mice were infected intraperitoneally (ip) with 10⁴ *T. b. rhodesiense* (STIB900) bloodstream forms. Experimental groups of four mice were treated ip or orally (per os [po]) with compounds on four consecutive days from day 3 to 6 post infection. A control group was infected but remained untreated. The tail blood of all mice was checked for parasitemia up to 60 days post infection. Surviving and aparasitemic mice at day 60 were considered cured and were euthanized. The day of relapse of the animals was recorded (including the cured mice, as >60) and data expressed as the mean day of relapse (MRD).

T. b. brucei (GVR35) CNS mouse model

The GVR35 mouse CNS model mimics the second stage of the disease. Five female NMRI mice per experimental group were inoculated ip with 2 x 10⁴ *T. b.*
brucei (GVR35) bloodstream forms. Treatment (i.p. or p.o.) with compound was given on five consecutive days from days 21 to 25 post infection. Some experimental groups were treated twice daily with a time interval of 7-8 h. In all experiments with fexinidazole a control group was treated on day 21 with a single intraperitoneal dose of diminazene aceturate at 40 mg/kg, which is sub-curative since it clears the trypanosomes only in the hemolymphatic system and not in the CNS, leading to a subsequent reappearance of trypanosomes in the blood (13). Parasitemia was monitored twice per week in the first five weeks after treatment followed by once a week up to 180 days post infection. Surviving and apasitemic mice at day 180 were considered cured and were euthanized. The day of relapse of the animals was recorded (including the cured mice, as >180) to calculate the MRD.

Results

In vitro activity of fexinidazole and its primary metabolites against African trypanosomes

Fexinidazole and its sulfoxide and sulfone metabolites and the reference drugs melarsoprol, pentamidine, eflornithine, nifurtimox and the veterinary compound diminazene aceturate have been assessed for in vitro efficacy against T. brucei subspecies isolates (Table 1). Fexinidazole showed in vitro trypanocidal activity against all tested T. brucei subspecies and strains in the range of 0.7 – 3.3 μM (0.2 to 0.9 μg/ml). The fexinidazole sulfoxide and sulfone metabolites were slightly more potent but within the same order of magnitude as the parent compound. Fexinidazole and its sulfoxide and sulfone metabolites showed
comparable activity to eflornithine and nifurtimox but were considerably less potent than the three other drugs tested.

*In vivo efficacy of fexinidazole in an experimental model of acute infection with African trypanosomes*

Fexinidazole showed dose related efficacy in the *T. b. rhodesiense* (STIB900) acute mouse model at intra-peritoneal (ip) doses of 20 – 50 mg/kg/day and oral (per os (po)) doses of 25 – 100 mg/kg/day given on four consecutive days with 100 mg/kg/day po being 100% curative (Table 2). In a separate experiment the two fexinidazole metabolites were less potent than fexinidazole when administered ip or orally in the acute model of infection. Fexinidazole sulfoxide cured one out of four infected mice at a dose of 50 mg/kg/day ip and two mice at 100 mg/kg/day po. Fexinidazole sulfone was not effective at 50 mg/kg/day ip and cured one mouse at a dose of 100 mg/kg/day.

*In vivo efficacy of fexinidazole in an experimental model for chronic infection with African trypanosomes, involving brain infection*

Fexinidazole was shown to be effective in the GVR35 mouse model which mimics the advanced and fatal stage of the disease when parasites have disseminated into the brain (Table 3). At ip doses of 50 mg/kg given twice per day (bid) or po doses of 100 mg/kg also given twice per day for 5 consecutive days, all mice were cured; at single doses of 200 mg/kg/day po for five consecutive days 7 out of 8 mice were cured and at single doses of 100 mg/kg/day po 3 out of 5 mice (DMSO/water vehicle), and 2 out of 8 mice (Tween/Methocel vehicle) were cured, respectively. In another experiment using
the same vehicle fexinidazole was compared to nifurtimox at the dose range of 50 - 200 mg/kg/day po given for five days. While fexinidazole resulted in partial cure at 100 mg/kg/day (2/8 mice cured) and almost complete cure at 200 mg/kg/day (7/8) (data from Ref. 33), nifurtimox had no curative effect at any dose tested. Significant levels of fexinidazole and the sulfoxide and sulfone metabolites can be detected in mice treated using the same protocol and assessed for plasma drug levels after day 5 (33). The plasma levels of both fexinidazole sulfoxide and fexinidazole sulfone following five days of once per day oral treatment with fexinidazole were found to be in the same range as that shown to kill all parasites in vitro indicating that these compounds probably provided the bulk of the trypanocidal activity of the administered parent compound.

**In vitro dynamic results**

In order to better understand the in vitro trypanocidal activity of fexinidazole, and the sulfoxide and sulfone metabolites pulse incubation experiments were performed and IC$_{50}$ and IC$_{90}$ values determined following compound wash out at various time points after exposure. The results are shown in Figure 1. A 48 hr period of exposure to the compounds is required to produce similar activities as in the standard 72 hr assay indicating that maximum killing effectiveness requires up to 48 hours exposure to the drugs.

**In vitro drug combination results**

Although NECT is currently the only available drug combination therapy to treat HAT the development of resistance to existing therapies is making the potential
use of combination therapies increasingly relevant. Data on the in vitro interaction of possible combinations has been proposed to support such development options (30). Fexinidazole and the biologically active sulfoxide and sulfone metabolites have been assessed in combination with several drugs currently available to patients. All drug combination studies were performed at three different ratios (1:3, 1:1 and 3:1) using the fixed-ratio isobologram method (10) and the data analysed using the IC_{50} results. Results of all drug interaction studies are shown in Table 5. Fexinidazole combined with its sulfoxide and sulfone metabolites as well as the combination of sulfoxide and sulfone all showed indifferent effects. The combinations of fexinidazole or either of its metabolites with melarsoprol, eflornithine or pentamidine also resulted in an indifferent effect. These data indicate that there are no cross-reactivities between these compounds which would preclude their use in, albeit unlikely, combination therapies.

Discussion

Only four drugs are registered for HAT treatment. Pentamidine and suramin are used against the early stage of the disease whilst treatment of the second stage depends on melarsoprol, eflornithine and the recently introduced combination therapy nifurtimox-eflornithine (NECT). Melarsoprol is an arsenical compound and is highly toxic with severe adverse effects (18). In addition there have been alarming reports of treatment failures with both melarsoprol and eflornithine, until recently the only available drugs for second stage treatment (1) and it is hoped that the broad implementation of the NECT regimen may avert the further
development of eflornithine resistance. New safe and effective drugs with simplified dosing regimens are urgently needed. Ideally, such new treatments would be effective in both acute and chronic disease stages. Such new treatment options would largely simplify disease management and, importantly, avoid the painful lumbar puncture procedure currently required for distinguishing between disease stages.

Fexinidazole has recently been identified as a promising new drug candidate for treatment of HAT(33) and data presented here provide in vitro and in vivo profiling of the anti-trypanosomal efficacy of fexinidazole and its two primary metabolites, the sulfoxide and sulfone.

Fexinidazole and the sulfoxide and sulfone metabolites were tested in vitro alongside reference drugs against a panel of African trypanosomes of the T. brucei spp. (Table 1) which included sensitive and resistant wild type, laboratory-induced melarsoprol and pentamidine resistant and P2-transporter knockout strains as well as new field isolates. The data showed that there is no evidence of innate resistance to fexinidazole or the two metabolites within any of the strains tested as all IC$_{50}$ values were in a similar range and varied by less than a factor of four. The new T. b. gambiense strains showed reduced IC$_{50}$ values for pentamidine but this is unlikely to indicate resistance in the field given the higher blood levels and long terminal half-life of the drug found in patients after standard treatment (4).

Fexinidazole showed in vivo efficacy in both the acute mouse model and, more importantly, the chronic mouse model with established brain infection. In the
STIB900 acute mouse model fexinidazole demonstrated 100% efficacy at an ip dose of 50 mg/kg/day and an oral dose of 100 mg/kg/day both given for 4 days (Table 2). Whilst a dose of 50 mg/kg/day ip fexinidazole was fully effective, the sulfoxide only partially cured with the same dose and route of administration and the sulfone was ineffective. After oral administration at a dose of 100 mg/kg/day both the sulfoxide and sulfone metabolites were only partially effective whereas fexinidazole cured 100% of the animals. Although no pharmacokinetic data are currently available to formally demonstrate oral absorption of the sulfoxide or sulfone metabolites in mice, it may be that neither are as readily absorbed as fexinidazole via the oral route. However, it is apparent that, even using the ip route of administration which should maximize the systemic bioavailability of the compounds, neither metabolite was as effective as the parent fexinidazole in this acute model of disease. In addition it is unlikely that protein binding could account for the lack of effectiveness of the metabolites when given orally as, whilst fexinidazole is highly protein bound in plasma (93% in mice; 95% in man) neither metabolite is highly protein bound, at least in human plasma (26% and 42% respectively for the sulfoxide and sulfone metabolites) (Data on file at DNDi). Overall these data support the view that the use of fexinidazole itself, acting as a biologically active pro-drug, whilst rapidly metabolized to the sulfoxide and sulfone metabolites in all animals tested (33), is likely to be the more useful compound for oral treatment compared to either of the two metabolites given alone.
In 1983, Jennings and Urquhart reported that fexinidazole, given in combination with suramin, cured a *T. brucei* CNS infection in mice (14). We have tested fexinidazole as monotherapy in the GVR35 mouse model of stage 2 HAT involving brain infection using two different vehicle formulations (Table 3). Using the optimized methocel/Tween vehicle, fexinidazole showed a dose related increase in efficacy and cured 7 out of 8 infected mice at a single oral daily dose of 200 mg/kg/day for 5 days. In comparison, nifurtimox was ineffective in the GVR35 mouse model up to a dose of 200 mg/kg/day for 5 days. It is of interest to note that the presumed trough levels of the two metabolites after 24 h are reported to be around 1 µg/ml (33) which would allow for a daily dosing schedule to be maintained with systemic drug levels near to those required to kill the parasite *in vitro*. Clearly, in this model, the drug levels in the CNS are of key importance and, whilst no data are available from the experiments presented, published data indicate that, in mice, brain levels of fexinidazole, the sulfoxide and the sulfone metabolites are approximately 0.8, 5 and 1 µg/ml respectively 60 minutes post oral dosing with fexinidazole (33). Further experiments are underway to more fully assess the brain levels of the compounds in mice at different times. Whilst the most effective oral dose of 200 mg/kg may seem high fexinidazole is well tolerated in laboratory animals at significantly higher doses (32) and, although no data are available in mice regarding a no toxic effect level an LD$_{50}$ of >10,000 mg/kg has been reported (DNDi data on file).
It is important to note that, of the drugs currently in clinical use, only melarsoprol has been shown to be effective in this experimental stage 2 HAT model.

Pulse incubation of *T. b. rhodesiense* with fexinidazole and the sulfoxide and sulfone metabolites shows that a 48 hours period of exposure is required to produce irreversible effects on trypanosomal survival for all three compounds (Fig 1). This result has implications for *in vivo* efficacy as it suggests that plasma or CSF concentrations may need to be maintained at or above optimal trypanocidal concentrations for >48 hrs to achieve elimination of all parasites. As discussed above it is apparent, at least in mice, that, whilst plasma levels of fexinidazole may not be maintained at a sufficient killing concentration, both the sulfoxide and sulfone metabolites are present in plasma and in brain, at concentrations sufficient to kill all parasites. In addition the data indicate that a five day dosing schedule would ensure sufficient trough levels of these metabolites at 24 h to maintain effective killing concentrations, in plasma. Concentrations in brain reach several \( \mu g/ml \) one hour after oral application (33), information on the persistence of fexinidazole and its metabolites is not available. It can be assumed that the metabolites and mainly the sulfone are responsible for the trypanocidal effect in the brain. The CSF is often used as surrogate for the brain since it is accessible without the need to kill the animal (6). Thus these data provide support to the observations in both mouse models that oral treatment with fexinidazole for 4 days (acute model) or 5 days (CNS model) can achieve cure. This time-dose relationship has been previously described for diamidines such as diminazene aceturate which are able to kill trypanosomes after a short
exposure time of 15 min at 1 µg/ml (16), whilst other trypanocidal agents (e.g. trybizine hydrochloride) with an in vitro potency similar to or greater than diminazene aceturate require a much longer exposure time of >8 hrs at 10 µg/ml to lead to death of the parasites (17).

Fexinidazole and the sulfoxide and sulfone metabolites have similar in vitro trypanocidal activity (Tables 1, 2 and Ref, 33). The in vivo activity of fexinidazole is likely to be due to the concerted action of the three molecules. The in vitro combination studies performed support this hypothesis. All combinations of fexinidazole and its metabolites were investigated using the fixed-ratio isobologram method (10). The IC$_{50}$ values for fexinidazole, the sulfoxide and the sulfone in combination did not differ from those of each drug alone, resulting in indifferent mean ΣFICs values between 1 and 1.4 for the combinations.

In several foci, melarsoprol treatment failures have reached 30% of those treated (19, 22, 28, 31) and treatment failures of up to 16% with eflorentine have been recently reported (1, 25). A strategy to prevent the development of resistance is the use of drugs in combination and the introduction of nifurtimox-eflorentine combination therapy (NECT) is an important development in the treatment of T. b. gambiense infections (26). The rationale behind combination treatments in general is that the likelihood of developing resistance to a single drug is relatively high, but much lower with a drug combination (34). Although in vitro cross-resistance studies have yet to be fully validated as predictive of human drug resistance the recently published study on cross resistance of fexinidazole and its sulfoxide and sulfone metabolites in a nifurtimox-resistant T. b. brucei strain
supports the approach of utilizing chemically unrelated drug combinations (30). The same authors also showed that resistance against fexinidazole could easily be generated in the laboratory thus underlining the potential need for a combination partner for fexinidazole. In the present study fexinidazole and the sulfoxide and sulfone metabolites were tested in vitro in combination with three existing drugs - pentamidine, melarsoprol and eflornithine. All combinations resulted in indifferent mean ΣFICs values. This observation supports the proposition that fexinidazole could be a candidate for combination with existing drugs that are currently acceptable treatments such as pentamidine, eflornithine and NECT or, more likely, with other new drug candidates that may become available in the future.

In conclusion the data presented in this paper demonstrate that fexinidazole and the sulfoxide and sulfone metabolites rapidly formed in vivo are effective at killing the parasites responsible for human African trypanosomiasis. Fexinidazole is effective in both acute and chronic mouse models of HAT at doses and dosing regimens which are expected to be practicable for human treatment. Time-dose studies indicate that effective drug levels need to be maintained for at least 48 hours and interaction data show that there is no cross-inhibition between fexinidazole and the sulfoxide or sulfone metabolites or other, chemically unrelated, treatment modalities. Overall these data provide evidence that fexinidazole has the potential to be an effective oral treatment for both T. b. gambiense and T. b. rhodesiense forms of human sleeping sickness and both stages of the disease.
Acknowledgements

These studies were funded via a grant from DNDi who received financial support from the following donors for this work: the Department for International Development (DFID) of the UK, the German Agency for technical Cooperation (GTZ), Medecins Sans Frontieres (MSF), Ministry of Foreign and European Affairs of France, the Spanish Agency for International Cooperation and Development, and a Swiss foundation.

None of these donors had any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
References


TABLE 1: In vitro trypanocidal activity against different *T. brucei* subspecies. IC\(_{50}\) values (µM) are the mean ± standard deviation (SD) from 3-5 cultures.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Strain</th>
<th>Fexinidazole MW**</th>
<th>Fexsulfone MW 295.3</th>
<th>Fexsulfoxide MW 311.3</th>
<th>Melarsoprol MW 398.3</th>
<th>Pentamidine MW 592.7</th>
<th>Eflornithine DMFO MW 236.7</th>
<th>Nifurtimox MW 287.3</th>
<th>Diminazene MW 515.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. brucei</em> rhodesiense</td>
<td>STIB900 wt</td>
<td>2.17 ± 0.29</td>
<td>1.44 ± 0.22</td>
<td>1.64 ± 0.36</td>
<td>0.011 ± 0.003</td>
<td>0.002 ± 0.0003</td>
<td>8.58 ± 2.7</td>
<td>1.09 ± 0.33</td>
<td>0.009 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>STIB900 mel</td>
<td>5.56 ± 1.9*</td>
<td>3.2 ± 0.15*</td>
<td>3.2 ± 0.44*</td>
<td>0.092 ± 0.028</td>
<td>0.095 ± 0.035</td>
<td>nd</td>
<td>nd</td>
<td>0.019 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>STIB900 pent</td>
<td>2.66 ± 0.57</td>
<td>1.26 ± 0.51</td>
<td>1.16 ± 0.29</td>
<td>0.043 ± 0.022</td>
<td>0.058 ± 0.019</td>
<td>nd</td>
<td>nd</td>
<td>0.011 ± 0.004</td>
</tr>
<tr>
<td><em>T. brucei</em></td>
<td>BS221 wildtype</td>
<td>2.38 ± 0.88</td>
<td>1.63 ± 0.92</td>
<td>1.49 ± 0.61</td>
<td>0.013 ± 0.004</td>
<td>0.002 ± 0.0003</td>
<td>nd</td>
<td>nd</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>BS221 AT1KO</td>
<td>1.33 ± 0.21</td>
<td>0.56 ± 0.04</td>
<td>0.85 ± 0.32</td>
<td>0.034 ± 0.003</td>
<td>0.008 ± 0.002</td>
<td>nd</td>
<td>nd</td>
<td>0.060 ± 0.016</td>
</tr>
<tr>
<td></td>
<td>STIB950 mdr</td>
<td>2.44 ± 0.99</td>
<td>0.99 ± 0.34</td>
<td>1.21 ± 0.14</td>
<td>0.038 ± 0.011</td>
<td>0.002 ± 0.0002</td>
<td>nd</td>
<td>nd</td>
<td>0.062 ± 0.005</td>
</tr>
<tr>
<td><em>T. gambiense</em></td>
<td>STIB930</td>
<td>1.84 ± 1.17</td>
<td>0.91 ± 0.27</td>
<td>0.94 ± 0.39</td>
<td>0.012 ± 0.005</td>
<td>0.016 ± 0.001</td>
<td>2.85 ± 0.98</td>
<td>2.24 ± 0.66</td>
<td>0.021 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>DAL898R</td>
<td>1.01 ± 0.36</td>
<td>0.76 ± 0.30</td>
<td>1.03 ± 0.13</td>
<td>0.009 ± 0.002</td>
<td>0.002 ± 0.0002</td>
<td>nd</td>
<td>nd</td>
<td>0.014 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>K3048</td>
<td>0.95 ± 0.19</td>
<td>nd</td>
<td>nd</td>
<td>0.032 ± 0.012</td>
<td>0.084 ± 0.015</td>
<td>7.63 ± 2.5</td>
<td>0.99 ± 0.12</td>
<td>0.076 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>45R</td>
<td>2.47 ± 1.59</td>
<td>nd</td>
<td>0.95 ± 0.47</td>
<td>1.24 ± 0.60</td>
<td>0.033 ± 0.011</td>
<td>0.069 ± 0.044</td>
<td>9.98 ± 2.4</td>
<td>1.06 ± 0.38</td>
</tr>
<tr>
<td></td>
<td>40R</td>
<td>2.61 ± 1.03</td>
<td>0.67 ± 0.35</td>
<td>0.95 ± 0.33</td>
<td>0.032 ± 0.006</td>
<td>0.088 ± 0.024</td>
<td>11.4 ± 5.8</td>
<td>1.46 ± 0.20</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>349Pi</td>
<td>1.07 ± 0.14</td>
<td>nd</td>
<td>nd</td>
<td>0.043 ± 0.011</td>
<td>0.066 ± 0.012</td>
<td>16.7 ± 3.6</td>
<td>0.78 ± 0.19</td>
<td>0.043 ± 0.025</td>
</tr>
<tr>
<td></td>
<td>349R</td>
<td>3.31 ± 0.88</td>
<td>nd</td>
<td>nd</td>
<td>0.033 ± 0.015</td>
<td>0.095 ± 0.012</td>
<td>22.8 ± 13.9</td>
<td>2.73 ± 0.66</td>
<td>0.064 ± 0.031</td>
</tr>
<tr>
<td></td>
<td>139R</td>
<td>2.37 ± 1.14</td>
<td>nd</td>
<td>nd</td>
<td>0.055 ± 0.023</td>
<td>0.074 ± 0.011</td>
<td>9.4 ± 2.19</td>
<td>1.34 ± 0.17</td>
<td>0.051 ± 0.013</td>
</tr>
</tbody>
</table>

* IC\(_{90}\) values ± standard deviation in µM
** MW: molecular weight
TABLE 2: *In vivo* anti-trypanosomal activity in the STIB900 acute mouse model

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose mg/kg</th>
<th>Route</th>
<th>Cured/Infected</th>
<th>Mean day of relapse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>0/12</td>
<td>8.75*</td>
</tr>
<tr>
<td>Fexinidazole</td>
<td>4x 20</td>
<td>ip</td>
<td>0/4</td>
<td>11± 2</td>
</tr>
<tr>
<td>Fexinidazole</td>
<td>4x 50</td>
<td>ip</td>
<td>0/4</td>
<td>60</td>
</tr>
<tr>
<td>Fexinidazole**</td>
<td>4x 25</td>
<td>po</td>
<td>0/4</td>
<td>12± 2</td>
</tr>
<tr>
<td>Fexinidazole**</td>
<td>4x 50</td>
<td>po</td>
<td>1/4</td>
<td>&gt;27</td>
</tr>
<tr>
<td>Fexinidazole**</td>
<td>4x 100</td>
<td>po</td>
<td>4/4</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Fexinidazole sulfoxide</td>
<td>4x 50</td>
<td>ip</td>
<td>1/4</td>
<td>&gt;24.5</td>
</tr>
<tr>
<td>Fexinidazole sulfoxide</td>
<td>4x 100</td>
<td>po</td>
<td>2/4</td>
<td>&gt;38.25</td>
</tr>
<tr>
<td>Fexinidazole sulfone</td>
<td>4x 50</td>
<td>ip</td>
<td>0/4</td>
<td>11± 2</td>
</tr>
<tr>
<td>Fexinidazole sulfone</td>
<td>4x 100</td>
<td>po</td>
<td>1/4</td>
<td>&gt;31.5</td>
</tr>
<tr>
<td>Melarsoprol</td>
<td>4x 4</td>
<td>ip</td>
<td>4/4</td>
<td>60</td>
</tr>
</tbody>
</table>

* Mean survival days post infection of untreated control animals, the value given is the average of three experiments

10% DMSO was used as vehicle

** Data published E. Torreele PLoS Negl Trop Dis. 4(12): e923 (Ref 33)
TABLE 3: *In vivo* anti-trypanosomal activity in the GVR25 chronic disease mouse model.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose mg/kg Route</th>
<th>Vehicle</th>
<th>Cured/Infected</th>
<th>Mean day of relapse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fexinidazole</td>
<td>5 x 50 ip</td>
<td>DMSO/water</td>
<td>1/5</td>
<td>73.8</td>
</tr>
<tr>
<td>Fexinidazole</td>
<td>5 x 50 bid</td>
<td>DMSO/water</td>
<td>5/5</td>
<td>&gt;180</td>
</tr>
<tr>
<td>Fexinidazole</td>
<td>5 x 100 po</td>
<td>DMSO/water</td>
<td>3/5</td>
<td>&gt;127</td>
</tr>
<tr>
<td>Fexinidazole</td>
<td>5 x 100 bid</td>
<td>DMSO/water</td>
<td>11/15</td>
<td>&gt;156.5*</td>
</tr>
<tr>
<td>Fexinidazole*</td>
<td>5 x 50 po</td>
<td>MethocelTween</td>
<td>0/8</td>
<td>41.3± 9</td>
</tr>
<tr>
<td>Fexinidazole*</td>
<td>5 x 100 po</td>
<td>MethocelTween</td>
<td>2/8</td>
<td>&gt;82.1</td>
</tr>
<tr>
<td>Fexinidazole*</td>
<td>5 x 200 po</td>
<td>MethocelTween</td>
<td>7/8</td>
<td>&gt;163.8</td>
</tr>
<tr>
<td>Nifurtimox</td>
<td>5 x 50 po</td>
<td>MethocelTween</td>
<td>0/8</td>
<td>31.0± 2</td>
</tr>
<tr>
<td>Nifurtimox</td>
<td>5 x 100 po</td>
<td>MethocelTween</td>
<td>0/8</td>
<td>31.0± 2</td>
</tr>
<tr>
<td>Nifurtimox</td>
<td>5 x 200 po</td>
<td>MethocelTween</td>
<td>0/8</td>
<td>37.4± 5</td>
</tr>
<tr>
<td>Diminazene</td>
<td>1 x 40 ip</td>
<td>DMSO/water</td>
<td>0/24</td>
<td>49.8± 6**</td>
</tr>
<tr>
<td>Eflornithine</td>
<td>10 x 2% po</td>
<td>Water</td>
<td>0/4</td>
<td>76.3± 8***</td>
</tr>
<tr>
<td>Melarsoprol</td>
<td>5 x 5 ip</td>
<td>Propyleneglycol/H2O</td>
<td>0/5</td>
<td>57.6± 14****</td>
</tr>
<tr>
<td>Melarsoprol</td>
<td>5 x 10 ip</td>
<td>Propyleneglycol/H2O</td>
<td>1/5</td>
<td>&gt;103.4****</td>
</tr>
<tr>
<td>Melarsoprol</td>
<td>5 x 15 ip</td>
<td>Propyleneglycol/H2O</td>
<td>4/5</td>
<td>&gt;180****</td>
</tr>
</tbody>
</table>

** An optimized suspension medium for oral administration comprising 5% w/v Tween 80/0.5% w/v Methocel in water to maximize absorption. These data have been previously published and are reproduced here for comparative purposes (33).
*** A 2% solution of eflornithine provided in drinking water for 10 days.

* Mean result from 3 separate experiments (n = 15)
** Mean result from 5 separate experiments (n = 24)
*** Data from 1 experiment (n = 4)
**** Representative data from 1 experiment (n =5/group)
FIGURE 1: (A) Growth inhibition curves after compound wash-out at specified times and viability assessment at 72 hr. (B) IC$_{50}$ and IC$_{90}$ values calculated from compound wash-out procedure. Values and standard deviations in µg/ml are means of 4 experiments (n = 4).