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

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19 **Abstract**

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

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40 **Introduction**

41 Human African trypanosomiasis (HAT), also know as sleeping sickness, is
42 caused by two subspecies of the protozoan parasite *Trypanosoma brucei* and is
43 transmitted through the bite of infected tsetse flies. In west and central Africa *T.*
44 *b. gambiense* is responsible for the chronic form of the disease whereas *T. b.*
45 *rhodesiense* is responsible for a more acute form of the disease endemic in
46 eastern Africa. Poor and neglected populations living in remote rural areas of
47 sub-Saharan Africa are at risk for HAT and in 2006, it was estimated that 50 -
48 70,000 individuals were infected (35). In recent years the reported HAT cases
49 have decreased to approximately 10,000 (29, 36) with over 95% of the reported
50 cases due to *T. b. gambiense* infection.

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

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63 toxicity and treatment duration of NECT when compared to melarsoprol or
64 eflornithine, the requirements for seven days of intravenous administration is still
65 a limitation.

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

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

75 **Materials and Methods**

76 **Materials**

77 Fexinidazole (1-methyl-2-((p-(methylthio)phenoxy)methyl)-5-nitroimidazole)
78 manufactured under GMP conditions (Axyntis), its sulfoxide and sulfone
79 derivatives (1-Methyl-2-(4-methylsulfonyl phenoxymethyl)-5-nitro imidazole and
80 1-Methyl-2-(4-methylsulfonyl phenoxymethyl)-5-nitro imidazole) at laboratory
81 grade (Axyntis) and nifurtimox (Bayer) were provided by DNDi, pentamidine
82 isethionate and diminazene aceturate were purchased from Sigma-Aldrich;
83 melarsoprol (Aventis) was provided by the WHO. The chemical structures of the

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84 experimental drug fexinidazole and the two metabolites fexinidazole sulfoxide
85 and fexinidazole sulfone have been previously published (33).

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

88 **Preparation of compounds**

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

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

98 **Parasites and cell culture conditions**

99 (i) *T. b. rhodesiense*: The STIB900 strain is a derivative of the STIB704 strain
100 isolated from a patient in Ifakara, Tanzania, in 1982 (5). STIB900mel and
101 STIB900pent are melarsoprol and pentamidine resistant lines, respectively which
102 were generated by growing STIB900 in increasing sub-curative drug
103 concentrations (3).

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104 (ii) *T. b. gambiense*: The STIB930 strain is a derivate of the TH1/78E(031) strain
105 isolated from a patient in Côte d'Ivoire in 1978 (9). The DAL 898R strain was also
106 isolated from a patient in Côte d'Ivoire in 1985 (5).

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

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

116 *T. b. rhodesiense* and *T. b. brucei* parasites were cultured at 37°C under a
117 humidified 5% CO_2 atmosphere in Minimum Essential Medium (MEM) with
118 Earle's salts, supplemented according to the protocol of Baltz et al. (2) with the
119 following modifications: 0.2mM 2-mercaptoethanol, 1 mM Na-pyruvate, 0.5mM
120 hypoxanthine, and 15% heat-inactivated horse serum as supplement. *T. b.*
121 *gambiense* strains were grown in HMI-9 medium (11) supplemented with 15%
122 heat-inactivated fetal bovine serum (FBS) and 5% human serum. To ensure
123 maintenance of a log growth phase, parasites were sub cultured into fresh
124 medium at appropriate dilutions every 2 to 3 days.

125 ***In vitro* growth inhibition assays**

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126 The compounds were tested in a serial drug dilution assay in order to determine
127 the IC_{50} values (concentration of drug causing 50% growth inhibition) by using
128 the Alamar Blue assay (27).

129 Serial drug dilutions were prepared in 96-well microtitre plates containing
130 appropriate culture medium as described above for each parasite strain, and
131 wells were inoculated with either 2,000 bloodstream forms for *T. b. rhodesiense*
132 or *T. b. brucei* assay or 10,000 trypanosomes for *T. b. gambiense* assay.
133 Cultures were incubated for 70 h at 37°C under a humidified 5% CO_2
134 atmosphere. After this time ten microliters of resazurin (12.5 mg resazurin
135 [Sigma] dissolved in 100 ml phosphate buffered saline) was added to each well.
136 The plates were incubated for an additional 2 to 4 h for *T. b. rhodesiense* and *T.*
137 *b. brucei* and an additional 6-8 h for *T. b. gambiense* isolates. The plates were
138 read in a Spectramax Gemini XS microplate fluorescence scanner (Molecular
139 Devices) using an excitation wavelength of 536 nm and an emission wavelength
140 of 588 nm. The IC_{50} values were calculated by linear regression (12) from the
141 sigmoidal dose inhibition curves using SoftmaxPro software.

142 ***In vitro* dynamic assays**

143 *T.b.rhodesiense* (STIB900) was seeded in clear 96-well V-bottom plates at a
144 density of 10,000 parasites per well in 100 µl medium and incubated for 1, 6, and
145 24 h with serially diluted test compounds. One plate was prepared for each time
146 point. At the designated time point a plate was spun at 650 rcf (relative
147 centrifugal force) for 5 min to sediment the parasites. The supernatant was
148 removed and 100 µL of warmed MEM media was added to each well to

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149 resuspend the parasites. The wash process was repeated four more times. After
150 the washing procedure the parasites were resuspended in 100 μL media and
151 transferred into new culture plates and further incubated. After a total of 70 h
152 incubation resazurin was added and the trypanocidal activity (IC_{50} and IC_{90}
153 values) determined as described for the *in vitro* growth inhibition assays.

154 ***In vitro* combination assays**

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

165 Isobolograms were constructed by plotting the FIC of drug A against that of drug
166 B for each of the three drug ratios, with concave curves indicating synergism,
167 straight lines indicating addition and convex curves indicating antagonism. To
168 obtain numeric values for the interactions, results were expressed as the sum
169 FICs (ΣFICs) of the FIC-A and FIC-B. Cutoff ranges were determined by mixing
170 the same drug at various ratios and accounting for experimental variation.
171 Changes in FIC values indicate the nature of the interactions as follows:

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172 ΣFIC<0.5 is synergism; ΣFIC 0.5 to 4.0 is indifferent, ΣFIC>4 is antagonism
173 (8,23). Mean ΣFICs were used to classify the overall nature of the interaction.

174 ***In vivo* experiments**

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

180 ***T. b. rhodesiense* (STIB900) acute mouse model**

181 The STIB900 acute mouse model mimics the first stage of the disease.
182 Experiments were performed as previously described (32), with minor
183 modifications. Female NMRI mice were infected intraperitoneally (ip) with 10^4 *T.*
184 *b. rhodesiense* (STIB900) bloodstream forms. Experimental groups of four mice
185 were treated ip or orally (per os [po]) with compounds on four consecutive days
186 from day 3 to 6 post infection. A control group was infected but remained
187 untreated. The tail blood of all mice was checked for parasitemia up to 60 days
188 post infection. Surviving and aparasitemic mice at day 60 were considered cured
189 and were euthanized. The day of relapse of the animals was recorded (including
190 the cured mice, as >60) and data expressed as the mean day of relapse (MRD).

191 ***T. b. brucei* (GVR35) CNS mouse model**

192 The GVR35 mouse CNS model mimics the second stage of the disease. Five
193 female NMRI mice per experimental group were inoculated ip with 2 x 10^4 *T. b.*

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194 *brucei* (GVR35) bloodstream forms. Treatment (i.p. or p.o.) with compound was
195 given on five consecutive days from days 21 to 25 post infection. Some
196 experimental groups were treated twice daily with a time interval of 7-8 h. In all
197 experiments with fexinidazole a control group was treated on day 21 with a single
198 intraperitoneal dose of diminazene aceturate at 40 mg/kg, which is sub-curative
199 since it clears the trypanosomes only in the hemolymphatic system and not in the
200 CNS, leading to a subsequent reappearance of trypanosomes in the blood (13).
201 Parasitemia was monitored twice per week in the first five weeks after treatment
202 followed by once a week up to 180 days post infection. Surviving and
203 aparasitemic mice at day 180 were considered cured and were euthanized. The
204 day of relapse of the animals was recorded (including the cured mice, as >180)
205 to calculate the MRD.

206 **Results**

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

209 Fexinidazole and its sulfoxide and sulfone metabolites and the reference drugs
210 melarsoprol, pentamidine, eflornithine, nifurtimox and the veterinary compound
211 diminazene aceturate have been assessed for *in vitro* efficacy against *T. brucei*
212 subspecies isolates (Table 1). Fexinidazole showed *in vitro* trypanocidal activity
213 against all tested *T. brucei* subspecies and strains in the range of 0.7 – 3.3 μM
214 (0.2 to 0.9 μg/ml). The fexinidazole sulfoxide and sulfone metabolites were
215 slightly more potent but within the same order of magnitude as the parent
216 compound. Fexinidazole and its sulfoxide and sulfone metabolites showed

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217 comparable activity to eflornithine and nifurtimox but were considerably less
218 potent than the three other drugs tested.

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

221 Fexinidazole showed dose related efficacy in the *T. b. rhodesiense* (STIB900)
222 acute mouse model at intra-peritoneal (ip) doses of 20 – 50 mg/kg/day and oral
223 (per os (po)) doses of 25 – 100 mg/kg/day given on four consecutive days with
224 100 mg/kg/day po being 100% curative (Table 2). In a separate experiment the
225 two fexinidazole metabolites were less potent than fexinidazole when
226 administered ip or orally in the acute model of infection. Fexinidazole sulfoxide
227 cured one out of four infected mice at a dose of 50 mg/kg/day ip and two mice at
228 100 mg/kg/day po. Fexinidazole sulfone was not effective at 50 mg/kg/day ip and
229 cured one mouse at a dose of 100 mg/kg/day.

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

232 Fexinidazole was shown to be effective in the GVR35 mouse model which
233 mimics the advanced and fatal stage of the disease when parasites have
234 disseminated into the brain (Table 3). At ip doses of 50 mg/kg given twice per
235 day (bid) or po doses of 100 mg/kg also given twice per day for 5 consecutive
236 days, all mice were cured; at single doses of 200 mg/kg/day po for five
237 consecutive days 7 out of 8 mice were cured and at single doses of 100
238 mg/kg/day po 3 out of 5 mice (DMSO/water vehicle), and 2 out of 8 mice
239 (Tween/Methocel vehicle) were cured, respectively. In another experiment using

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240 the same vehicle fexinidazole was compared to nifurtimox at the dose range of
241 50 - 200 mg/kg/day po given for five days. While fexinidazole resulted in partial
242 cure at 100 mg/kg/day (2/8 mice cured) and almost complete cure at 200
243 mg/kg/day (7/8) (data from Ref. 33), nifurtimox had no curative effect at any dose
244 tested. Significant levels of fexinidazole and the sulfoxide and sulfone
245 metabolites can be detected in mice treated using the same protocol and
246 assessed for plasma drug levels after day 5 (33). The plasma levels of both
247 fexinidazole sulfoxide and fexinidazole sulfone following five days of once per
248 day oral treatment with fexinidazole were found to be in the same range as that
249 shown to kill all parasites *in vitro* indicating that these compounds probably
250 provided the bulk of the trypanocidal activity of the administered parent
251 compound.

252 ***In vitro* dynamic results**

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

260 ***In vitro* drug combination results**

261 Although NECT is currently the only available drug combination therapy to treat
262 HAT the development of resistance to existing therapies is making the potential

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

277 **Discussion**

278 Only four drugs are registered for HAT treatment. Pentamidine and suramin are
279 used against the early stage of the disease whilst treatment of the second stage
280 depends on melarsoprol, eflornithine and the recently introduced combination
281 therapy nifurtimox-eflornithine (NECT). Melarsoprol is an arsenical compound
282 and is highly toxic with severe adverse effects (18). In addition there have been
283 alarming reports of treatment failures with both melarsoprol and eflornithine, until
284 recently the only available drugs for second stage treatment (1) and it is hoped
285 that the broad implementation of the NECT regimen may avert the further

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286 development of eflornithine resistance. New safe and effective drugs with
287 simplified dosing regimens are urgently needed. Ideally, such new treatments
288 would be effective in both acute and chronic disease stages. Such new treatment
289 options would largely simplify disease management and, importantly, avoid the
290 painful lumbar puncture procedure currently required for distinguishing between
291 disease stages.

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

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

307 Fexinidazole showed *in vivo* efficacy in both the acute mouse model and, more
308 importantly, the chronic mouse model with established brain infection. In the

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309 STIB900 acute mouse model fexinidazole demonstrated 100% efficacy at an ip
310 dose of 50 mg/kg/day and an oral dose of 100 mg/kg/day both given for 4 days
311 (Table 2). Whilst a dose of 50 mg/kg/day ip fexinidazole was fully effective, the
312 sulfoxide only partially cured with the same dose and route of administration and
313 the sulfone was ineffective. After oral administration at a dose of 100 mg/kg/day
314 both the sulfoxide and sulfone metabolites were only partially effective whereas
315 fexinidazole cured 100% of the animals. Although no pharmacokinetic data are
316 currently available to formally demonstrate oral absorption of the sulfoxide or
317 sulfone metabolites in mice, it may be that neither are as readily absorbed as
318 fexinidazole via the oral route. However, it is apparent that, even using the ip
319 route of administration which should maximize the systemic bioavailability of the
320 compounds, neither metabolite was as effective as the parent fexinidazole in this
321 acute model of disease. In addition it is unlikely that protein binding could
322 account for the lack of effectiveness of the metabolites when given orally as,
323 whilst fexinidazole is highly protein bound in plasma (93% in miice; 95% in man)
324 neither metabolite is highly protein bound, at least in human plasma (26% and
325 42% respectively for the sulfoxide and sulfone metabolites) (Data on file at
326 DNDi). Overall these data support the view that the use of fexinidazole itself,
327 acting as a biologically active pro-drug, whilst rapidly metabolized to the sulfoxide
328 and sulfone metabolites in all animals tested (33), is likely to be the more useful
329 compound for oral treatment compared to either of the two metabolites given
330 alone.

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331 In 1983, Jennings and Urquhart reported that fexinidazole, given in combination
332 with suramin, cured a *T. brucei* CNS infection in mice (14). We have tested
333 fexinidazole as monotherapy in the GVR35 mouse model of stage 2 HAT
334 involving brain infection using two different vehicle formulations (Table 3). Using
335 the optimized methocel/Tween vehicle, fexinidazole showed a dose related
336 increase in efficacy and cured 7 out of 8 infected mice at a single oral daily dose
337 of 200 mg/kg/day for 5 days. In comparison, nifurtimox was ineffective in the
338 GVR35 mouse model up to a dose of 200 mg/kg/day for 5 days. It is of interest to
339 note that the presumed trough levels of the two metabolites after 24 h are
340 reported to be around 1 µg/ml (33) which would allow for a daily dosing schedule
341 to be maintained with systemic drug levels near to those required to kill the
342 parasite *in vitro*. Clearly, in this model, the drug levels in the CNS are of key
343 importance and, whilst no data are available from the experiments presented,
344 published data indicate that, in mice, brain levels of fexinidazole, the sulfoxide
345 and the sulfone metabolites are approximately 0.8, 5 and 1 µg/ml respectively 60
346 minutes post oral dosing with fexinidazole (33). Further experiments are
347 underway to more fully assess the brain levels of the compounds in mice at
348 different times. Whilst the most effective oral dose of 200 mg/kg may seem high
349 fexinidazole is well tolerated in laboratory animals at significantly higher doses
350 (32) and, although no data are available in mice regarding a no toxic effect level
351 an LD_{50} of >10,000 mg/kg has been reported (DNDi data on file).

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353 It is important to note that, of the drugs currently in clinical use, only melarsoprol
354 has been shown to be effective in this experimental stage 2 HAT model.

355 Pulse incubation of *T. b. rhodesiense* with fexinidazole and the sulfoxide and
356 sulfone metabolites shows that a 48 hours period of exposure is required to
357 produce irreversible effects on trypanosomal survival for all three compounds
358 (Fig 1). This result has implications for *in vivo* efficacy as it suggests that plasma
359 or CSF concentrations may need to be maintained at or above optimal
360 trypanocidal concentrations for >48 hrs to achieve elimination of all parasites. As
361 discussed above it is apparent, at least in mice, that, whilst plasma levels of
362 fexinidazole may not be maintained at a sufficient killing concentration, both the
363 sulfoxide and sulfone metabolites are present in plasma and in brain, at
364 concentrations sufficient to kill all parasites. In addition the data indicate that a
365 five day dosing schedule would ensure sufficient trough levels of these
366 metabolites at 24 h to maintain effective killing concentrations, in plasma.
367 Concentrations in brain reach several µg/ml one hour after oral application (33),
368 information on the persistence of fexinidazole and its metabolites is not available.
369 It can be assumed that the metabolites and mainly the sulfone are responsible
370 for the trypanocidal effect in the brain. The CSF is often used as surrogate for the
371 brain since it is accessible without the need to kill the animal (6). Thus these data
372 provide support to the observations in both mouse models that oral treatment
373 with fexinidazole for 4 days (acute model) or 5 days (CNS model) can achieve
374 cure. This time-dose relationship has been previously described for diamidines
375 such as diminazene aceturate which are able to kill trypanosomes after a short

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376 exposure time of 15 min at 1 μg/ml (16), whilst other trypanocidal agents (e.g.
377 trybizine hydrochloride) with an *in vitro* potency similar to or greater than
378 diminazene aceturate require a much longer exposure time of >8 hrs at 10 μg/ml
379 to lead to death of the parasites (17).

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

388 In several foci, melarsoprol treatment failures have reached 30% of those treated
389 (19, 22, 28, 31) and treatment failures of up to 16% with eflornithine have been
390 recently reported (1, 25). A strategy to prevent the development of resistance is
391 the use of drugs in combination and the introduction of nifurtimox-eflornithine
392 combination therapy (NECT) is an important development in the treatment of *T.*
393 *b. gambiense* infections (26). The rationale behind combination treatments in
394 general is that the likelihood of developing resistance to a single drug is relatively
395 high, but much lower with a drug combination (34). Although *in vitro* cross-
396 resistance studies have yet to be fully validated as predictive of human drug
397 resistance the recently published study on cross resistance of fexinidazole and
398 its sulfoxide and sulfone metabolites in a nifurtimox-resistant *T. b. brucei* strain

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399 supports the approach of utilizing chemically unrelated drug combinations (30).
400 The same authors also showed that resistance against fexinidazole could easily
401 be generated in the laboratory thus underlining the potential need for a
402 combination partner for fexinidazole. In the present study fexinidazole and the
403 sulfoxide and sulfone metabolites were tested *in vitro* in combination with three
404 existing drugs - pentamidine, melarsoprol and eflornithine. All combinations
405 resulted in indifferent mean ΣFICs values. This observation supports the
406 proposition that fexinidazole could be a candidate for combination with existing
407 drugs that are currently acceptable treatments such as pentamidine, eflornithine
408 and NECT or, more likely, with other new drug candidates that may become
409 available in the future.

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

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554 TABLE 1: *In vitro* trypanocidal activity against different *T. brucei* subspecies. IC_{50} values (μM) are the mean ± standard

555 deviation (SD) from 3-5 cultures.

556

Parasite	Strain	Fexinidazole MW** 279.3	Fex-sulfone MW 295.3	Fex-sulfoxide MW 311.3	Melarsoprol MW 398.3	Pentamidine MW 592.7	Eflornithine /DMFO MW 236.7	Nifurtimox MW 287.3	Diminazene MW 515.5
T.b. rhodesiense	STIB900 wt	2.17 ± 0.29 5.56 ± 1.9*	1.44 ± 0.22 3.2 ± 0.15*	1.64 ± 0.36 3.2 ± 0.44*	0.011 ± 0.003	0.002 ± 0.0003	8.58 ± 2.7	1.09 ± 0.33	0.009 ± 0.002
	STIB900 mel	2.66 ± 0.57	1.26 ± 0.51	1.16 ± 0.29	0.092 ± 0.028	0.095 ± 0.035	nd	nd	0.019 ± 0.002
	STIB900 pent	2.71 ± 0.87	1.16 ± 0.39	1.48 ± 0.75	0.043 ± 0.022	0.058 ± 0.019	nd	nd	0.011 ± 0.004
T.b. brucei	BS221 wildtype	2.38 ± 0.88	1.63 ± 0.92	1.49 ± 0.61	0.013 ± 0.004	0.002 ± 0.0003	nd	nd	0.005 ± 0.001
	BS221 AT1KO	1.33 ± 0.21	0.56 ± 0.04	0.85 ± 0.32	0.034 ± 0.003	0.008 ± 0.002	nd	nd	0.060 ± 0.016
	STIB950 mdr	2.44 ± 0.99	0.99 ± 0.34	1.21 ± 0.14	0.038 ± 0.011	0.002 ± 0.0002	nd	nd	0.062 ± 0.05
T.b. gambiense	STIB930	1.84 ± 1.13	0.91 ± 0.27	0.94 ± 0.39	0.012 ± 0.005	0.016 ± 0.001	2.85 ± 0.98	2.24 ± 0.66	0.021 ± 0.009
	DAL 898R	1.01 ± 0.36	0.76 ± 0.30	1.03 ± 0.13	0.009 ± 0.002	0.002 ± 0.0002	nd	nd	0.014 ± 0.001
	K3048	0.95 ± 0.19	nd	nd	0.032 ± 0.012	0.084 ± 0.015	7.63 ± 2.5	0.99 ± 0.12	0.076 ± 0.03
	45R	2.47 ± 1.59	0.95 ± 0.47	1.24 ± 0.60	0.033 ± 0.011	0.069 ± 0.044	9.98 ± 2.4	1.06 ± 0.38	0.074 ± 0.033
	40R	2.61 ± 1.03	0.67 ± 0.35	0.95 ± 0.33	0.032 ± 0.006	0.088 ± 0.024	11.4 ± 5.8	1.46 ± 0.20	0.12 ± 0.02
	349Pi	1.07 ± 0.14	nd	nd	0.043 ± 0.011	0.066 ± 0.012	16.7 ± 3.6	0.78 ± 0.19	0.043 ± 0.025
	349R	3.31 ± 0.88	nd	nd	0.033 ± 0.015	0.095 ± 0.012	22.8 ± 13.9	2.73 ± 0.66	0.064± 0.031
	130R	2.37 ± 1.14	nd	nd	0.055 ± 0.023	0.074 ± 0.011	9.4 ± 2.19	1.34 ± 0.17	0.051 ± 0.013

557 * IC_{90} values ± standard deviation in μM
558 ** MW: molecular weight
559

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560 TABLE 2: *In vivo* anti-trypanosomal activity in the STIB900 acute mouse model

561

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

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

564 10% DMSO was used as vehicle

565 ** Data published E.Torreele PLoS Negl Trop Dis. 4(12): e923 (Ref 33)

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567 TABLE 3: *In vivo* anti-trypanosomal activity in the GVR25 chronic disease mouse
568 model.
569

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

570
571 * Data published E.Torreele PLoS Negl Trop Dis. 4(12): e923 (Ref. 33).
572 ** An optimized suspension medium for oral administration comprising 5% w/v
573 Tween 80/0.5% w/v Methocel in water to maximize absorption. These data have
574 been previously published and are reproduced here for comparative purposes
575 (33).
576 *** A 2% solution of eflornithine provided in drinking water for 10 days.
577
578 $^{+}$ Mean result from 3 separate experiments (n = 15)
579 $^{++}$ Mean result from 5 separate experiments (n = 24)
580 $^{+++}$ Data from 1 experiment (n = 4)
581 $^{++++}$ Representative data from 1 experiment (n =5/group)
582

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583 FIGURE 1: (A) Growth inhibition curves after compound wash-out at specified
584 times and viability assessment at 72 hr. (B) IC_{50} and IC_{90} values calculated from
585 compound wash-out procedure. Values and standard deviations in μg/ml are
586 means of 4 experiments (n = 4)

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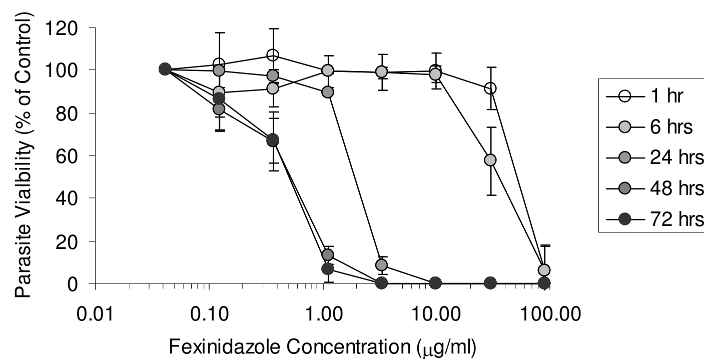

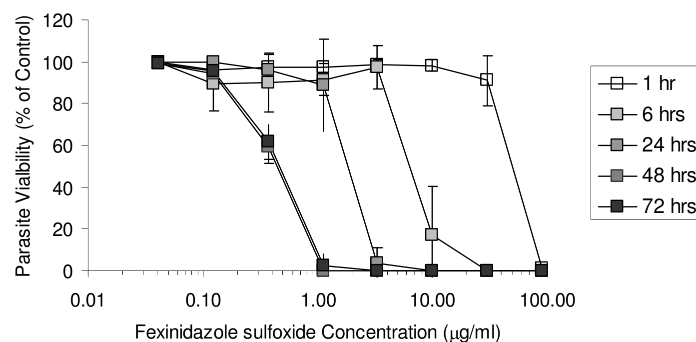

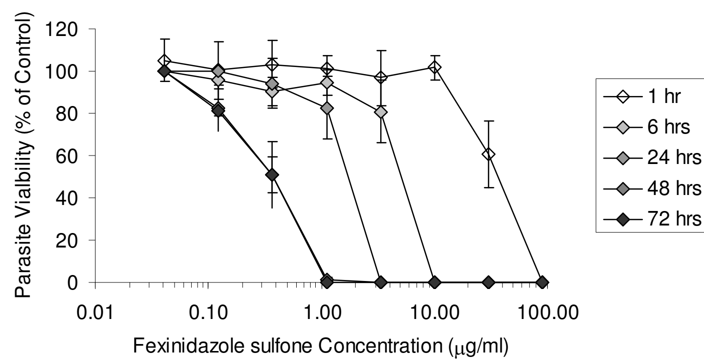

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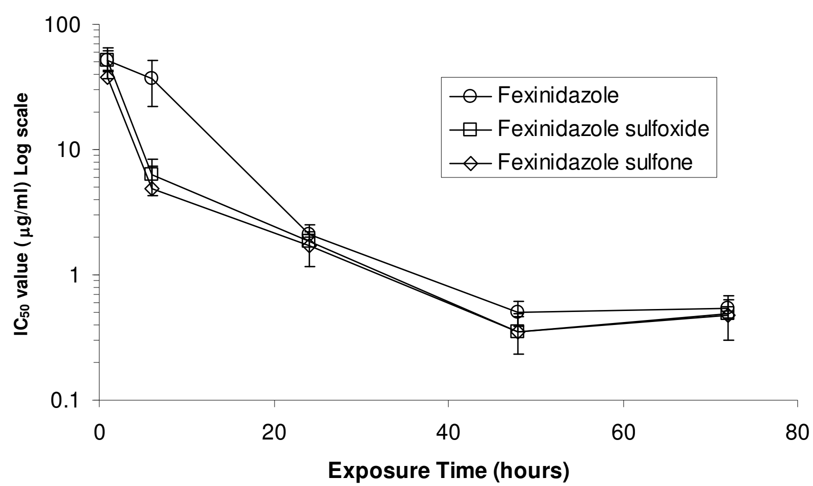

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