1   **The limited ability of posaconazole to cure both acute and chronic *Trypanosoma cruzi***

2   **infections revealed by highly sensitive *in vivo* imaging**

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20   **Short running title:** Limited efficacy of posaconazole against Chagas disease.

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ABSTRACT

The anti-fungal drug posaconazole has shown significant activity against *Trypanosoma cruzi in vitro* and in experimental murine models. Despite this, in a recent clinical trial it displayed limited curative potential. Drug testing is problematic in experimental Chagas disease because of difficulties in demonstrating sterile cure, particularly during the chronic stage of infection when parasite burden is extremely low and tissue distribution ill-defined. To better assess posaconazole efficacy against acute and chronic Chagas disease, we have exploited a highly sensitive bioluminescence imaging system which generates data with greater accuracy than other methods, including PCR-based approaches. Mice inoculated with bioluminescent *T. cruzi* were assessed by *in vivo* and *ex vivo* imaging, with cyclophosphamide-induced immunosuppression used to enhance the detection of relapse. Posaconazole was found to be significantly inferior to benznidazole as a treatment for both acute and chronic *T. cruzi* infections. Whereas 20 days treatment with benznidazole was 100% successful in achieving sterile cure, posaconazole failed in almost all cases. Treatment of chronic infections with posaconazole did however significantly reduce infection-induced splenomegaly, even in the absence of parasitological cure. The imaging-based screening system also revealed that adipose tissue is a major site of recrudescence in mice treated with posaconazole in the acute, but not the chronic stage of infection. This *in vivo* screening model for Chagas disease is predictive, reproducible and adaptable to diverse treatment schedules. It should provide greater assurance that drugs are not advanced prematurely into clinical trial.

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INTRODUCTION

Chagas disease is a major public health problem in Latin America and is increasingly prevalent in other regions as a result of migration patterns (1-2). The causative agent, *Trypanosoma cruzi*, is transmitted to humans predominantly by hematophagous triatomine bugs, although other routes include contaminated food and drink, blood transfusion and congenital transmission. Following infection, patients progress to the acute stage of the disease, where parasites are readily detectable in the bloodstream by microscopic examination. In most individuals, immune-mediated responses suppress parasitemia within 4-6 weeks and the majority of patients then remain asymptomatic, despite a life-long low-level infection. However, years or often decades later, about 30% of those infected develop chronic Chagas disease pathology, typically cardiomyopathy and/or digestive megasyndromes (3). Because of the complex and long-term course of the infection, vaccine development is considered to be extremely challenging and most biomedical research has focussed on improving chemotherapy.

For the last 40 years, the nitroheterocyclic compounds benznidazole and nifurtimox have been the only drugs available to treat Chagas disease (4-5). This is despite the fact that therapeutic schedules are long, treatment failures have been frequently reported, and both drugs exhibit toxicity. In addition, their efficacy in preventing or alleviating chronic disease pathology remains to be conclusively demonstrated (6-7). Benznidazole and nifurtimox are prodrugs and both are activated within *T. cruzi* by the same mitochondrial nitroreductase (TcNTR) (8) leading to the generation of reactive metabolites which mediate parasite killing (9-11). This shared activation mechanism provides potential for cross-resistance (8, 12, 13) and highlights the need to identify additional therapeutic agents which target distinct biochemical pathways. In this context, sterol metabolism in *T. cruzi* has generated

76   considerable interest, particularly the enzymes involved in ergosterol biosynthesis (14, 15).

77   The anti-fungal drug posaconazole for example, is a potent inhibitor of the *T. cruzi* sterol

78   14α-demethylase (CYP51) and has shown significant anti-parasitic activity *in vitro* and *in*

79   *vivo* (16-18). Furthermore, combination therapy with benznidazole has demonstrated that

80   murine infections can be cured with a reduced dosing regime (19, 20) However, in a recent

81   randomised clinical trial against chronic *T. cruzi* infection, posaconazole was shown to have

82   limited curative potential (21) and *in vitro* studies have found it to be less active than

83   benznidazole (22).

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85   The vast majority of Chagas disease patients are only diagnosed once they begin to display

86   chronic disease pathology, or after testing prior to blood donation or surgical procedures.

87   Drug trials against chronic stage infections are particularly challenging because it is difficult

88   to unequivocally demonstrate sterile cure. In addition, lack of knowledge of the sites of

89   parasite persistence can be a confounding factor that impacts on the reproducibility of PCR-

90   based methodologies, making it difficult to accurately assess parasite burden in real time. To

91   streamline the drug discovery process, we sought to improve the utility of current predictive

92   models of experimental Chagas disease by developing an enhanced *in vivo* imaging system.

93   This was achieved by engineering trypanosomes to express a red-shifted luciferase reporter

94   which emits tissue-penetrating orange-red light (λ_{em} 617 nm) (23, 24). In *T. cruzi*, the

95   bioluminescent reporter is expressed at similar levels in different parasite life-cycle stages,

96   has no effect on growth properties or virulence, and is maintained at constant levels for more

97   than 12 months in the absence of selective drug pressure. Importantly, this *in vivo* imaging

98   system has a limit of detection of between 100 and 1000 parasites, and has allowed parasite

99   burden to be assessed in real time during experimental chronic infections in individual mice

100   (24). Throughout chronic infections, dynamic bioluminescence foci can appear and disappear

101   over a period of less than 24 hours (24), consistent with a scenario where infected cells are

102   being trafficked to and from peripheral sites. In BALB/c mice infected with the CL Brener

103   strain, the gastrointestinal tract was found to be the major site of parasite persistence.

104   Unexpectedly, in this model, infection of the heart was rarely observed in the chronic stage,

105   even though these mice continued to exhibit cardiac inflammation and diffuse fibrosis,

106   signatures of chronic Chagas disease pathology.

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108   The enhanced sensitivity of this red-shifted luciferase based reporter system has the potential

109   to provide new approaches for monitoring the effectiveness of drugs against experimental

110   Chagas disease and should be a valuable addition to the drug discovery pipeline. Here, we

111   describe its use to assess the efficacy of posaconazole to treat acute and chronic experimental

112   infections. In line with a recent clinical trial, our predictive model suggests major limitations

113   in the utility of this drug.

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MATERIALS AND METHODS

Mice and infections. Female BALB/c mice were purchased from Charles River (UK) and CB17 SCID mice were bred in-house. Animals were maintained under specific pathogen-free conditions in individually ventilated cages, where they experienced a 12 hour light/dark cycle and had access to food and water *ad libitum*. All experiments were carried out under UK Home Office licence PPL 70/6997 and approved by the LSHTM Animal Welfare and Ethical Review Board. Mice were aged 8 – 12 weeks when infected with a bioluminescent reporter clone derived from the genome reference strain CL Brener (24). In standard experiments, 1 x 10^4 *in vitro* derived tissue-culture trypomastigotes (TCTs) or thawed cryopreserved bloodstream trypomastigotes (BTs) in 0.2 ml PBS were first used to infect SCID mice via intraperitoneal (i.p.) inoculation. Parasitaemic blood from these SCID mice was obtained 2 – 3 weeks later and adjusted to 5 x 10^3 BTs/ml with PBS. BALB/c mice were then infected with 1 x 10^3 BTs via i.p injection (24).

Treatment. For drug treatment, benznidazole (Hoffmann-La Roche AG) was prepared from powder at 10 mg/ml in 7% Tween-80, 3% ethanol (v/v) and 90% (v/v) water. Posaconazole (Sequoia Research Products Ltd) was prepared at 2 mg/ml in 5% (v/v) dimethyl sulphoxide and 95% (v/v) HPMC-SV (0.5% (w/v) hydroxypropyl methylcellulose, 0.5% (v/v) benzyl alcohol and 0.4% (v/v) Tween 80). Noxafil (MSD Ltd.), a liquid formulation of posaconazole (40 mg/ml), was diluted to 2 mg/ml in water. Mice were treated with standard doses of benznidazole (100 mg/kg/day) or posaconazole (20 mg/kg/day) by oral gavage for consecutive days, as required. To facilitate detection of residual infection after treatment, in some experiments BALB/c mice were immunosuppressed with cyclophosphamide (200 mg/kg) by i.p. injection at 3 – 4 day intervals, for a maximum of 3 doses.

150 ***In vivo* bioluminescence imaging.** Mice were injected i.p. with 150 mg/kg d-luciferin

151 (Perkin-Elmer) in Dulbecco's Ca^{2+}/Mg^{2+} free PBS, then anaesthetized using 2.5% (v/v)

152 gaseous isofluorane in oxygen. To measure bioluminescence, mice were placed in an IVIS

153 Lumina II system (Caliper Life Science) and both dorsal and ventral images were acquired 10

154 – 20 minutes after d-luciferin administration using LivingImage 4.3. Exposure times varied

155 between 30 seconds and 5 minutes, depending on signal intensity. Anaesthesia was

156 maintained throughout via individual nose-cones. After imaging was complete, mice were

157 revived and returned to cages. To estimate parasite burden, whole body regions of interest

158 (ROIs) were drawn using LivingImage v.4.3 to quantify bioluminescence expressed as total

159 flux (photons/second; p/s). The detection threshold was established previously using data

160 from control uninfected mice (24). Animals where bioluminescence intensity was

161 consistently below 5 x 10^3 p/s/sr/cm^2 in both dorsal and ventral images following

162 immunosuppression, were regarded as cured, subject to confirmation by *ex vivo* assessment

163 (below).

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165 **Assessment of treatment efficacy by *ex vivo* imaging.** Selected organs and tissue samples

166 from all mice were assessed for infection by *ex vivo* imaging (Figure 1e), as described

167 previously (24). Briefly, mice were injected with 150 mg/kg d-luciferin i.p., then sacrificed

168 by ex-sanguination under terminal anaesthesia 7 minutes later. Mice were perfused with 10

169 ml 0.3 mg/ml d-luciferin in PBS via the heart. Organs and tissues were excised, transferred to

170 a Petri dish or culture dish, soaked in 0.3 mg/ml d-luciferin in PBS, and then imaged as per

171 live mice. Routinely, the rest of the carcass was also assessed for bioluminescence associated

172 with skin, skeletal muscle or remaining adipose tissue. As with *in vivo* imaging,

173 bioluminescence intensity of 5 x 10^3 p/s/sr/cm^2 was used as the threshold to designate cure.

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175　**PCR-based detection.** Heart, large intestine and blood tissues were snap frozen on dry ice

176　and stored at -80°C until required for DNA extraction. In the case of the gut, three one

177　centimetre sections were pooled from the proximal colon, the mid-colon region and the

178　rectum of each mouse. Samples were then thawed and immediately homogenized in at least

179　200 µl lysis buffer (4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA, pH 7.4) per 50

180　mg of tissue, using a BulletBlender Storm instrument (Next Advance). Proteinase K (Sigma)

181　was added to the tissue suspension at 0.6 mg per 200 µl and incubated at 56°C for 1 hour,

182　then at 37°C overnight. DNA was extracted from lysates using a HighPure PCR template

183　preparation kit (Roche), according to the manufacturer's instructions. Real-time PCR

184　reactions were prepared using the QuantiTect SYBR Green PCR Kit (Qiagen) and run on a

185　RotorGene 3000 instrument. Each reaction contained 50 ng DNA and 0.5 µM of each primer.

186　*T. cruzi*-specific primers TCZ-F/TCZ-R (25) targeted at the 195 bp satellite repeat (10^4 copies

187　in the CL Brener genome), or mouse-specific primers GAPDHf/GAPDHr (26) targeted at the

188　murine *gapdh* gene, were used.

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190　*T. cruzi*-specific qPCR Ct values were converted to inferred numbers of parasite equivalents

191　(p.e.) by reference to a standard curve with a range of 2.5 x 10^6 – 2.5 x 10^{-1} p.e./ml of tissue

192　lysate. The *T. cruzi* standard curve was established from serial dilution of a DNA sample

193　derived from 75 mg homogenised muscle tissue, spiked with 2 x 10^7 epimastigotes, using

194　DNA from unspiked equivalent samples as the diluent. Murine DNA content was determined

195　by normalizing mouse-specific qPCR Ct values by reference to a standard curve with a range

196　of 2.5 x 10^1 – 2.5 x 10^{-4} µg/ml. The murine standard curve was established from serial

197　dilution of a mouse DNA sample using herring sperm DNA as the diluent. Due to the non-

198　specific fluorescence inherent to this SYBR green qPCR method, we defined parasite

199　detection limits as the mean +3SDs for samples from uninfected control mice.

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201   **Statistics.** Results are shown as mean ±SD or SEM where sample sizes are equal or non-

202   equal, respectively. Individual animals were used as the unit of analysis for *in vivo* and *ex*

203   *vivo* experiments. For spleen mass, means were compared using Student's *t*-test.

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RESULTS

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Benznidazole and posaconazole efficacy against chronic stage *T. cruzi* infections.

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225 BALB/c mice, infected i.p. with 10^3 bioluminescent bloodstream form *T. cruzi*

226 trypomastiogotes (CL Brener strain), were monitored by *in vivo* imaging (Fig. 1A, Materials

227 and Methods). In this experimental model, peak parasitemia occurs after 14 days, and is

228 followed by an immune-mediated reduction in parasite load during progress to the chronic

229 stage, 40-50 days post-infection (dpi) (Fig. 2B) (24). After 74 days, cohorts of mice were

230 treated daily for 20 days by the oral route with benznidazole (100 mg/kg), or with one of two

231 posaconazole formulations (20 mg/kg). These dosing regimes have been widely used for

232 experimental purposes (19, 20, 27, 28). Benznidazole acted rapidly and the whole body

233 bioluminescence of each mouse fell to undetectable levels within 5 days (Fig. 1B and 2A).

234 Posaconazole was slower acting, but by the conclusion of the treatment period, the inferred

235 parasite load had also dropped to background levels. The bioluminescence profile during

236 treatment was very similar with both posaconazole formulations (Fig. 1C and D, and 2A). 20

237 days after the cessation of treatment (113 dpi), half the mice in each cohort were

238 immunosuppressed (Materials and Methods). No signs of infection were observed in any of

239 the benznidazole treated mice in either the immunosuppressed or immunocompetent groups

240 (Fig. 1B). However, in the posaconazole treated group, the infection relapsed in all of the

241 cyclophosphamide treated mice (Fig. 1C and D, and 2a; Table 1).

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243 Organs from all of the mice were assessed for infection by *ex vivo* imaging at the

244 experimental end-point (Fig. 1e, Materials and Methods). In accordance with experiments

245 using this and other mouse-parasite combinations (24; M.L., unpublished observations),

246 persistent bioluminescent foci at this point of the infection (~148 dpi) were associated

247 predominantly with the gastrointestinal tract (mainly the colon and stomach) in untreated

248   mice, and only sporadically with other major organs/tissues. Mice were designated as cured,

249   if they were bioluminescence negative by both *in vivo* and *ex vivo* imaging, following

250   cyclophosphamide treatment (Materials and Methods). Based on these criteria, none of the

251   nine   chronically   infected   mice   treated   with   posaconazole   and   subsequently

252   immunosuppressed were cured. In contrast, all five mice that were treated with benznidazole

253   and then immunosuppressed were inferred to be parasite-free.

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255   Quantitative PCR (qPCR) after immunosuppression has until now been the most accurate

256   technique for defining parasitological cure in *T. cruzi* infections (20, 28, 29). However, when

257   we assessed the efficacy of this method in our chronic infection model, we found that PCR

258   methodology had a tendency to overestimate cure-rate, particularly with posaconazole

259   treatment. In chronically infected untreated mice, pooled gut tissue (Materials and Methods)

260   was PCR-positive in each case, and negative when mice were treated with benznidazole for

261   20 days, including the group that was subsequently immunosuppressed (Fig. 3A). With the

262   posaconazole treated non-immunosupressed mice, gut issue was PCR-negative in 9 out of 10

263   cases, also consistent with a high rate of cure. This inferred cure rate was reduced when tissue

264   derived from mice that had also been cyclophosphamide treated was analysed. The number of

265   PCR-negative (cured) animals fell to 4 out of 9, indicating that some low level infections only

266   become detectable by PCR after immunosuppression. However, even this reduced cure rate is

267   at odds with data from bioluminescence imaging, which demonstrated unequivocally that

268   posaconazole failed to eradicate parasites in any of the treated mice (Fig. 1, Table 1). In the

269   case of cardiac tissue, with one exception, results were PCR-negative in all cases (Fig. 3B), in

270   accordance with bioluminescence imaging of chronic stage infections (Fig. 1, ref. 25). When

271   blood samples were analysed, they were predominantly negative, even when mice were non-

272   treated (Fig. 3c). Collectively, these results highlight the limitations of using PCR-based

273   approaches to define parasitological cure in this dynamic chronic stage model. The low level

274   and sporadic nature of bloodstream parasitemia and the focal and highly dynamic nature of

275   tissue infection, even within the gastro-intestinal tract, appear to be the confounding factors

276   which result in an overestimation of the cure-rate when determined by qPCR alone.

277

278   To further assess the ability of benznidazole to cure chronically infected mice, we reduced

279   the treatment period to 10 and 5 oral doses (100 mg/kg/day) over consecutive days. In each

280   case, bioluminescence fell below the level of detection by the completion of treatment, and

281   subsequent immunosuppression of these mice did not lead to a relapse, as assessed by either

282   *in vivo* and *ex vivo* imaging (Fig. 4). In this experimental model therefore, there is scope to

283   reduce the length of benznidazole treatment of chronic *T. cruzi* infections and still achieve a

284   curative outcome.

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286   Splenomegaly is frequently observed in experimental *T. cruzi* infections, both acute and

287   chronic. Here, we observed that the spleens of chronically infected mice were approximately

288   twice the mass of those from non-infected mice (Fig. 5). This spleen enlargement could be

289   reversed by curative treatment with benznidazole (assessed by *in vivo* and *ex vivo* imaging,

290   with an immunosuppressed group in parallel, Fig. 1). Interestingly, there was also a reversal

291   of splenomegaly following posaconazole treatment. In these mice, there was a major

292   reduction in parasite burden, but a curative outcome was not achieved (Fig. 1). Therefore,

293   splenomegaly in this model is linked with parasite load and can be largely reversed, at least in

294   the short term, without having to achieve a sterile cure.

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296   **Benznidazole and posaconazole efficacy against acute stage *T. cruzi* infections.** Using the

297   same experimental model as above, we compared the ability of benznidazole and

298   posaconazole to cure acute stage *T. cruzi* infections. Treatment was started at the peak of

299   parasite burden (14 dpi) with standard oral doses (benznidazole, 100 mg/kg; posaconazole, 20

300   mg/kg) administered daily for 20 days. Similar to the chronic stage infections, benznidazole

301   treatment resulted in a rapid fall in parasite load, with bioluminescence reduced to

302   background levels within 5 days (Fig. 2B and 6B). There was no relapse of infection, when

303   mice were assessed by *in vivo* or *ex vivo* imaging following immunosuppression (Fig. 6B and

304   E), and all mice treated with benznidazole were therefore designated as cured.

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306   With posaconazole treatment, the reduction in bioluminescence was much more rapid than

307   had been observed with chronic stage infections (compare Fig. 2A and B), and only

308   marginally slower than with benznidazole. Again, there were no significant differences in the

309   efficacy of the two posaconazole formulations. Bioluminescence remained close to, or only

310   slightly above background levels, until the mice were treated with cyclophosphamide

311   (initiated 49 dpi) (Fig. 2B and 6). At this point, there was a rapid rebound in parasite load in

312   most cases, with 16 out of 19 mice displaying a clear bioluminescence signal (Fig. 6, Table

313   1). Of the three mice judged to be cured, one had been treated with the Noxafil and two with

314   the HPMC-SV posaconazole formulation (Materials and Methods). These results therefore

315   suggest that although posaconazole is more effective at reducing the parasite load during the

316   acute stage than it is during the chronic stage, it has only a limited ability to achieve a sterile

317   cure in this experimental model, in either stage of the disease.

318

319   In 9 out of the 16 posaconazole treated mice which relapsed after cyclophosphamide

320   treatment, we observed that adipose tissue was the major site of recrudescence (Fig. 7A, as

321   example). This suggests that the ability of parasites to persist in this location following acute

322   stage posaconazole treatment is a common phenomenon. In contrast ($P<0.05$), when mice in

323　the chronic stage of infection were treated and then immunosuppressed, only 1 out of 9

324　animals displayed a significant parasite burden in this tissue (shown Fig. 1E (iv)), with the

325　gastro-intestinal tract being the major site of parasite recrudescence (Fig. 7B).

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345   **DISCUSSION**

346   Progress in developing new drugs for chronic *T. cruzi* infections has been limited by

347   difficulties in unambiguously demonstrating parasitological cure. An underlying cause, as

348   inferred from murine infections, could be the discrete nature of infection foci during chronic

349   Chagas disease, and their highly dynamic spatiotemporal distribution (24). As a consequence,

350   there is a risk of overestimating cure rates associated with unguided tissue sampling, even

351   when using PCR-based technology. Highly sensitive bioluminescence imaging circumvents

352   some of these issues by facilitating the real time evaluation of parasite burden throughout

353   long term infections, with minimal tissue sampling bias.

354

355   Several studies have reported on the efficacy of the CYP51 inhibitor posaconazole, and its

356   ability to cure *T. cruzi* infections in murine models (17-20). Despite this, when the drug was

357   advanced into clinical trials, it failed to provide significant benefit to chronically infected

358   patients, in terms of parasitological cure (21). In line with this, data from our predictive

359   model imply that posaconazole has limited potential against both stages of Chagas disease

360   (Table 1). *In vivo* imaging revealed that although posaconazole is highly effective at reducing

361   parasite burden, it does not readily cure acute or chronic *T. cruzi* infections. When mice in the

362   acute stage were treated, the bioluminescence-inferred parasite burden was reduced by more

363   than 3 orders of magnitude within 7 days, however sterile cure was rarely achieved (Fig. 2

364   and 6). With chronic infections, posaconazole failed to cure any of the mice and the reduction

365   in parasite load occurred more slowly (Fig. 1 and 2). This inability, in the vast majority of

366   cases, to eradicate parasites has parallels with *in vitro* studies. These showed that although the

367   EC_{50} of posaconazole against intracellular *T. cruzi* is in the nanomolar range, it often fails to

368   eliminate parasite infection (22). One reason for the faster rate of parasite knockdown in the

369   acute stage (Fig. 2) might be that parasites replicate more rapidly, and are therefore more

370 susceptible to drugs that perturb lipid metabolism. Alternatively, in the chronic stage, when

371 parasites are restricted predominantly to gastro-intestinal sites (24), they may be less

372 accessible compared to the acute stage, when parasites can be targeted in all organs, although

373 this explanation is less likely given the pharmacokinetic and distribution properties of

374 posaconazole (30).

375

376 Typically, treatment with posaconazole reduced whole body bioluminescence to background

377 levels, with few infection foci detectable in the absence of immunosuppression. Given the

378 sensitivity of the imaging procedure (24), this suggests that the remaining parasites survive in

379 low numbers within small groups of infected cells. As a result, detection of residual parasites

380 by PCR-based methods is problematic (Fig. 3). In the past, this may have led to an over-

381 estimation of the ability of posaconazole to cure chronic infections. Posaconazole treatment

382 has been shown to reverse spleen enlargement, a characteristic of murine *T. cruzi* infections.

383 In these experiments, curative outcome was inferred on the basis of several criteria (27).

384 However, here we demonstrate that reversal of splenomegaly is not indicative of sterile cure

385 (Fig. 5), but is linked merely with a reduction in parasite burden.

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387 In more than 50% of cases (9/16), end-point *ex vivo* analysis of acute stage infections

388 identified visceral fat as the tissue with the highest parasite burden following relapse (Fig. 7).

389 There are several reasons why posaconazole could be less effective at eliminating parasites

390 from this site. Parasite load may be higher in adipose tissues (24), differential drug

391 accessibility may be an issue, or parasites could be less susceptible in a lipid/sterol rich

392 environment. When mice treated during the chronic stage were examined after relapse, only 1

393 mouse out of 9 displayed a detectable level of bioluminescence in visceral fat (Fig. 7). At this

394 stage of an infection, parasites are restricted mainly to the gastro-intestinal tract, and only

395 sporadically detected in the visceral fat, or other tissues, by bioluminescence. In chronic

396 infections therefore, this tissue is less likely to be relevant as a reservoir for parasite survival

397 following drug treatment. Previous studies have identified parasites localised in adipose

398 tissue in some chronic human infections (31, 32) In non-treated mice however,

399 bioluminescence imaging did not identify the visceral fat as a primary site of recrudescence

400 during a chronic infection (see Fig. 7).

401

402 In summary, we have shown that benznidazole is significantly more effective at curing both

403 acute and chronic *T. cruzi* infections than posaconazole. The utility and flexibility of the *in*

404 *vivo* imaging procedure we have developed has potential for making a valuable contribution

405 to the Chagas disease drug discovery pipeline. It can also, as shown here, add value to the

406 screening process by providing new information on drug efficacy. Importantly, the

407 availability of such a sensitive *in vivo* technique should provide greater assurance that drugs

408 are not advanced prematurely into clinical trial.

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563 **FIG 1.** Benznidazole, but not posaconazole, cures mice chronically infected with

564 *Trypanosoma cruzi*. Mice infected with bioluminescent *T. cruzi* were injected with 150

565 mg/kg d-luciferin, anaesthetised and imaged using an IVIS Lumina II system (Materials and

566 Methods). (a-d) Ventral (V) and dorsal (D) images of individual representative infected mice.

567 (A) Non-treated mouse. (B) Mouse treated with 100 mg/kg benznidazole on days 74 – 93

568 post-infection, then immunosuppressed by 200 mg/kg cyclophosphamide treatment on days

569 113, 118 and 128. (C) Mouse treated with 20 mg/kg posaconazole (Noxafil formulation) on

570 days 74 - 93, and then immunosuppressed, as above. (D) Mouse treated with posaconazole

571 (HPMC-SV formulation) on days 74 - 93, and immunosuppressed, as above. (E) Tissue-

572 specific *ex vivo* imaging. (i) Non-treated mouse 132 days post-infection (dpi). (ii) Mouse 147

573 dpi, which had been treated with benznidazole, and then immunosuppressed, as described

574 above. (iii and iv) Mice 147/148 dpi, which had been treated with posaconazole (Noxafil and

575 HPMC-SV formulations respectively), then immunosuppressed, as above. (v) Schematic

576 which identifies the positions of organs displayed in insets (i-iv). (Gut Mes, gut mesentery

577 tissue; OES, oesophagus; SKM, skeletal muscle; STM, stomach; VIS FAT, visceral

578 fat/adipose tissue). The heat-map is on a log10 scale and indicates intensity of

579 bioluminescence from low (blue) to high (red); the minimum and maximum radiances for the

580 pseudocolour scale are shown.

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588 **FIG 2.** Quantification of whole animal bioluminescence (ventral and dorsal) following

589 treatment with benznidazole and posaconazole. (A) Infected mice treated starting 74 dpi for

590 20 days by the oral route. (B) Mice treated starting 14 dpi for 20 days by the oral route. Red

591 squares, untreated mice; n=5, acute; n=6 chronic. Blue squares, treated with benznidazole at

592 100 mg/kg; n=10. Green squares, treated with posaconazole (Noxafil) at 20 mg/kg; n=9.

593 Purple squares, treated with posaconazole (HPMC-SV formulation) at 20 mg/kg; n=10.

594 Arrows indicate the start and end points of treatment. Grey lines indicate detection threshold

595 determined as the mean (solid line) and mean +2SD (dashed line) of background

596 bioluminescence of control uninfected mice. Crosses signify dates of cyclophosphamide

597 treatment (200 mg/kg). Inoculation failed to result in an infection in one mouse in each of the

598 acute and chronic infection studies. These mice were not treated (Noxafil cohort) and

599 excluded from the analysis.

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613   **FIG 3.** qPCR-inferred parasite loads in drug-treated chronically infected mice. DNA was

614   extracted from large intestine (A), heart (B) and blood (C) samples and the relative amounts

615   of *T. cruzi* and murine DNA were quantified by real-time PCR amplification of the multi-

616   copy 195 bp satellite repeat and of the *gapdh* gene, respectively (Materials and Methods).

617   The limit of detection is represented by the dotted line which corresponds to the mean +3SDs

618   of large intestine, heart or blood samples from uninfected cells. Above this line, there is a

619   linear correlation with parasite number as established by a standard curve (Materials and

620   Methods). In (A) and (B), the base-line is equivalent to <1 parasite per 5 million murine

621   cells). In (C), the base-line established with the multi-copy 195 bp repeat corresponds to 1

622   parasite equivalent per µl blood. NI: non-infected; INT: infected non-treated; IBZ: infected,

623   benznidazole treated (as in Fig. 1); IBZ, CYP: infected, benznidazole treated, then

624   immunosupressed with cyclophosphamide; IP1: infected, posaconazole (Noxafil) treated;

625   IP1, CYP: infected, posaconazole (Noxafil) treated, then immunosupressed with

626   cyclophosphamide; IP2: infected, posaconazole (HPMC-SV formulation) treated; IP2, CYP:

627   infected, posaconazole (HPMC-SV formulation) treated, then immunosuppressed.

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638 **FIG 4.** Assessing the ability of benznidazole to cure mice chronically infected with

639 *Trypanosoma cruzi* using 5 and 10 day treatment regimes. (A) Outline of experimental

640 protocol. Cohorts of 6 mice were infected with bioluminescent *T. cruzi* (Materials and

641 Methods). 103 days post-infection (dpi), they were treated with benznidazole (daily by the

642 oral route, 100 mg/kg) for 5 days or 10 days. As indicated, mice were subsequently

643 immunosuppressed with 3 i.p. doses of cyclophosphamide (200 mg/kg). (B) Ventral images

644 of 3 representative mice from each cohort taken at the indicated dpi. (C) Representative *ex*

645 *vivo* imaging of organs from infected mice 140 dpi (Materials and Methods). Organs are

646 displayed in accordance with schematic in Fig. 1E (v). Heat-maps indicate intensity of

647 bioluminescence from low (blue) to high (red) (log10 scales); the minimum and maximum

648 radiances for the pseudocolour scale are shown

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663   **FIG 5.** Effect of chronic *Trypanosoma cruzi* infection and drug treatment on spleen mass. 74

664   days post-infection, mice were treated with benznidazole (100 mg/kg) (IBZ) or posaconazole

665   (20 mg/kg) (IP1, IP2; Noxafil and HPMC-SV formulations, respectively) by the oral route,

666   daily for 20 days (see also Fig. 1). Spleens were harvested and weighed at the end of the

667   experiment. Spleen weights in infected, non-treated mice (INT) were significantly greater

668   than those in non-infected mice (NI) ($P<0.0001$), or infected mice which had been treated

669   ($P<0.005$, in each case).

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688   **FIG 6.** Posaconazole has limited efficacy as a treatment for acute *Trypanosoma cruzi*

689   infections. Mice (n = 10) were infected with bioluminescent *T. cruzi* (Fig. 1, Materials and

690   Methods) and treatment initiated at the peak of the acute stage, day 14. (A-D) Ventral (V) and

691   dorsal (D) images of representative individual mice. (A) Infected, non-treated. (B) Treated

692   with 100 mg/kg benznidazole on days 14 – 33 post-infection, then immunosuppressed by 200

693   mg/kg cyclophosphamide treatment on days 49, 53, and 57. (C) Treated and cured with 20

694   mg/kg posaconazole (Noxafil formulation) on days 14 – 33, and immunosuppressed as above.

695   (D) Treated with posaconazole (Noxafil formulation) on days 14 - 33, and

696   immunosuppressed as above. 16 out of 19 posaconazole treated mice were assessed as non-

697   cured. One mouse did not become infected and was excluded from the study. (E) *Ex vivo*

698   imaging of organs and tissues obtained from mice on days 74 – 79, as indicated, following

699   drug treatment and immunosuppression. Organs and tissues were arranged as in Fig. 1E (v).

700   Heat-maps are on log10 scales and indicate intensity of bioluminescence from low (blue) to

701   high (red).

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713 **FIG 7.** *Ex vivo* imaging of cyclophosphamide-induced parasite recrudescence following

714 posaconazole treatment of mice in the acute and chronic stages of infection. (A) Mice in the

715 acute stage of *T. cruzi* infection were treated with posaconazole for 20 days and then treated

716 with cyclophosphamide (see legend to Fig. 6 for details). Images were taken 74 dpi. For

717 comparison, the lower image shows parasite recrudescence following immunosuppression of

718 a non-treated, chronically infected mouse (imaged 173 dpi). (B) *T. cruzi* infected mice,

719 treated with posaconazole during the chronic stage of infection and then treated with

720 cyclophosphamide (see legend to Fig. 1). Images taken 147 (upper) and 148 (lower) dpi. The

721 schematic identifies the positions of organs (Gut Mes, gut mesentery tissue; OES,

722 oesophagus; SKM, skeletal muscle; STM, stomach; VIS FAT, visceral fat/adipose tissue).

723 The location of the visceral fat tissue is highlighted by an arrow. Heat-maps are on log10

724 scales and indicate intensity of bioluminescence from low (blue) to high (red).

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738 **Table 1.** Summary of drug efficacy against acute and chronic *Trypanosoma cruzi* infections

739 inferred from bioluminescence. Data were collated from experiments illustrated in Fig. 1, 4

740 and 6. Mice were designated as cured only when bioluminescence negative by both *in vivo*

741 and *ex vivo* imaging following immunosuppression (Materials and Methods). In the

742 posaconazole treatment, data from both formulations were pooled.

743

Drug	Disease state	Treatment time	Daily dose	Number cured
Benznidazole	chronic	20 days	100 mg/kg	5/5
Benznidazole	acute	20 days	100 mg/kg	5/5
Benznidazole	chronic	10 days	100 mg/kg	6/6
Benznidazole	chronic	5 days	100 mg/kg	6/6
Posaconazole	chronic	20 days	20 mg/kg	0/9
Posaconazole	acute	20 days	20 mg/kg	3/19

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Fig. 1

(A) Chronic infection
Treated days 74 - 93

(B) Acute infection
Treated days 14 - 33

Legend:
- ■ Untreated
- ■ Benznidazole
- ■ Posaconazole (Noxafil)
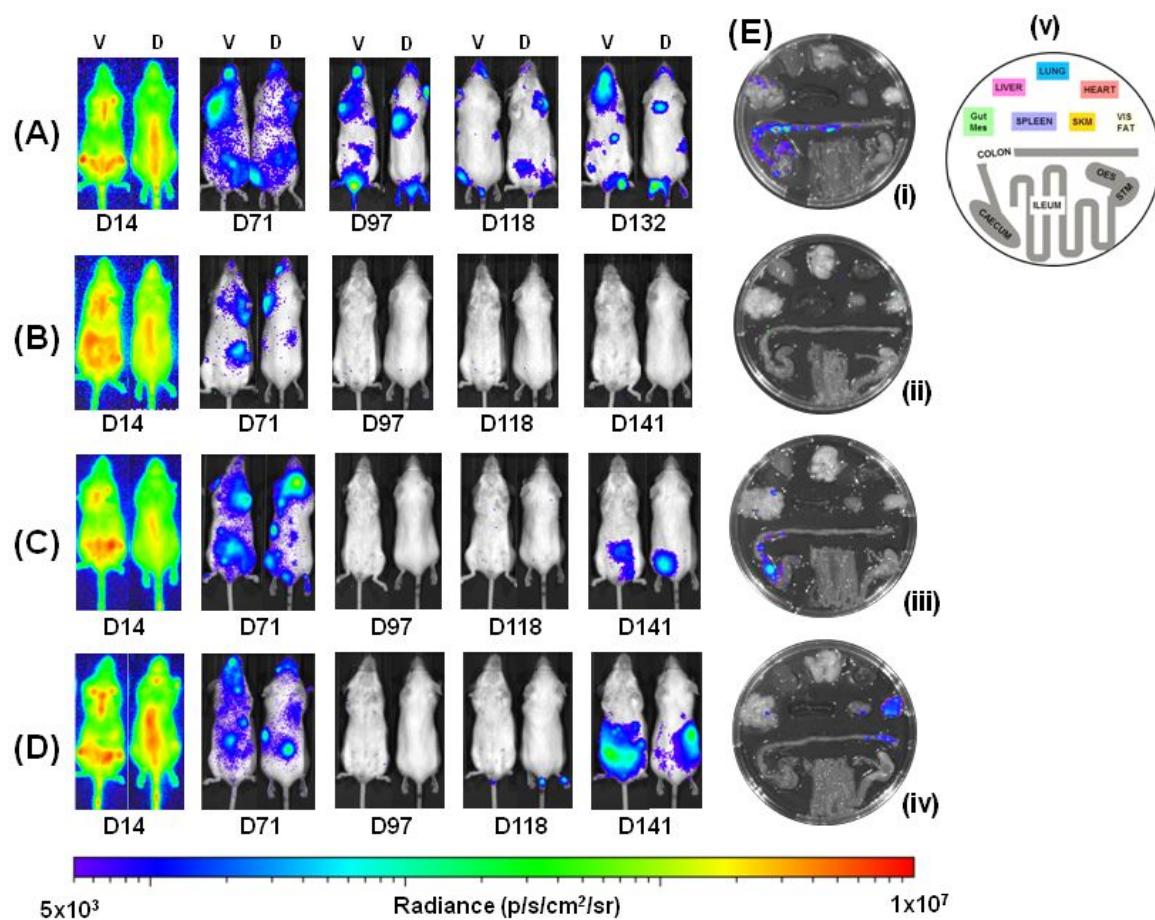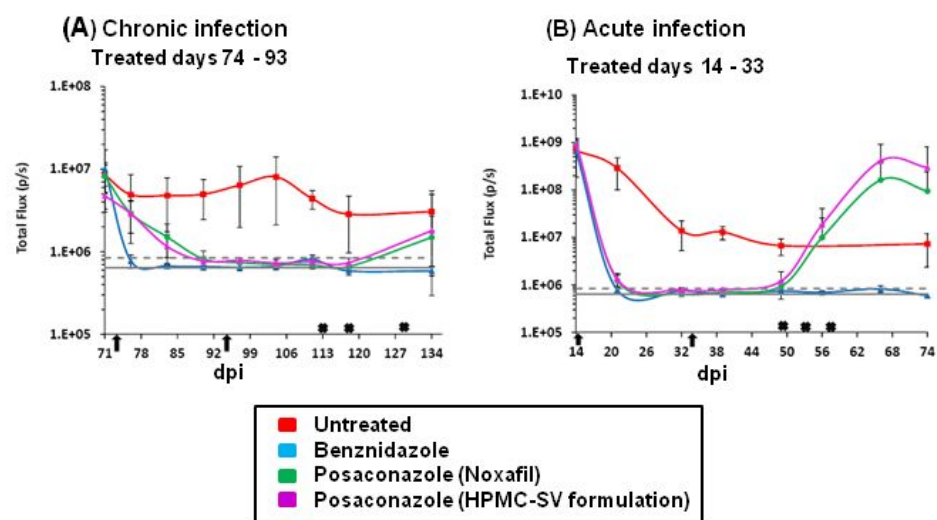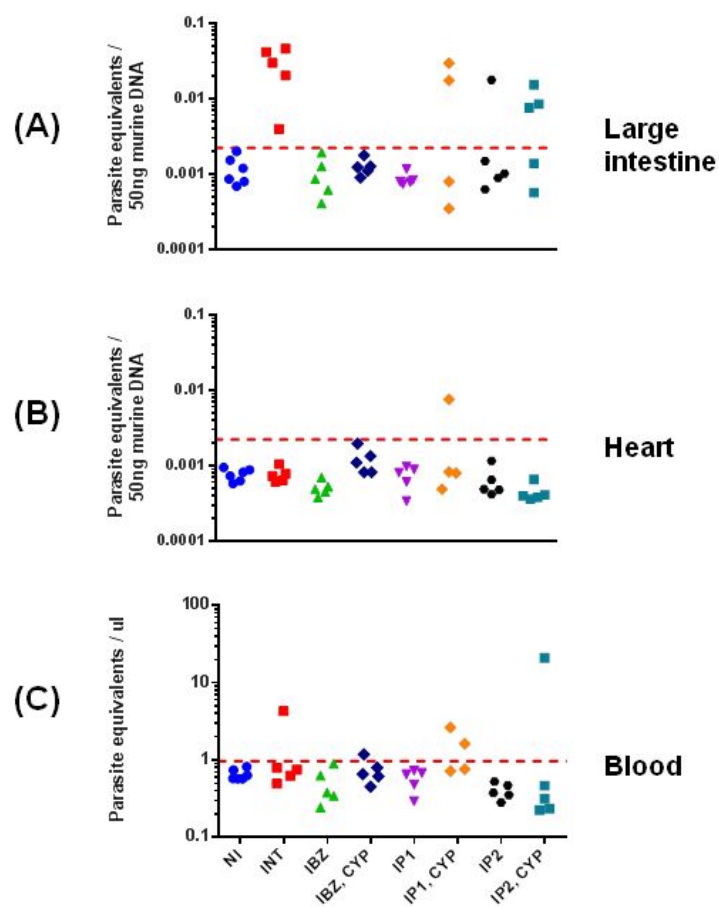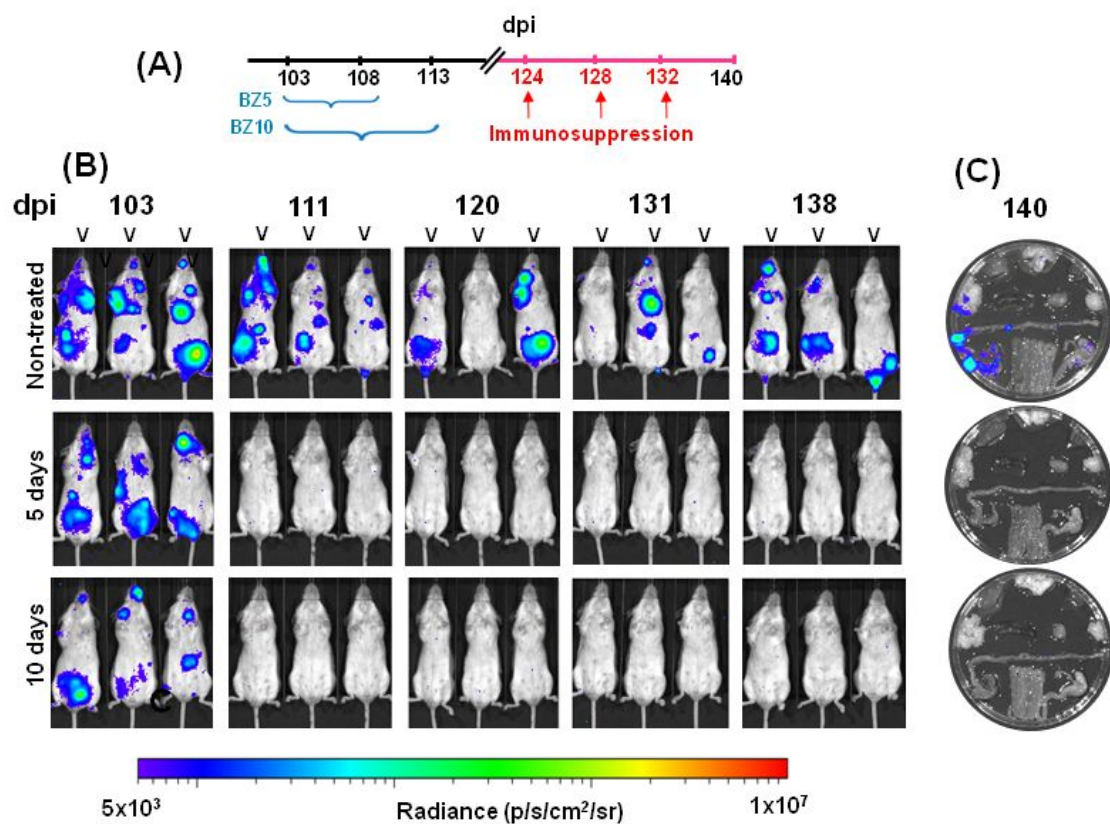- ■ Posaconazole (HPMC-SV formulation)

Fig. 2

(A)

Large
intestine

(B)

Heart

(C)

Blood

Fig. 3

Fig. 4

Fig. 5

Fig. 6

(A)
treated
acute stage

(B)
treated
chronic stage

non-treated
chronic stage

Radiance (p/s/cm²/sr)

1×10^7

2×10^4

3×10^4

3×10^3

LUNG

LIVER

HEART

Gut
Mes

SPLEEN

SKM

VIS
FAT

COLON

OES

STM

CAECUM

ILEUM

Fig. 7