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A sensitive and reproducible *in vivo* imaging mouse model for evaluation of drugs against late-stage human African trypanosomiasis

Hollie Burrell-Saward[1], Jean Rodgers[2], Barbara Bradley[3], Simon L. Croft[1] and Theresa H. Ward[1]*

[1]Department of Immunology and Infection, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London WC1E 7HT, UK; [2]Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G61 1QH, UK; [3]Institute of Infection and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G61 1QH, UK

*Corresponding author. Tel: +020-7927-2649; E-mail: theresa.ward@lshtm.ac.uk

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Objectives: To optimize the *Trypanosoma brucei brucei* GVR35 VSL-2 bioluminescent strain as an innovative drug evaluation model for late-stage human African trypanosomiasis.

Methods: An IVIS® Lumina II imaging system was used to detect bioluminescent *T. b. brucei* GVR35 parasites in mice to evaluate parasite localization and disease progression. Drug treatment was assessed using qualitative bioluminescence imaging and real-time quantitative PCR (qPCR).

Results: We have shown that drug dose–response can be evaluated using bioluminescence imaging and confirmed quantification of tissue parasite load using qPCR. The model was also able to detect drug relapse earlier than the traditional blood film detection and even in the absence of any detectable peripheral parasites.

Conclusions: We have developed and optimized a new, efficient method to evaluate novel anti-trypanosomal drugs *in vivo* and reduce the current 180 day drug relapse experiment to a 90 day model. The non-invasive *in vivo* imaging model reduces the time required to assess preclinical efficacy of new anti-trypanosomal drugs.

Keywords: *Trypanosoma brucei brucei*, bioluminescence imaging, luciferase

Introduction

Human African trypanosomiasis (HAT), or sleeping sickness, is caused by the vector-borne protozoan parasites *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense,* with the subspecies giving rise to different clinical features and progression of this multi-stage disease. Fewer than 8000 cases were reported in 2013,[1] but almost 70 million people are exposed to the risk of the parasite infection within the African continent every year.[2,3]

The disease manifests in two stages: (i) an early stage, which is often referred to as the haemolymphatic stage, with parasites present in the blood, lymph and peripheral tissue; and (ii) a later second stage that follows invasion of the CNS. Early and late stages are treated by different anti-trypanosomal drugs.[4] Patients typically present with late-stage HAT due to the non-specific nature of early-stage symptoms. However, chemotherapeutic intervention at the late stage is not straightforward, being limited by the requirement of hospitalization for parenteral dosing and inherent drug toxicity.[5]

Drug development has been slow, partially due to the length of time that is involved in *in vivo* testing. Two new compounds are in clinical trials (fexinidazole and SCYX-7158). Currently the mouse models involved in HAT drug development are either for the early stage of the infection, using the monomorphic line *Trypanosoma brucei brucei* Lister 427 (or a derivative), or for the late stage, using the gold standard pleiomorphic strain *T. b. brucei* GVR35.[6] GVR35 is commonly used to study CNS infection and assess drug therapies for the late stage of the disease, as these trypanosomes cross the blood–brain barrier and limit outbred mice to a survival time of 30 days post-infection. The standard drug relapse method follows a 180 day infection protocol.[7]

Bioluminescence imaging can provide highly sensitive, non-invasive detection of parasite distribution in a mouse model that can be followed in a single animal for the entirety of the experiment and removes the need for extra animal groups for each timepoint. Parasites labelled with bioluminescent reporters that emit light in the blue and green parts of the spectrum have encountered problems with high levels of tissue absorption, resulting in poor signal detection in deep tissue.[8] By using red-shifted proteins, an improved level of signal can be detected as light absorption by tissues is minimized, therefore providing more sensitive parasite detection than traditional blood films.[9,10]

An initial bioluminescent model using green light-emitting luciferase expressed in GVR35 was developed to study HAT in a mouse

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model.[10] Further to the use of GVR35 LUC2, a red-shifted bioluminescent *T. b. brucei* strain, GVR35 VSL-2, was developed.[9] This strain was shown to have a bioluminescence 1000-fold higher than the GVR35 LUC2 strain, with a much higher sensitivity that allows detection of ≤100 trypanosomes *in vivo.*[9]

We describe here a highly sensitive bioluminescence model, utilizing the GVR35 VSL-2 strain to evaluate trypanocidal compounds and to determine drug relapse earlier than the current 180 day gold-standard model. In addition, this system reduces the number of animals required in comparison with the WT model and is able to evaluate and give a dose–response on drugs by 35 days post-infection. Overall, use of this improved system shortens experiments from 180 to 90 days, which could significantly enhance drug development for HAT.

Methods

Animals and ethics

Female CD1 mice weighing 20–25 g (Charles River, Margate, UK) were housed in groups of up to five animals in specific pathogen-free individually vented cages, and were fed *ad libitum*. All work was carried out under the approval of the UK Home Office Animals (Scientific Procedures) Act 1986 and the London School of Hygiene & Tropical Medicine Ethics Committee. ARRIVE guidelines are followed in this report.[11]

Parasites

The strains used were WT *T. b. brucei* GVR35 and the transfected *T. b. brucei* strains GVR35 VSL-2 and GVR35 LUC2.[9,10] The WT produces a chronic infection in mice,[12] and was used to validate the bioluminescent transfected strain.

Infection

Mice were infected as per Kennedy *et al*.[6] with minor modifications. Trypanosomes (5×10^3/mL) from a donor mouse were diluted in phosphate-buffered glucose saline, pH 8.0, as described[7] and injected into mice by the intravenous route. The mice were randomized into cages of five animals. For neuropathological evaluation, mice were infected by intraperitoneal injection of 3×10^4 *T. b. brucei* GVR35 WT, LUC2 or VSL-2 strains.

At the end of an experiment, blood was collected via cardiac puncture and mixed with a chaotropic salt (guanidine HCl) in a 50/50 solution and stored at 4°C for DNA extraction and real-time quantitative PCR (qPCR). For *ex vivo* imaging and qPCR analysis of the brains, mice were perfused with PBS to remove blood, and the brains were snap frozen on dry ice and stored at −80°C for later DNA extraction and qPCR.

In vivo bioluminescence imaging

Infected mice were monitored every 7 days via blood microscopy and by whole-animal imaging (all VSL-2 mice and a control group of WT mice). Mice were injected intraperitoneally with the luciferase substrate D-luciferin at 150 mg/kg (Perkin Elmer, Beaconsfield, UK; diluted in Dulbecco's PBS) and imaged using a standard set of exposure times in an IVIS® Lumina II (Perkin Elmer) under anaesthesia (2% isoflurane/O_2 mix at 2.5 L/min). All images were acquired using exposure times of 1, 3, 10, 30, 60 and 180 s, with medium binning, 1 f/stop and an open filter, and field of view E (12.5×12.5 cm). Bioluminescence was quantified with Living Image® v.4.2 software (Perkin Elmer) and corrected for background bioluminescence.

To confirm CNS infection, *ex vivo* imaging of perfused brains was performed. Prior to imaging, 50 μL of 15 mg/mL luciferin was pipetted onto the brain and imaged using the settings as above.

qPCR to determine parasite load

qPCR was used to determine parasite load in cardiac blood and perfused brain material, using a primer sequence to target the invariant surface glycoprotein (ISG-75).

DNA was extracted from whole-brain homogenate and cardiac blood using Roche High Pure PCR template according to the manufacturer's instructions (Roche, Burgess Hill, UK) and quantified by Nanodrop (ThermoFisher Scientific, Loughborough, UK).

Standards for the gene ISG-75 were generated by conventional PCR from *T. b. brucei* GVR35-derived cDNA, followed by purification using the QIAquick PCR Purification Kit (Qiagen, Crawley, UK). Copy numbers were calculated based on the fragment length, and standards were used in subsequent assays to generate standard curves ranging from 10^8 to 10 copies/reaction. The levels of ISG-75 in the indicated samples were measured by qPCR using SYBR® Green (Applied Biosystems by Life Technologies, Paisley, UK) incorporation in an ABI Prism 7000 sequence detection system (Applied Biosystems) relative to the standard curve. Each reaction was performed in duplicate with 4 μL of DNA, 10 μL of SYBR® Green master mix, 0.2 μL of each primer (final concentration 200 nM) and 5.6 μL of water, to produce a reaction volume of 10 μL. PCR conditions were a 10 min 95°C denaturation step followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 1 s.

Primer sequences were as follows: ISG forward, 5′-TTGCTGATAAA GTTGCAGAGGA-3′; and ISG reverse, 5′-CAACTCGAACTCTATATAACCAGCA-3′.

Neuropathological evaluation

The severity of the pathological reaction within the brain of mice infected with GVR35 WT was evaluated and compared with that in the brain of mice infected with the VSL-2 strain as well as the recently described LUC2 strain.[10] This was carried out to ensure that there were no overt differences in neuropathogenesis between the genetically modified strains and the WT parasite. The mice were euthanized at 7, 14, 21 and 28 days post-infection and the brains were extracted and fixed in 4% neutral buffered formalin. The tissue was then embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. The severity of the reaction was assessed by two independent assessors in a blinded fashion using a previously defined grading scale.[6]

In vivo drug efficacy and relapse evaluation

Mice were infected as described above. A CNS control group treated with analytical grade diminazene aceturate (DA) (Sigma-Aldrich, Poole, UK) at 40 mg/kg intraperitoneally, which clears peripheral parasitaemia, and a negative control group of untreated infected mice was included for both strains. Mice were randomized into groups of five with a single group per drug/dose.

Melarsoprol is difficult to dissolve for administration; therefore, with advice from Pharmidex (Stevenage, UK), we used an intravenous formulation consisting of 10% (v/v) 1,3-dimethyl-2-imidazolidinone (Sigma-Aldrich), 10% (v/v) 1-methyl-2-pyrrolidinone (Sigma-Aldrich), 35% (v/v) propylene glycol (Sigma-Aldrich) and 45% (v/v) 50 mM glycine/HCl (Sigma-Aldrich), which was then diluted to 10% (v/v) in 5% dextrose solution. Melarsoprol (Sanofi, Paris, France) was diluted in this intravenous formulation and administered at four dose–response concentrations (10, 6, 3 and 1 mg/kg) over a 4 day period from day (D) 21 to D24 inclusive. Mice were monitored via imaging and blood films (from the tail vein) every 4–5 days until D35. At D35 mice were culled and qPCR analysis was carried out on blood and brain samples to determine ED_{50} and ED_{90} values.

To assess relapse, two doses (10 mg/kg and 6 mg/kg) from the drug efficacy experiment were used and, following the same protocol as described above, mice were infected and dosed. Mice were imaged and blood films (from the tail vein) taken every 7 days from D35 to D180. Mice were culled when humane endpoints (weight loss >10% total body weight and hind-leg paralysis) of the experiment were reached.

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Results

Validation of bioluminescence model

To ensure that the VSL-2 model exhibits the same kinetics of infection as the WT GVR35, a direct comparison between the two strains was carried out using the standard 35 day study as described by Kennedy.[13]

At D7 post-infection the bioluminescence signal was patchy throughout the mouse, but resided predominantly in the lower back region and lymph nodes (Figure 1a). The trypomastigotes continued to multiply and spread throughout the blood and lymphatic system and on D14 the signal was observed throughout the animal. By D21 a similar signal distribution was seen, although the bioluminescence was much higher, peaking at 1×10^8 photons/s. From the heat-map scale it can be seen that the liver/spleen area displays the highest bioluminescence (Figure 1a), providing further evidence that the lymphatic system is heavily infected.

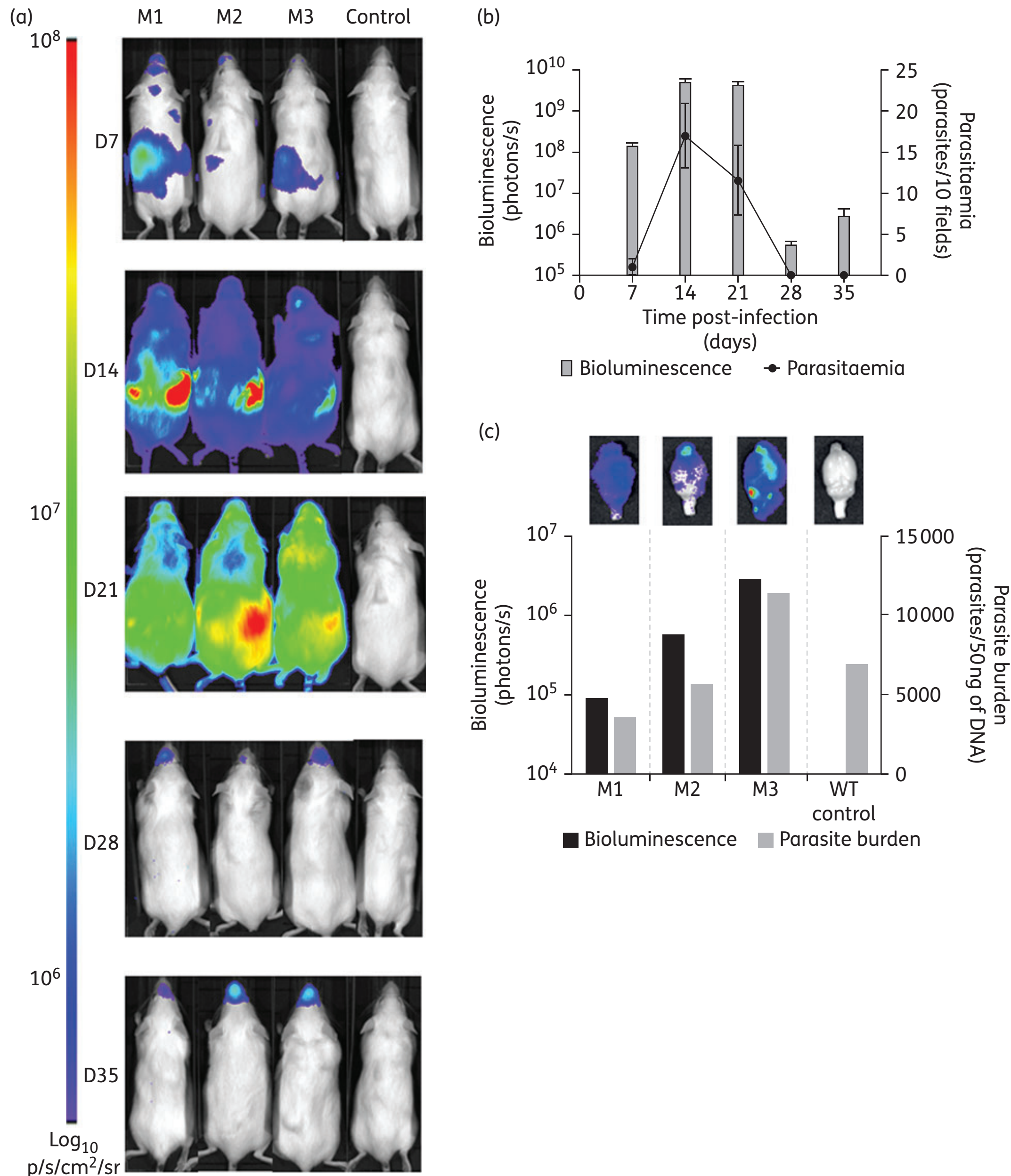

Figure 1. *In vivo* validation of *T. b. brucei* GVR35 VSL-2. Six CD1 mice were infected with VSL-2 via the intravenous route and treated with DA at D21. They were monitored by imaging and blood film acquisition every 7 days until D35 post-infection. (a) Three representative animals of six (M1, M2 and M3) imaged at the indicated timepoints compared with a mouse infected with WT GVR35, showing bioluminescence on a heat-map scale (total flux) where red is an area of high flux, i.e. high bioluminescence activity. (b) Quantification of average total flux and parasite number from the six mice in (a). Each data point is the mean ± SD of the bioluminescence analysis and microscopic counts. (c) Mice from (a) were perfused and brains excised and imaged. Excised brains were homogenized and extracted DNA was subjected to qPCR, and compared with the bioluminescence.

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Following treatment with DA at D21, overall bioluminescence in infected animals imaged at D28 was greatly reduced, with a focal signal found only in the head region, as the clearance of peripheral parasitaemia revealed possible CNS infection. By D35, the bioluminescence signal in the head region became more intense, as the cranial concentration of parasites appeared to increase, with a 10-fold increase in flux between D28 and D35. The control WT mouse showed no bioluminescence.

Quantification of total flux revealed that as the infection progressed the bioluminescence increased from 10^8 to just under 10^{10} total flux until DA treatment was administered (Figure 1b). In comparison, parasitaemia was not detectable via microscopy until D14 and then was undetectable following treatment with DA (Figure 1b). It remained undetectable until the end of the experiment at D35 despite the bioluminescence signal being clearly apparent in the head.

To confirm the presence of trypanosomes in brain tissue following whole-animal imaging at D35, the mice were perfused to clear the tissue of blood, and brains were excised and imaged *ex vivo* (Figure 1c). Bioluminescence was detectable in all VSL-2-infected mouse brains and was comparable to the signal seen in the head region of the respective intact mice at D35 (Figure 1a). In the brain of Mouse 2 the signal was most prominent in the olfactory bulb of the brain, whereas in Mice 1 and 3 the signal non-specifically disseminated through the entire brain, with Mouse 3 showing a high signal (red 'hot spot') in the cerebellum. The remainder of the infected group showed a similarly non-specific distribution of bioluminescence in the brain (not shown).

Following *ex vivo* imaging, DNA was prepared from the perfused brains and qPCR was used to determine the parasite load in each brain (Figure 1c). Comparing qPCR with the bioluminescence signal, the total flux was representative of the number of trypanosomes present. The brain of Mouse 3 had areas of higher flux and therefore had an overall higher quantity of trypanosomes present. However, in the brains of Mice 1 and 2, despite showing differences in the distribution of parasites, a trend could be identified between parasite burden as determined by qPCR and bioluminescence. The control WT-infected brain had no bioluminescence but high parasitaemia.

To determine whether parasite transfection had interfered with invasion and neuropathological effects in the brain,[6] a well-established neuropathology grading scale was used to assess the severity of the neuropathological response over the course of infection of 28 days (Figure 2). VSL-2 was compared with the current bioluminescence model LUC2 and with WT. The results showed that there was no significant difference between the neuropathological response to infection for the VSL-2 and WT strains.

Drug efficacy of melarsoprol in the treatment of *T. b. brucei GVR35 VSL-2*

To extend the potential of a drug evaluation model, the ability to determine dose–response effect, as well as relapse, could provide improved knowledge of drug efficacy. Infected mice were treated with melarsoprol at various doses as indicated to provide a dose–response. Bioluminescence and parasitaemia were measured at D21, D24, D28, D30 and D35 (Figure 3). The bioluminescence and parasitaemia fell (Figure 3b) immediately after treatment (D24 post-infection). Mice treated with 1 mg/kg melarsoprol demonstrated an immediate clearance after treatment, but by D30 the bioluminescence signal was detectable and was focused to the head and spinal region of the mouse (Figure 3a). The bioluminescence continued to increase and at D35 reached 100-fold greater than background (as determined by the average bioluminescence obtained from WT mice) at 10^8 photons/s and was

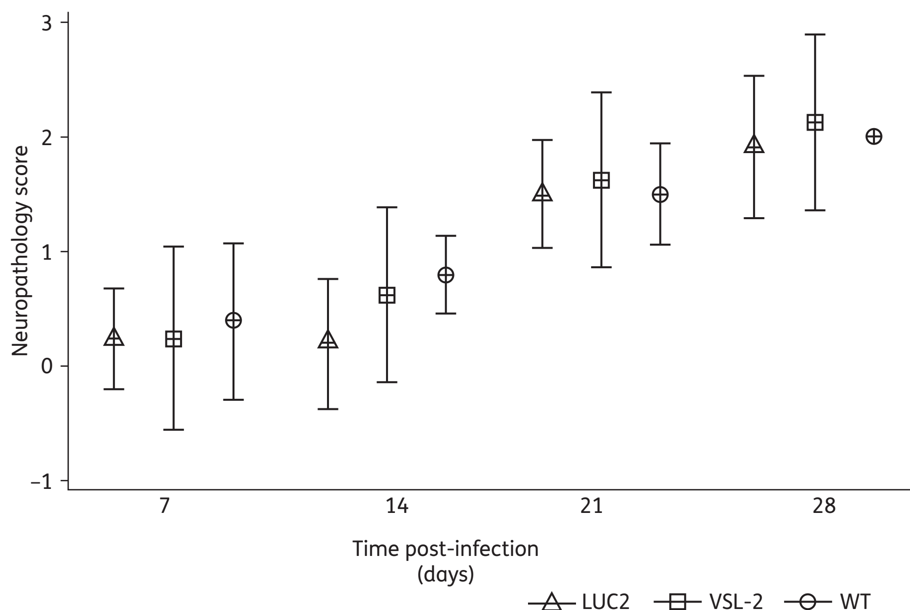

Figure 2. Comparison of the neuropathological effect in bioluminescent strains LUC2 and VSL-2 with the effect in WT. Using the standard neuropathological scoring method,[6] groups of 11 or 12 brains were stained with haemolysin and eosin, and sections were evaluated and scored. The graph shows the mean and 95% CI for each group. A Student's *t*-test showed that there is no significant difference between the strains at all timepoints; $P > 0.05$.

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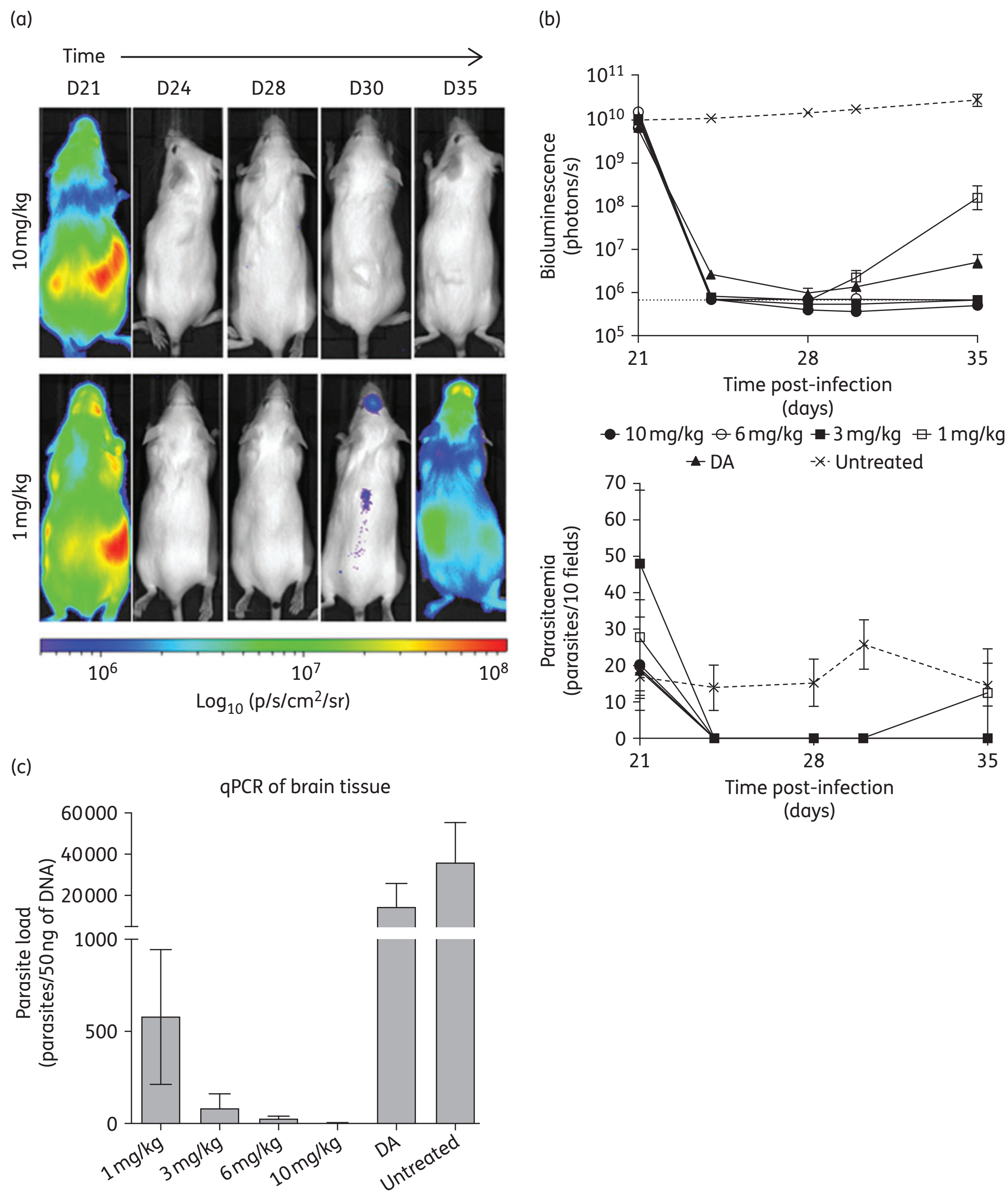

Figure 3. Determining melarsoprol drug efficacy using bioluminescence and qPCR. Infected CD1 mice were split into groups of six and treated with various doses of melarsoprol from D21 and monitored until D35. (a) A single representative mouse (one mouse from the group of six) treated with the highest dose (10 mg/kg) and the lowest dose (1 mg/kg), showing bioluminescence on a heat-map scale (red representing maximum flux) before and after treatment. (b) Average (of six mice) quantification of total flux and parasite number. Each data point is the mean ± SD of the bioluminescence analysis and microscopic counts. Background bioluminescence is indicated by the horizontal broken line. (c) Brain homogenates from each of the mice from (b) were extracted for DNA and analysed using qPCR. The graph shows the mean ± SD for six mice of the number of parasites in 50 ng of DNA.

disseminated throughout the entire animal. Although bioluminescence was detectable from D30 in the low-dose melarsoprol treatment, peripheral parasitaemia could only be detected in these mice when the infection re-disseminated throughout the animals at D35 (Figure 3a and b).

A single 40 mg/kg dose of DA was followed by an initial drop in bioluminescence, with signal limited to the head region (as shown in Figure 1a), before increasing over the remaining 11 days (Figure 3b). Unlike what was seen in the low-dose melarsoprol-treated mice, peripheral parasitaemia was undetectable in blood films, suggesting the signal originated from organs or the lymphatic system. No recovery of bioluminescence was seen in mice treated at higher doses of melarsoprol within this time course.

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At D35 mouse brains were removed and qPCR analysis was carried out to determine the quantity of trypanosomes present after drug treatment. The detection limit of the qPCR was 50 trypanosomes/50 ng of DNA. A clear dose–response effect was observed (Figure 3c), with the increasing dose of melarsoprol reducing the number of trypanosomes present to below the detection limit. Mice treated with DA showed a high number of trypanosomes in the brain, although with a reduction in parasite load compared with the untreated control, further confirming that DA was unable to clear parasites in the CNS to a curative level. The qPCR data of melarsoprol-treated brains correlated with the whole-animal and *ex vivo* imaging data, and provided evidence that bioluminescence can be used to determine the dose–response effect.

Non-invasive monitoring of drug relapse

To investigate the potential of the VSL-2 model in the study of drug relapse, mice infected with GVR35 VSL-2 were treated with melarsoprol at curative (10 mg/kg) and non-curative (6 mg/kg) doses administered intravenously. The bioluminescence and parasitaemia for each individual mouse were then followed through a 180 day study (Figure 4).

Parasitaemia for the mice dosed with 6 mg/kg (Figure 4a) was undetectable prior to D63 in any animal, at which point low numbers of parasites were detectable in the blood of four of the five animals, after which parasitaemia increased. Mouse 5 remained aparasitaemic (according to blood films) during the 119 days, showing few symptoms other than splenomegaly before being culled. In comparison, the bioluminescence data for this group of mice appeared very different. The signal was detected as early as D49 for three of the five mice, and by D63 the signal was observed in the remaining mice. Unlike the parasitaemia data for the same mice, the bioluminescence fluctuated during the 56 day period prior to the termination of the group at D119. Therefore, despite there being no detectable peripheral parasitaemia for Mouse 5, bioluminescence was still detected in all mice in the 6 mg/kg group.

Immediately after treatment with 10 mg/kg melarsoprol, blood films indicated that the parasitaemia had cleared in all mice. Four of the five mice remained negative throughout the 180 days, but Mouse 2 had parasitaemia at D91 of 14 trypanosomes/10 fields of view before decreasing and then reappearing again at D112 before being culled at D119 as the mouse became lethargic (Figure 4b). In contrast, the bioluminescence data for

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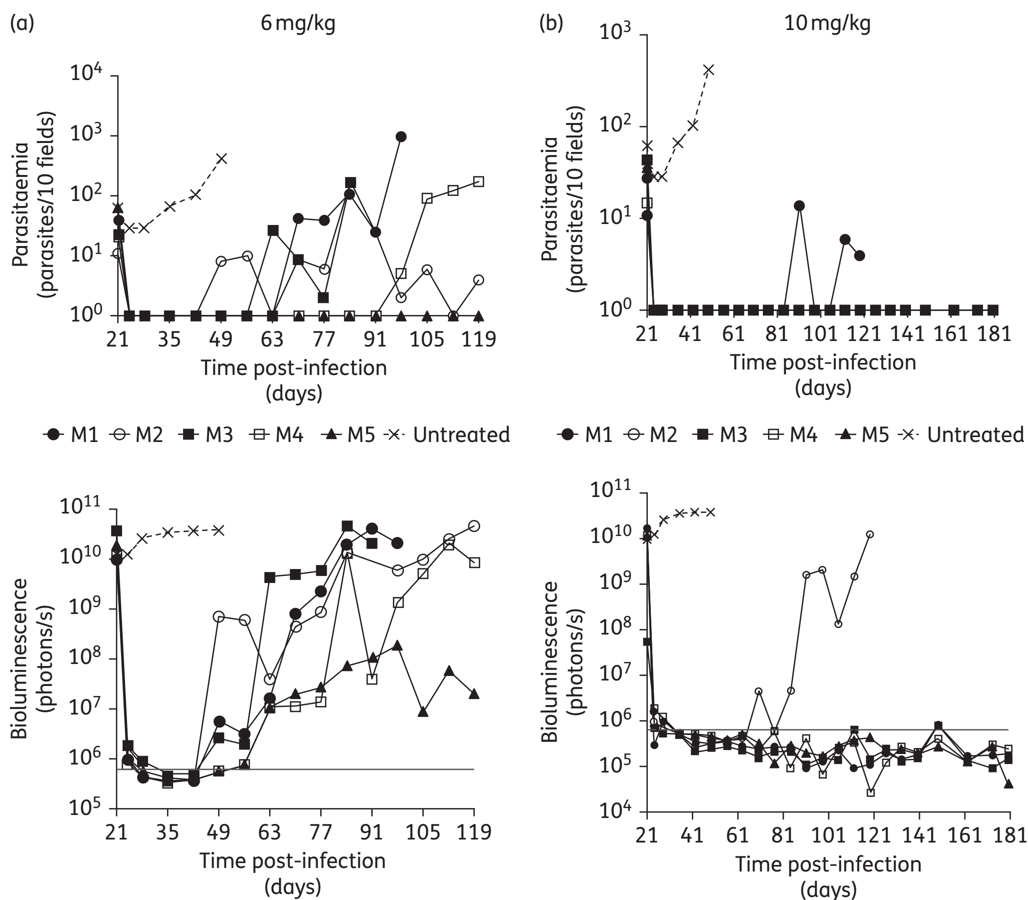

Figure 4. Early detection of drug relapse of melarsoprol using VSL-2. Mice infected with VSL-2 via the intravenous route were imaged and blood filmed at weekly intervals after melarsoprol dosing. Graphs show the bioluminescence and peripheral parasitaemia from a group of five mice (M1, M2, M3, M4 and M5) dosed with (a) 6 mg/kg melarsoprol and (b) 10 mg/kg melarsoprol. Values of zero were manually altered to 1 in the parasitaemia counts to enable plotting on a logarithmic scale. Background bioluminescence is indicated by the horizontal line.

10 mg/kg shows that relapse occurred in Mouse 2 much earlier, at D70 (Figure 4b), with a total flux of 4.64×10^6 photons/s (10-fold increase on the background) before falling below background detection, and then following a pattern of decreasing and increasing bioluminescence before a spike at D119 of 1.27×10^{10} photons/s, when the mouse reached its humane endpoint. The bioluminescence approach was therefore able to detect drug relapse much earlier than traditional blood film.

Discussion

The current preclinical drug evaluation model requires mice to be monitored for up to 180 days post-infection, with relapse being detected when parasites re-establish an infection in the periphery, detectable in blood films.[6] This model is unable to detect viable parasites in other organs, such as the brain, without mice being sacrificed and the tissues removed for further analysis. The experimental time period of 6 months is not appropriate for drug development and a new innovative approach is required to speed up the process and provide data on time-to-kill and differential effects on parasite distribution. At present there is no current model for assessing dose–response drug efficacy, as it is not possible to determine the level of parasite persistence within tissues using contemporary techniques.

Bioluminescent model systems utilizing luciferase have been described for the assessment of drugs for other diseases and infections.[14–16] The progress made with both the LUC2 model and development of the red-shifted VSL-2 strain has paved the way for a new mouse model of HAT that is both highly sensitive and non-invasive. Preliminary studies on GVR35 VSL-2 showed that the model produces similar infection to the WT strain and that bioluminescence can be detected in the brain by both whole-animal and *ex vivo* imaging.[9]

VSL-2 provides higher imaging sensitivity than previous transfected trypanosome strains,[9,10] and it is this that enables a dose–response effect to be detected in whole-mouse imaging after treatment with melarsoprol. While the LUC2 model provided a substantial improvement on the currently used GVR35 WT model,[10] the greater sensitivity and detection limit of the VSL-2 model as determined in this study allows bioluminescence to be used to determine a dose–response effect, and analysis of excised brains using PCR corroborates this. Furthermore, the intravenous infection route provides a tightly reproducible infection determined by bioluminescence detection, even in the outbred mouse line used. A shortened experimental time of 35 days is sufficient to confirm anti-trypanosomal activity on stage 2 HAT by loss of bioluminescence in the brain, something that has been difficult to determine *in vivo* before now.

The greater sensitivity of the bioluminescence approach is demonstrated further in the analysis of drug relapse. By D60 there is a clear indication of subcurative clearance (in this case 6 mg/kg) and by 90 days relapse is evident, resulting in the reduction of experimental time by 50%. The model also provides an option to extend the time required to follow the animals with minimal invasion due to the non-invasive nature of bioluminescence imaging. Although 10 mg/kg appeared as a curative dose in four of the five mice through measurement of peripheral parasitaemia and bioluminescence, in one mouse (Mouse 2) it was clear from the bioluminescence that parasites could persist long after treatment with increasing flux around D60 and then again at D81–D112, suggesting relapse. Early studies on melarsoprol also showed that the regimen of 10 mg/kg×3 days intravenously gave no relapse in peripheral parasitaemia detected by blood films, but two-thirds of the mice in the study died.[17] Poltera *et al.*[17] concluded that melarsoprol was curative as no parasites were found in the circulating blood or brain tissue in all of the mice, as determined by histopathological study and immunofluorescence. The data we have shown here indicate that peripheral parasitaemia was not detected but that bioluminescence re-emerged after treatment in one of the five mice through the 180 day experiment, lending further evidence that the lymphatic system is involved in parasite invasion and also raising the question of how ‘cure’ can be defined in the mouse model.

In summary, we have presented evidence here that the red-shifted bioluminescent GVR35 VSL-2 model has the capability of providing a far more sensitive drug assessment system by non-invasive imaging, with the ability to reduce initial screening time by 120 days. In addition to this, the high sensitivity of the imaging, combined with quantitative methods of qPCR, has allowed a new methodology to be developed that can assess drug efficacy in terms of ED_{50} and ED_{90} values of novel drugs for treatment of late-stage HAT. The overall reduction in drug evaluation time and the more comprehensive data that can now be determined for preclinical drugs will speed up drug development and aid the ongoing progress to HAT elimination.

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Transparency declarations

None to declare.

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