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RESEARCH ARTICLE

A simple, robust, and affordable bioluminescent assay for drug discovery against infective African trypanosomes

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Abstract

African trypanosomiasis is a major problem for human and animal health in endemic countries, where it threatens millions of people and affects economic development. New drugs are needed to overcome the toxicity, administration, low efficacy, and resistance issues of the current chemotherapy. Robust, simple, and economical high-throughput, whole-cell-based assays are required to accelerate the identification of novel chemical entities. With this aim, we generated a bioluminescent cell line of the bloodstream stage of *Trypanosoma brucei brucei* and established a screening assay. Trypanosomes were stably transfected to constitutively express a thermostable red-shifted luciferase. The growth phenotype and drug sensitivity of the reporter cell line were essentially identical to that of the parental cell line. The endogenous luciferase activity, measured by a simple bioluminescence assay, proved to be proportional to parasite number and metabolic status. The assay, optimized to detect highly potent compounds in a 96-well-plate format, was validated by screening a small compound library (inter-assay values for Z' factor and coefficient variation were 0.77 and 5.8%, respectively). With a hit-confirmation ratio of ~97%, the assay was potent enough to identify several hits with $EC_{50} \leq 10$ μM. Preliminary tests indicated that the assay can be scaled up to a 384-well-plate format without compromising its robustness. In summary, we have generated reporter trypanosomes and a simple, robust, and affordable bioluminescence screening assay with great potential to speed up the early-phase drug discovery against African trypanosomes.

KEYWORDS

African trypanosomiasis, high-throughput screening, luciferase, *Trypanosoma brucei*, genetically encoded biosensor

1 | INTRODUCTION

Human African trypanosomiasis (HAT) is endemic in 36 sub-Saharan countries, where about 70 million people are at risk of infection. *Trypanosoma brucei gambiense* and *T. brucei rhodesiense* cause sleeping sickness, a disease that initially develops as a hematolymphatic infection and may further progress to a meningoencephalitic infection. The duration of the first stage varies from a few weeks in *T. b. rhodesiense* to months or years in *T. b. gambiense* (Simarro et al., 2012). The disease is fatal if left untreated.

Trypanosoma brucei brucei, *Trypanosoma vivax*, and *Trypanosoma congolense* are the etiological agents of animal African trypanosomiasis or Nagana cattle disease, which remains a major obstacle for the nutritional and economic development of the rural communities from endemic areas (Shaw, Cecchi, Wint, Mattioli, & Robinson, 2014). The economic losses due to cattle death or productive impairment have been estimated in several US$ billions per year.

Vaccine development is hampered by the successful mechanisms employed by the pathogens to evade the host immune response

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(Stijlemans et al., 2016); hence, chemotherapy is the only effective choice to fight these diseases.

The early stage of HAT is treated with pentamidine (a large aromatic diamidine) and suramin (a high molecular weight polysulfonated naphthylurea). Both drugs are delivered by intramuscular injection and their remarkable side effects account for the high rates of treatment discontinuation, in particular for suramin.

Only melarsoprol (melaminophenyl arsenic derivative), nifurtimox (nitrofuran derivative), and eflornithine (α-difluoromethylornithine) are efficacious against the second stage of the disease produced by *T. b. gambiense*. Despite its high toxicity (5% patients die during treatment), melarsoprol is still used and, for decades, has been the only choice for treating the second stage of HAT caused by *T. b. rhodesiense*.

In 2009, the coadministration of eflornithine and nifurtimox was accepted for the treatment of the second stage HAT caused by *T. b. gambiense*. More recently (2018), fexinidazole (a nitroimidazole) was approved for the treatment of both stages of HAT caused by *T. b. gambiense* (Mesu et al., 2018). Unfortunately, African trypanosomes have been shown to be capable of developing cross-resistance against nitroheterocyclic compounds such as nifurtimox and fexinidazole (Sokolova et al., 2010).

To sum up, affordable new drugs with improved pharmacological profile (safety, effectiveness, capacity to cross the blood brain barrier) are needed to treat HAT.

The main approach to find new drugs relies on the phenotypic screening against whole target cells. The bloodstream form of *T. brucei brucei*, which is nonpathogenic to human and, from the genetic point of view, almost identical or similar to *T. b. rhodesiense* and *T. b. gambiense*, respectively, is the preferred model for *in vitro* and *in vivo* drug screening. Different high-throughput methods to assess *T. brucei* viability have been established and applied to screen compounds libraries. The assays are based on the determination of the ATP (Mackey et al., 2006; Sykes & Avery, 2009a), nucleic acid content (Faria et al., 2015; Gould, Vu, Seebeck, & de Koning, 2008) or the metabolic state of the cells (Sykes et al., 2012; Sykes & Avery, 2009b), and use luminescent or fluorometric/colorimetric reagents such as: luciferase/D-Luciferine (CellTiter-Glo®, EnduRen™, and ONE-Glo™ from Promega), SYBR Green and propidium iodide or Alamar Blue™, respectively. All these assays have different limitations, such as long incubation times, accuracy, or costs.

Different strains and subspecies of African trypanosomes have been genetically modified to express luciferase and employed to study *in vivo* and/or *in vitro* parasite pathogenesis and drug efficacy (Burrell-Saward, Rodgers, Bradley, Croft, & Ward, 2015; McLatchie et al., 2013; Myburgh et al., 2013; Van Reet, Pyana, Rogé, Claes, & Büscher, 2013; Van Reet, Van de Vyver, Pyana, Van der Linden, & Büscher, 2014). However, none of these cell lines was used to establish and run a high-throughput amenable, and affordable bioluminescence screening assay.

With this goal in mind, we have engineered a bloodstream *T. b. brucei* to constitutively express a red-shifted firefly luciferase (Branchini et al., 2010) and developed a simple and robust 96-well-plate HTS phenotypic assay. The assay was employed successfully for the screening of a small but structurally diverse compound library (alkaloids, rare sugars, peptides, polyheterocyles, quinones, selenosugars, aromatic linker derivatives, and singletons). The assay has the potential to be scaled up to a 384-well-plate format while the transgenic cell line, specially adapted for tetracycline(tet)-inducible expression systems (e.g., gene RNAi silencing or overexpression), may have applications for therapeutic efficacy and drug target validation studies based on the *in vivo* imaging technology.

2 | METHODS

2.1 | Design and preparation of construct pHD1034_VSG-RE9HLUC-TUBULIN

The vector (pTb-AMLuc) containing the codon-optimized gene of the red-shifted *Photinus pyralis luciferase* (PpyRE9H or RE9HLUC; (Branchini et al., 2010) flanked upstream by a variant surface glycoprotein (VSG) 5′-untranslated region (UTR) and downstream by a tubulin 3´-UTR (VSG-RE9HLUC-TUBULIN) (McLatchie et al., 2013) was a kind gift of Dr. John Kelly (London School of Hygiene and Tropical Medicine, London, UK). The vector selected for the constitutive expression of the red-shifted luciferase in *T. brucei* was pHD1034, which harbors a puromycin resistance gene, drives the constitutive expression of the transgene from a ribosomal DNA (rDNA) promoter, and integrates into the nontranscribed RRNA spacer, a multicopy loci of the *T. brucei* genome (Quijada et al., 2002). Expression of the transgene is mediated by the endogenous RNA Polymerase I (Zomerdijk, Kieft, & Borst, 1991). Cloning of the VSG-RE9HLUC-TUBULIN fragment (2,180 bp) into the vector pHD1034 was ordered to GenScript® and the new construct was named pHD1034_LUC.

2.2 | Parasite culture and transfection

The bloodstream form of the monomorphic *T. b. brucei* strain *427*, cell line 514–1313 (parental cell line) (Alibu, Storm, Haile, Clayton, & Horn, 2005), was cultured *in vitro* with HMI-9 medium (Hirumi & Hirumi, 1989), supplemented with 10% fetal bovine serum, 10 U/ml penicillin (Gibco), 10 μg/ml streptomycin (Gibco), 0.2 μg/ml bleomycin (Jena Bioscience), and 4 μg/ml G418 disulfate salt (Sigma-Aldrich), inside a humidified incubator (Thermo Fisher Scientific) with controlled temperature (37 °C) and CO_2 (5%) in 25 or 75 cm^2 vented-cap culture-flask. This cell line expresses constitutively the tetracycline repressor protein (bleomycin resistance) and a T7-RNA polymerase (G418 resistance), which allows the use of tet-inducible vectors for the down- (RNA interference; Comini et al., 2004) or up- (overexpression, dominant negative; Manta et al., 2013) regulation of target genes.

In a total volume of 100 μl, 15 μg of DNA from pHD1034_LUC was linearized with NotI-HF® (1 U/μl), in Cut Smart® Buffer (New England Biolabs) at 37 °C during 14–18 hr. Thereafter, DNA was precipitated with sodium acetate (Applichem Panreac; 0.3 M final concentration) and isopropanol (0.7 volumes) and resuspended in 5 μl of water. For all molecular biology procedures UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen) was used. Parasites (3×10^7)

in the exponential growth phase were resuspended in 100 μl of Basic Parasite Nucleofector™ Kit1 solution (Lonza) and electroporated with an Amaxa™ Nucleofector™ II (Lonza), program X-001 after DNA addition. The selection of transgenic clones was achieved by growing parasites in the presence of 0.8 μg/ml puromycin (Invitrogen). The new transgenic cell line was called LUC.

2.3 | Counting of viable cells

The number of viable parasites in cultures from the parental and LUC cell lines was assessed by counting cells under the light microscope using a Neubauer chamber or by flow cytometry in a CyAn™ADP (DakoCytomation), according to the protocol described by Maiwald et al. (2014)).

2.4 | Culture synchronization

In order to obtain a more homogenous population of cells in a similar metabolic status and growth stage (log-phase), parasites cultures (from the LUC or parental cell line) were synchronized by at least two consecutive passages every 24 hr at an initial cell density ≤ 3×10^4 parasites/ml in 10 or 50 ml fresh culture medium in 25 or 75 cm^2 vented-cap culture-flask, respectively. Otherwise stated, all experiments were performed with samples from synchronized cultures.

2.5 | Commercial luciferase assay

The Luciferase Assay System (Cat. # E4530, Promega) was used for the preliminary characterization of the LUC cell line. One million cells from a synchronized culture were pelleted and carefully washed twice in Phosphate-Buffered Saline (PBS) glucose 1% wt/vol upon centrifugation (2000*g* for 10 min at room temperature). Different amounts of parasites were lysed by resuspending them in 200 μl of Cell Culture Lysis Reagent diluted 1:5 in distilled water and vortexing during 2 min. After clarification (12,000*g* for 2.5 min at room temperature), 20 μl of the supernatants were transferred per well to a 96-well black plate (Greiner F-BOTTOM, 655209), then 50 μl of Luciferase Assay Reagent were added per well and the plate was incubated inside the luminometer for 2 min at 25 °C. Blank controls corresponded to samples from culture medium alone that were processed as indicated above. The plate was read in a LUMIstar OPTIMA Microplate (BMG LABTECH) luminometer using the following settings: 10 s shaking, 10 s/well acquisition, 0.2 s measurement delay, maximum gain value (4,095 in our instrument), and 25 °C.

2.6 | Bioluminescent assay development and optimization

For the assays described below, stock (15 mg/ml) and working (1.5 or 3 mg/ml) solutions of D-Luciferin (Perkin Elmer, Cat. # 122799) were prepared by dissolving or diluting the reagent in PBS glucose 1% wt/vol.

Parasites from synchronized cultures were diluted into fresh culture medium lacking bleomycin, G418, and puromycin at cell densities ranging from 2.5 to 15×10^4 cells/ml. DMSO (2.2 μl) followed by 220 μl of the corresponding parasites´ suspensions or culture medium alone (blank control) were added per well into a 96-well culture plate. At least three biological replicates were assayed for each condition. The plates were incubated at 37 °C and 5% CO_2 for 24 hr. Thereafter, the samples (~ 220 μl) were transferred to a 96-well black plate and 20 μl of a solution of D-luciferin (3 mg/ml or 1.5 mg/ml) with or without Triton X-100 (0.01 or 0.05% vol/vol) was added. The plate was read in a luminometer by recording luminescence every 5 min for a total of 55 min using the following settings: 10 s shaking, 5 s/well acquisition, 0.2 s measurement delay, maximum gain, and 37 °C.

Whenever required, cell density of viable parasites was determined by flow cytometry as indicated above (Section 2.3). At least triplicate samples were prepared for each condition tested.

For testing assay linearity, parasites from synchronized cultures were harvested, concentrated, and transferred into a 96-well black plate at cell densities ranging from 7×10^6 to 0.11×10^6 cells/ml (twofold serial dilutions; total volume 220 μl/well). Wells containing culture medium alone were used as blank controls. Four biological replicates were tested. Upon addition of 20 μl/well of a D-Luciferin (1.5 mg/ml) and Triton X-100 (0.05% vol/vol) solution, bioluminescence was measured using the device and acquisition settings described above. The limit of detection was calculated as the number of parasites equivalent to a bioluminescence signal of threefold the *SD* corresponding to the blank sample.

For testing the suitability of the bioluminescent assay to be scaled up, 60 μl of a parasite suspension (1×10^6 cells/ml from a synchronized culture) or medium alone were transferred to a 384-well black plate (Greiner 781,900) and 5.5 μl of a solution containing D-Luciferin (3 mg/ml in PBS glucose 1% wt/vol) and Triton (0.05% vol/vol) was added. At least 16 replicates were prepared. Bioluminescence signal was measured with a luminometer as described above.

2.7 | Standardized bioluminescence assay

A suspension of 1×10^5 LUC parasites/ml from a synchronized culture is added (220 μl/well) to a 96-well culture plate containing 2.2 μl/well DMSO (negative control) or drugs/compounds dissolved in DMSO at different concentrations (see next sections). Wells containing culture medium (220 μl) and DMSO (2.2 μl) corresponded to blank controls. At least triplicate samples were tested. The plates were incubated at 37 °C and 5% CO_2 for 24 hr. Next, ~220 μl from each well is transferred to a 96-well black plate and 20 μl of a solution containing D-Luciferin (1.5 mg/ml in PBS glucose 1% wt/vol) and Triton X-100 (0.05% vol/vol) was added. Bioluminescence signal was measured every 8 min for a total of 32 min in a LUMIstar OPTIMA Microplate luminometer using the following settings: 10 s shaking, 5 s/well acquisition, 0.2 s measurement delay, maximum gain, and 37 °C.

2.8 | Drug sensitivity assays

The half maximal effective concentration (EC_{50}) of nifurtimox (Lampit®, Bayer), suramin (Sigma), and diminazene aceturate (DAC, Santa Cruz Biotechnology) was determined for the LUC cell line using the *Standardized bioluminescence assay*. Stock and serial dilutions from each drug were prepared in 100% vol/vol DMSO. For each drug, parasite viability at 2 ×, 1 ×, and 0.5 × EC_{50} values was assessed for both the LUC and the parental cell lines using flow cytometry analysis. The experimental setup was identical to that described for the *Standardized bioluminescence assay* except that instead of determining luminescence, the samples were processed for flow cytometry analysis as indicated in Maiwald et al. (2014). At least three biological replicates were assayed for each sample and control.

2.9 | Screening assay

The screening of a library of 141 compounds was performed according to the *Standardized bioluminescence assay* protocol. The compounds (prepared at 1 mM in 100% vol/vol DMSO) were assayed at a final concentration of 10 μM (1% vol/vol DMSO) and in three biological replicates. According to guidelines from The Drugs for Neglected Diseases initiative (DNDi), an organization leading early discovery strategies for neglected diseases, a hit compound for human African trypanosomiasis must have an $EC_{50} \leq 10$ μM against the bloodstream form of *T. brucei* subspecies (Don & Ioset, 2014). For a subset of hits (i.e., compounds reducing parasite viability to ≤50%) identified by the primary screening (see Section 3.4), a concentration-response analysis for at least seven different concentrations of the compounds was performed applying the same assay protocol. For all experiments, nifurtimox (added at its EC_{50} of 6 μM) and DMSO (1% vol/vol) were included as positive and negative controls, respectively. The EC_{50} values were calculated from the concentration-response curves as indicated below.

2.10 | Data analysis

For the bioluminescence assay, parasite viability was calculated according to the following formula: Viability (%) = $(BL_{cpd} - BL_{blank})/(BL_{neg} - BL_{blank}) \times 100$, where BL refers to the mean of bioluminescence signal corresponding to the tested compound ($_{cpd}$), the blank ($_{blank}$, complete media containing 1% vol/vol DMSO), or the negative control ($_{neg}$, parasites treated with 1% vol/vol DMSO). The coefficient of variation (CV) was calculated from the negative control wells as: $CV = (SD_{neg} / BL_{neg}) \times 100$. The Z´ factor was determined from the formula: $Z' \text{ factor} = [1 - (3 \times SD_{blank} + 3 \times SD_{neg})/(BL_{neg} - BL_{blank})]$ (Zhang, 1999). The signal-to-background (S/B) ratio was estimated as BL_{neg}/BL_{blank}.

EC_{50} values were determined from concentration-response curves fitted to a four-parameter sigmoid equation using the GraphPad Prism software (version 6.0). All errors are expressed as one *SD*.

3 | RESULTS

3.1 | Characterization of luciferase-reporter cell line of monomorphic bloodstream *T. b. brucei*

We decided to generate a luciferase-reporter cell line of the monomorphic bloodstream *T. b. brucei* because it is easy to cultivate *in vitro*, it is safe for humans, it shows a high virulence *in vivo*, and it is a good model of species pathogenic for mammals. As a drawback, the monomorphic parasites are not competent to differentiate to the stage capable to colonize the insect vector. In order to extend the application of the reporter trypanosomes toward gene function studies, we selected a parental cell line of *T. b. brucei* suitable for genetic manipulation by tet-inducible systems depending on endogenous or T7-RNA Pol.

Despite transfections with the vector pTb-AMLuc (McLatchie et al., 2013) yielded parasites resistant to the selection antibiotic, none of the several clones analyzed expressed active luciferase (not shown). The technical or molecular reasons behind this result are unknown and were not investigated. Thus, the sequence encoding the red-shifted luciferase flanked by UTRs that were reported to yield maximal luciferase expression (McLatchie et al., 2013) was inserted downstream the rDNA promoter sequence of the vector pHD1034. The clone showing highest luciferase signal (not shown) was characterized.

First, the stability of the genomic reporter gene integration was evaluated by culturing the LUC clone in the absence of selection antibiotics for 60 days (Figure S1). The removal of the selective pressure increased the growth rate of the parasites (doubling time of 5.5 vs. 7 hr in the absence vs. presence of antibiotics, respectively) but did not affect the level of luciferase activity (Figure S1). The LUC cell line recultured in the presence of antibiotics retained full resistance to them, further confirming the highly stable genomic integration of the reporter DNA-cassette.

Furthermore, the growth phenotype of the LUC trypanosomes was almost identical to that of the parental cell line (Figure 1a), demonstrating that genetic manipulation and expression of the reporter gene did not entail major phenotypic changes. As expected for a protein expressed constitutively and whose activity depends on the level of cellular ATP, luciferase activity showed a good correlation with the growth phase/metabolic status of the transgenic trypanosomes (Figure 1b). For instance, parasites in mid-log phase presented maximal bioluminescence, whereas cells in late-log, stationary, and death phase, all of which undergo energy deprivation, showed a drastic decrease (>70%) in bioluminescence signal. In addition, the bioluminescence of mid-log LUC trypanosomes showed to be proportional to parasite number (Figure 1c).

Taken together, these data demonstrate that LUC trypanosomes can report very reliably parasite viability and number.

3.2 | Bioluminescence assay development

Although highly sensitive luciferase kits are commercially available, they are expensive and some of them have technical drawbacks

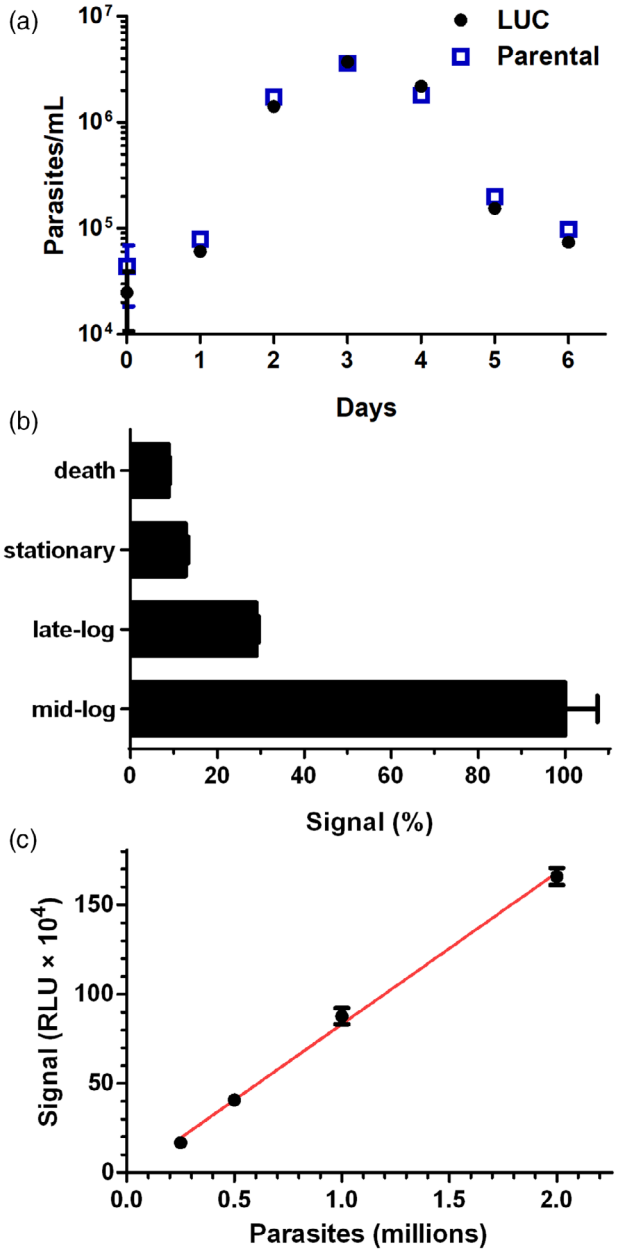

FIGURE 1 Phenotypic characterization of LUC cell line. (a) Growth phenotype. Synchronized bloodstream parasites from the parental (squares) and reporter (LUC; circles) cell lines cultured for 60 days in the absence of selection antibiotics were seeded at 4×10^4 cells/ml (Day 0) in a 96-well culture plate and cell density assessed every 24 hr by flow cytometry. Errors are expressed as one standard deviation. (b) Cell growth phase and bioluminescence. The bioluminescence produced by 1×10^6 LUC parasites grown in culture flask and harvested at different growth phases was measured employing the Luciferase Assay System (Promega). The bioluminescence signal is expressed as percentage relative to the highest signal obtained, which corresponds to samples from mid-log phase parasites, and errors refer to one standard deviation of the mean values. (c) Signal versus parasite number. The bioluminescence signal of different amounts of mid-log phase LUC parasites (synchronized cultures) was measured employing the Luciferase Assay System (Promega). RLU, Relative Light Units. Fitting the full set of data to a linear regression equation yielded an $r^2 = .9975$ (red line). Errors are expressed as one *SD* [Color figure can be viewed at wileyonlinelibrary.com]

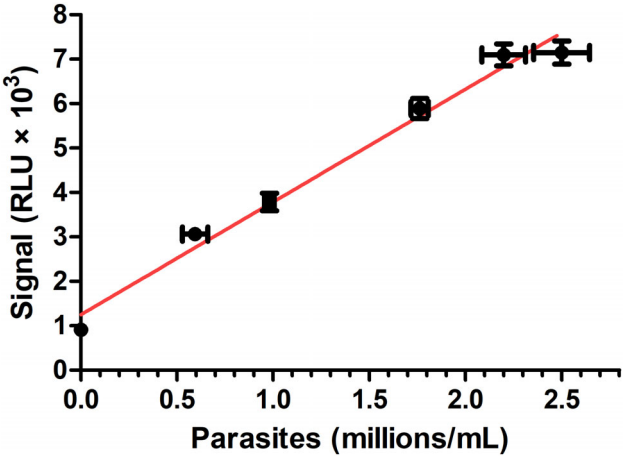

FIGURE 2 Cell growth and bioluminescence signal of LUC cell line in a 96-well plate. Synchronized LUC trypanosomes were seeded at 0.25, 0.5, 1.0, 1.25, 1.5×10^5 cells/ml in a 96-well culture plate and after 24 hr incubation, bioluminescence (250 μg/ml D-Luciferin) and cell density assessed. RLU, relative light units. Fitting the full set of data to a linear regression equation yielded an $r^2 = .984$ (red line). Errors are expressed as one *SD* [Color figure can be viewed at wileyonlinelibrary.com]

(e.g., signal stability, buffer-for-medium exchange). The bioassay was rationalized to test parasite viability upon 24 hr incubation in a 96-well plate and optimized to measure bioluminescence with a protocol that avoids washing steps, uses a homemade reagent, and has a reasonable readout time for HTS.

First, in order to identify the cell density of LUC trypanosomes that yields optimal bioluminescence and assay performance upon 24 hr growth in a 96-well plate, different initial inocula were tested. As shown in Figure 2, a good linearity between signal and cell density was obtained within the range 0.6–2.2 × 10^6 parasites/ml, which corresponds to initial inocula from 0.25 to 1.25×10^5 parasites/ml. This linear correlation is lost for samples from late-log phase parasites (2.5×10^6 cells/ml), which agrees with the results obtained for LUC trypanosomes grown in flasks (Figure 1b) and data reported in the literature (Mackey et al., 2006; Sykes & Avery, 2009a). This finding is relevant for phenotypic assays that assess viability by measuring cell metabolic status, since metabolically stressed cells may bias the results.

The following inocula yielded satisfactory quality parameters: 0.5×10^5 parasites/ml (i.e., S/B = 4.2, Z´ factor = 0.78, CV = 5.2%, signal/cell ratio = 5×10^{-3} RLU/cell), 1.0×10^5 parasites/ml (i.e., S/B = 6.5, Z´ factor = 0.85, CV = 3.8% and signal/cell ratio = 4×10^{-3} RLU/cell), and 1.25×10^5 parasites/ml (i.e., S/B = 7.8, Z´ factor = 0.87, CV = 3.4% and signal/cell ratio = 3×10^{-3} RLU/cell). Based on this analysis, and on the fact that parasites seeded at the latest inoculum are close to enter into late-log phase, we preferred starting the assay with an inoculum of 1.0×10^5 parasites/ml.

In order to further improve the performance of the bioluminescence assay, a time-course analysis of luciferase activity at different concentrations of D-Luciferin and the detergent Triton X-100 was

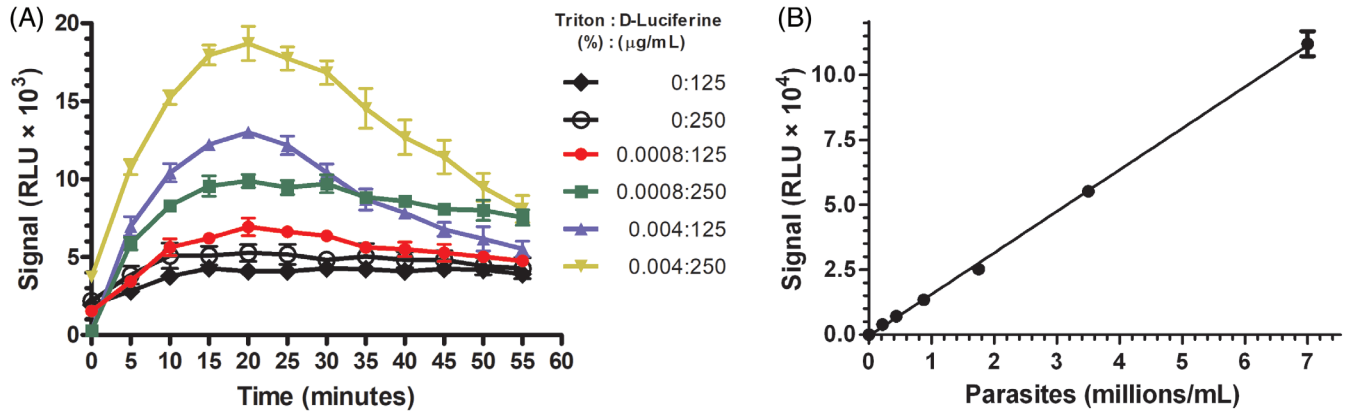

FIGURE 3 Optimization of 96-well bioluminescence assay. (a) Time-course analysis of bioluminescence. The bioluminescence signal produced by ~1.8 × 10^6 parasites/ml was evaluated at different concentrations of D-Luciferin (125 and 250 μg/ml) and of Triton X-100 (0, 0.0008 and 0.004% vol/vol) during a total of 55 min upon addition of reagents. Errors are expressed as one standard deviation. (b) Bioluminescence assay linearity. The bioluminescence signal produced by different amounts of mid-log phase LUC trypanosomes was evaluated at the standard assay conditions (D-Luciferin 125 μg/ml, Triton X-100 0.004% vol/vol, 37 °C for 20 min) Bioluminescence signal is expressed as Relative Light Units (RLU) with errors corresponding to one standard deviation of the mean values. Fitting the full set of data to a linear regression equation yielded r^2 = .999 [Color figure can be viewed at wileyonlinelibrary.com]

performed. In nonpermeabilized cells (black symbols, Figure 3a), doubling luciferin concentration from 125 to 250 μg/ml produced a minor and nonsignificant increase of bioluminescence. In contrast, addition of the detergent led to a significant increase in signal with kinetics and maximum values that were proportional to luciferin and Triton concentrations. For instance, compared to the condition with 250 μg/ml luciferin alone, the combination 250 μg/ml luciferin/0.004% vol/vol Triton increased by 3.5-fold and ≥ 4-fold the signal maximum and increase-rate (estimated for the 0–5 min window time) values, respectively. The kinetic of bioluminescence decay was also accelerated at the highest Triton concentration (0.004% vol/vol), whereas signal remained more stable for mildly permeabilized (0.0008% vol/vol Triton) and nonpermeabilized cells. Nonetheless, in the presence of detergent and independently of the combination tested, bioluminescence attained maximum values (i.e., > 90% signal) between 15 and 25 min upon addition of reagent, with a peak at time point 20 min. For endpoint HTS-based assays, these conditions provide a reasonable time window (10 min) for reliable measurements.

Although the combination 250 μg/ml luciferin/0.004% vol/vol Triton showed higher signal-to-background ratio (S/B = 22) than that calculated for the combination 125 μg/ml luciferin/0.004% vol/vol Triton (S/B = 15), the last one showed excellent average values for Z' factor (0.9) and CV (3.0%) for the 15–25 min time window. On this basis and taking into account that D-Luciferin has a major impact in the assay's costs, the combination D-luciferin 125 μg/ml and Triton 0.004% vol/vol was selected for further assay development.

Under this condition and for mid-log phase trypanosomes, the assay displayed a broad linearity range (from 0.22 to >7 million parasites/ml) and a low limit of detection of about 4 × 10^4 parasites/ml (Figure 3b).

Finally, a preliminary approximation to miniaturize the assay to a 384-well-plate format showed that it is possible as long as the final concentration of D-Luciferin is increased to 250 μg/ml. Under these conditions, the assay presented an acceptable performance with estimated values of S/B = 4.5, Z´ factor = 0.66, and CV = 7.4% (n = 16).

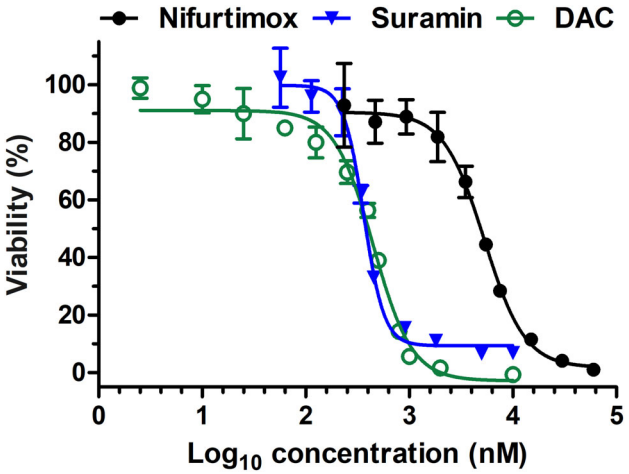

FIGURE 4 Drug sensitivity of LUC trypanosomes. Concentration-response curves obtained for LUC trypanosomes treated with different clinical or veterinary drugs. Parasite viability was assessed using the *Standardized bioluminescence assay* (see Materials and Methods). The EC_{50} values (5.31 ± 0.44 μM for nifurtimox, 0.36 ± 0.03 μM for suramin and 0.43 ± 0.07 μM for diminazene aceturate: DAC) were determined by fitting the data to a four-parameter sigmoidal equation. Errors are expressed as one *SD* [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Drug sensitivity analysis

Although the LUC cell line displayed a growth behavior that was undistinguishable from that of the parental cell line (Figure 1a), a comparative analysis of drug sensitivity was performed for both cell lines

with the aim to verify whether the integration and expression of the reporter gene alter parasite physiology. Three drugs, namely nifurtimox, suramin, and DAC, that are in clinical or veterinary use against African trypanosomiasis and that exert their anti-trypanosomal action by different modes were selected for this study.

Concentration-response curves with satisfactory fitting to sigmoidal equations were obtained for all three drugs tested against the LUC cell line by the bioluminescence assay (Figure 4). The corresponding EC_{50} values were 5.31 ± 0.44 μM for nifurtimox, 0.36 ± 0.03 μM for suramin, and 0.43 ± 0.07 μM for DAC. These values are similar to those determined by other methods—EC_{50} for DAC = 0.66 μM in Sykes and Avery (2009a); EC_{50} for nifurtimox = 15 μM in Maiwald et al. (2014); and EC_{50} for suramin = 78 nM in Franco et al. (2017). More relevant, trypanosomes from the parental and LUC cell lines exposed to different concentrations (2×, 1×, and 0.5× EC_{50}) of these drugs for 24 hr displayed an almost identical sensitivity (Table S1). This result confirms that the reporter transgene does not affect *T. brucei* physiology.

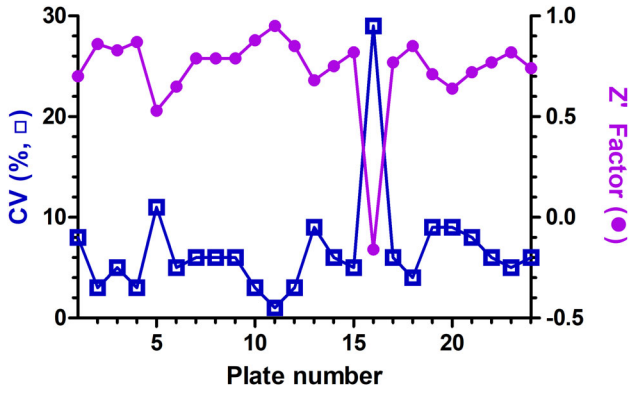

FIGURE 5 Performance of 96-well bioluminescence assay in a screening campaign. The coefficient of variation (CV %; square) and Z' factor (circle) values obtained for each assay plate (n = 24) analyzed during the screening campaign are shown. Plate 16 did not meet assay's quality control (see Section 3.4). Excluding this outlier plate, the Z' factor and CV presented average values of 0.77 and 5.8%, respectively [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Assay validation in a HTS setting

The bioluminescence assay was validated by screening a library of 141 compounds, belonging to different families (41 alkaloids, 5 rare sugars, 14 peptides, 25 polyheterocyles, 22 quinones, 6 selenosugars, 16 aromatic linker derivatives, and 12 singletons), in a 96-well format. The quality parameters Z´ factor and CV obtained during the screening campaign (Figure 5) presented mean values of 0.77 and of 5.8%, respectively, which met the assay quality criteria defined as Z' factor > 0.5 and CV <17% (Zhang, 1999; Sui, 2007).

Worth noting, and adding value to the relevance of cell culture synchronization prior to assay, only one plate (plate 16, Figure 5) presented outlier values for Z´ factor (< 0) and CV (29%), as a consequence of working with nonsynchronized parasite' cultures.

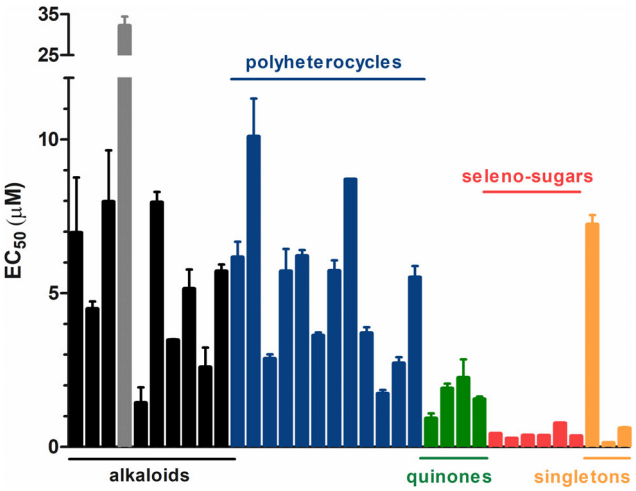

FIGURE 7 EC_{50} determination for selected hits. Concentration-response curve analysis was performed for 35 randomly selected hits from the primary screening. A single compound showed an EC_{50} higher than 10 μM (gray bar), which gives a hit confirmation rate for the assay of 97%. Errors are expressed as one *SD* [Color figure can be viewed at wileyonlinelibrary.com]

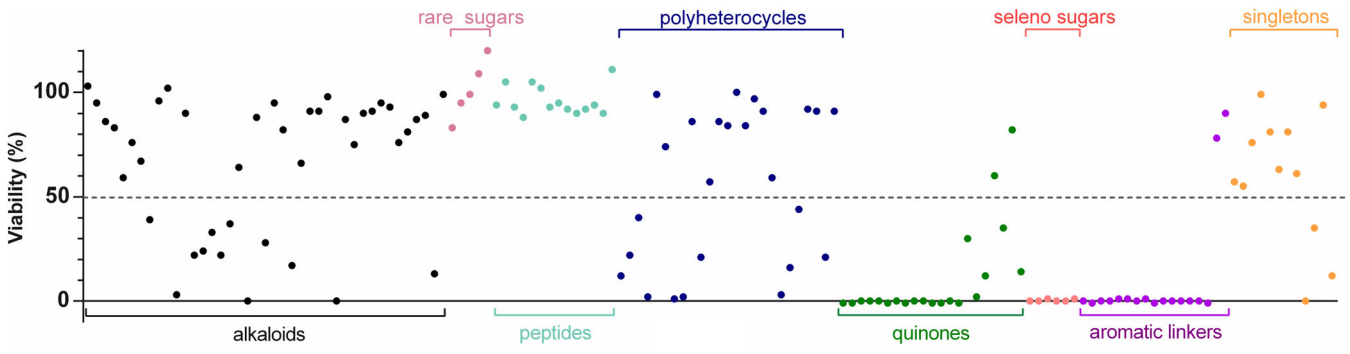

FIGURE 6 Primary screening using LUC trypanosomes. A library of 141 compounds was screened at a fixed concentration of 10 μM against LUC trypanosomes using the *Standardized bioluminescence assay*. Compounds reducing cell viability to ≤50% with respect to vehicle-treated (DMSO 1% vol/vol) parasites were considered hits. The error average expressed as one *SD* was 5% [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Main features and performance of cell viability assays employed in screening campaigns against *Trypanosoma brucei*

	CellTiter-Glo[a]	Alamar Blue[b]	SYBR Green[b]	Bioluminescence[c]
Total assay time	72 hr	96 hr	73 hr	48 hr
Compound incubation time	48 hr	72 hr	72 hr	24 hr
Readout time	12 min	24 hr	1 hr	32 min
Cost of readout reagent per plate (US$)	24.5[d]	1.77 (12.7)[e]	1.06[f]	1.17[g] (2.57)[h]
Z' factor	0.65	0.67 ± 0.06 (0.81)[i,j]	0.7[j]	0.77[j] (0.66)[h]
Coefficient of variation (%)	3.7	7.7 ± 1.9	2.3 ± 0.9	5.8 ± 2.4[j] (7.4)[h]
Signal to background	646	9–12[i]	NR	7.7 [j] (4.5)[h]
Parasites/ml at the screening time point	6×10^3	2.4×10^4	6×10^3	1×10^5
Detection limit (10^4 parasites/ml)	≤ 0.1[k]	7.77 ± 6.01	2.05 ± 0.31	4

Note: Data reported in the literature for assays used in 384-well format is compared to the assay of the present study (Bioluminescence).
[a]Sykes et al., 2012.
[b]Faria et al., 2015.
[c]This study, 96-well format assay.
[d]CellTiter-Glo® from Promega.
[e]Alamar Blue™ from Thermo-Fisher, using the resazurine reagent may reduce the cost by a factor of 100-fold.
[f]SYBR green from Thermo-Fisher.
[g]D-Luciferin from Perkin Elmer.
[h]Values estimated for the 384-well-plate assay.
[i]Sykes & Avery 2009a.
[j]Inter-assay average values obtained from the primary screening.
[k]Value inferred from data reported in the study.

The primary screening performed at a fixed compound concentration of 10 μM yielded 66 hits (cell viability ≤50%) and a hit rate of 47% (Figure 6). Except for one compound, concentration-response analysis performed for 35 randomly selected hits revealed EC_{50} values lower than 10 μM (Figure 7), which yields a hit confirmatory rate of 97%.

4 | DISCUSSION

Several methods used to assess cell viability have been successfully adapted for high-throughput whole-cell assays in the field of drug discovery against African trypanosomes (Bowling, Mercer, Don, Jacobs, & Nare, 2012; Faria et al., 2015; Lim, Zahari, Amanah, Zainuddin, & Adenan, 2016; Mackey et al., 2006; Mackey, Koupparis, Nishino, & McKerrow, 2011; Maiwald et al., 2014; Räz, Iten, Grether-Bühler, Kaminsky, & Brun, 1997; Suganuma, Allamanda, Hakimi, Zhou, & Angeles, 2014; Suganuma, Molefe, & Inoue, 2017; Sykes et al., 2012; Sykes & Avery, 2009b; Van Reet et al., 2013).

One of the most widely used methods is the resazurin-based assay (i.e., Alamar Blue™), for which the irreversible reduction of resazurin to resorufin (fluorescent product) is an indicator of cell metabolism/viability. Although the resazurin-assay is cost-effective, major drawbacks are related to its chemical nature (Eichler, 1934; O'Brien, Wilson, Orton, & Pognan, 2000; Prütz, Butler, & Land, 1996) and the long incubation times (from several hours to days) required for product formation (Pace & Burg, 2015). In fact, resazurin alone has been shown to exert an antiproliferative activity against *T. brucei* and mammalian cells or, in combination with compounds, to enhance or even ameliorate their cytotoxic effects (Faria et al., 2015; Gould et al., 2008; O'Brien et al., 2000; Sykes & Avery, 2009b). This explains the higher false-positive rate reported for Alamar Blue™ in a multimethod comparative study (Faria et al., 2015).

T. brucei proliferation could also be assessed reliably in a 96- or 384-well format (~72 hr incubation time) by quantifying the content of nucleic acids using propidium iodide (Gould et al., 2008) or the cyanine-based dye SYBR Green (Faria et al., 2015). The SYBR Green assay displays a lower detection limit (2×10^4 parasites/ml) than the PI-based method (<10^6 parasites/ml) (Table 1). Both assays are affordable and proved suitable to detect cytocidal and, though with a lower sensitivity, also cytostatic drugs or compounds.

Flow cytometry-based assays are statistically robust and suitable for multiparametric or high-content analysis (Franco et al., 2017; Franco, Medeiros, et al., 2017). However, it is a mid-throughput method (e.g., data acquisition and analysis) that needs costly instrumental and, thus, appears more suitable for the screening of small or target-focused libraries (Maiwald et al., 2014; Voyton, Morris, Ackroyd, Morris, & Christensen, 2018).

CellTiter-Glo® is an alternative endpoint assay that quantifies intracellular ATP by using recombinant luciferase and D-Luciferin. The assay is very sensitive, able to detect slow-killing inhibitors, and has a fast readout (Faria et al., 2015). The major limitations of this assay are its comparatively lower Z' factor and the reagents' costs (Sykes & Avery, 2009a), which, in part, preclude their implementation in large HTS campaigns (Table 1).

Here we reported the generation of a cell line from the infective stage of *T. b. brucei* with a stably integrated red-shifted luciferase gene (Branchini et al., 2010), and the development of a robust and sensitive HTS bioluminescence assay. In agreement with early observations employing a different *T. b. brucei* cell line (McLatchie et al., 2013), constitutive expression of luciferase proved stable for up to 2 months and did not entail alterations in the growth and drug-sensitivity phenotype of the transgenic trypanosomes. Bioluminescence signal proved to be a reliable measurement of parasite viability and number because, under the conditions tested, it is determined mostly by the intracellular energy status (ATP availability) and luciferase level. The quality parameters of the bioluminescence assay obtained in a 96-well screening campaign (S/B, CV, and Z' inter-assay values of 7.7, 0.77, and 5.8%, respectively) were equivalent to those from other methods (Table 1). The assay proved highly robust to identify true active compounds (97% hit ratio). The assay setup was primarily designed to favor the discovery of highly potent compounds (e.g., 1×10^5 parasites, with a doubling time of 7 hr, are incubated with compounds for 24 hr); however, by adjusting the initial cell density and extending the exposure time, the assay may be adapted for identifying slow-killing hits.

Scaling up of the assay from a 96- to a 384-well format was possible at the expense of increasing D-Luciferin concentration from 125 to 250 μg/ml, condition that yielded acceptable performance parameters (S/B = 4.5, Z´ factor = 0.66, and CV = 7.4%).

The bioluminescence assay here presented can be rated as a cost-effective and robust method for hit identification and hit-to-lead optimization applications. In addition, the genetic background of the LUC trypanosomes allows the use of tet-inducible vectors for the up- or down-regulation of specific genes. This bioluminescent-reporter cell line appears as a valuable tool to address all the stages of the early-phase drug discovery process, from target validation *in vitro* and *in vivo* (e.g., RNAi, dominant-negative or conditional KO approaches), through compound screening and up to drug efficacy test in animal models.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

Alibu, V. P., Storm, L., Haile, S., Clayton, C., & Horn, D. (2005). A doubly inducible system for RNA interference and rapid RNAi plasmid construction in Trypanosoma brucei. *Molecular and Biochemical Parasitology*, *139* (1), 75–82. https://doi.org/10.1016/J.MOLBIOPARA.2004.10.002

Bowling, T., Mercer, L., Don, R., Jacobs, R., & Nare, B. (2012). Application of a resazurin-based high-throughput screening assay for the identification and progression of new treatments for human African trypanosomiasis. *International Journal for Parasitology: Drugs and Drug Resistance*, *2*, 262–270. https://doi.org/10.1016/J.IJPDDR.2012.02.002

Branchini, B. R., Ablamsky, D. M., Davis, A. L., Southworth, T. L., Butler, B., Fan, F., … Pule, M. A. (2010). Red-emitting luciferases for bioluminescence reporter and imaging applications. *Analytical Biochemistry*, *396* (2), 290–297. https://doi.org/10.1016/J.AB.2009.09.009

Burrell-Saward, H., Rodgers, J., Bradley, B., Croft, S. L., & Ward, T. H. (2015). A sensitive and reproducible *in vivo* imaging mouse model for evaluation of drugs against late-stage human African trypanosomiasis. *The Journal of Antimicrobial Chemotherapy*, *70*(2), 510–517.

Comini, M. A., Guerrero, S. A., Haile, S., Menge, U., Lünsdorf, H., & Flohé, L. (2004). Valdiation of Trypanosoma brucei trypanothione synthetase as drug target. *Free Radical Biology and Medicine*, *36*(10), 1289–1302. https://doi.org/10.1016/J.FREERADBIOMED.2004.02.008

Don, R., & Ioset, J. R. (2014). Screening strategies to identify new chemical diversity for drug development to treat kinetoplastid infections. *Parasitology*, *141*(1), 140–146.

Eichler, H. (1934). Der Nachweis der hydrosulfite (sulfoxylate) und des nachierenden Wasserstoffs mit Resazurin. Fresenius. *Journal of Analytical Chemistry*, *99*, 270–272.

Faria, J., Moraes, C. B., Song, R., Pascoalino, B. S., Lee, N., Siqueira-neto, J. L., … Freitas-junior, L. H. (2015). Drug discovery for human African Trypanosomiasis: Identification of novel scaffolds by the newly developed HTS SYBR green assay for Trypanosoma brucei. *Journal of Biomolecular Screening*, *20*(1), 70–81. https://doi.org/10.1177/1087057114556236

Franco, J., Medeiros, A., Benítez, D., Perelmuter, K., Serra, G., Comini, M. A., & Scarone, L. (2017). In vitro activity and mode of action of distamycin analogues against African trypanosomes. *European Journal of Medicinal Chemistry*, *126*, 776–788. https://doi.org/10.1016/J.EJMECH.2016.12.002

Franco, J., Sardi, F., Szilágyi, L., Kövér, K. E., Fehér, K., & Comini, M. A. (2017). Diglycosyl diselenides alter redox homeostasis and glucose consumption of infective African trypanosomes. *International Journal for Parasitology: Drugs and Drug Resistance*, *7*(3), 303–313. https://doi.org/10.1016/J.IJPDDR.2017.08.001

Gould, M. K., Vu, X. L., Seebeck, T., & de Koning, H. P. (2008). Propidium iodide-based methods for monitoring drug action in the kinetoplastidae: Comparison with the Alamar blue assay. *Analytical Biochemistry*, *382*(2), 87–93. https://doi.org/10.1016/J.AB.2008.07.036

Hirumi, H., & Hirumi, K. (1989). Continuous cultivation of Trypanosoma brucei blood stream forms in a medium containing a low concentration

of serum protein without feeder cell layers. *The Journal of Parasitology*, *75*(6), 985–989. https://doi.org/10.2307/3282883

Lim, K. T., Zahari, Z., Amanah, A., Zainuddin, Z., & Adenan, M. I. (2016). Development of resazurin-based assay in 384-well format for high throughput whole cell screening of Trypanosoma brucei rhodesiense strain STIB 900 for the identification of potential anti-trypanosomal agents. *Experimental Parasitology*, *162*, 49–56. https://doi.org/10.1016/J.EXPPARA.2016.01.002

Mackey, Z. B., Baca, A. M., Mallari, J. P., Apsel, B., Hansell, E. J., Peter, K., … James, H. (2006). Discovery of Trypanocidal compounds by whole cell HTS of Trypanosoma brucei. *Chemical Biology & Drug Design*, *67*, 355–363. https://doi.org/10.1111/j.1747-0285.2006.00389.x

Mackey, Z. B., Koupparis, K., Nishino, M., & McKerrow, J. H. (2011). High-throughput analysis of an RNAi library identifies novel kinase targets in Trypanosoma brucei. *Chemical Biology & Drug Design*, *78*(3), 454–463. https://doi.org/10.1111/j.1747-0285.2011.01156.x

Maiwald, F., Benítez, D., Charquero, D., Dar, M. A., Erdmann, H., Preu, L., … Kunick, C. (2014). 9- and 11-substituted 4-azapaullones are potent and selective inhibitors of African trypanosoma. *European Journal of Medicinal Chemistry*, *83*, 274–283. https://doi.org/10.1016/J.EJMECH.2014.06.020

Manta, B., Pavan, C., Sturlese, M., Medeiros, A., Crispo, M., Berndt, C., … Comini, M. A. (2013). Iron-sulfur cluster binding by mitochondrial monothiol glutaredoxin-1 of Trypanosoma brucei: Molecular basis of iron-sulfur cluster coordination and relevance for parasite infectivity. *Antioxidants & Redox Signaling*, *19*(7), 665–682. https://doi.org/10.1089/ars.2012.4859

McLatchie, A. P., Burrell-saward, H., Myburgh, E., Lewis, M. D., Ward, T. H., Mottram, J. C., … Taylor, M. C. (2013). Highly sensitive *in vivo* imaging of Trypanosoma brucei expressing "' red-shifted '" luciferase. *PLoS Neglected Tropical Diseases*, *7*(11), e2571. https://doi.org/10.1371/journal.pntd.0002571

Mesu, V. K. B. K., Kalonji, W. M., Bardonneau, C., Mordt, O. V., Blesson, S., Simon, F., … Tarral, A. (2018). Oral fexinidazole for late-stage African Trypanosoma brucei gambiense trypanosomiasis: A pivotal multicentre, randomised, non-inferiority trial. *Lancet (London, England)*, *391*(10116), 144–154.

Myburgh, E., Coles, J. A., Ritchie, R., Kennedy, P. G. E., McLatchie, A. P., Rodgers, J., … Mottram, J. C. (2013). In vivo imaging of trypanosome-brain interactions and development of a rapid screening test for drugs against CNS stage trypanosomiasis. *PLoS Neglected Tropical Diseases*, *7*(8), e2384. https://doi.org/10.1371/journal.pntd.0002384

O'Brien, J., Wilson, I., Orton, T., & Pognan, F. (2000). Investigation of the Alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry*, *267*(17), 5421–5426. https://doi.org/10.1046/j.1432-1327.2000.01606.x

Pace, R. T., & Burg, K. J. L. (2015). Toxic effects of resazurin on cell cultures. *Cytotechnology*, *67*(1), 13–17. https://doi.org/10.1007/s10616-013-9664-1

Prütz, W. A., Butler, J., & Land, E. J. (1996). Photocatalytic and free radical interactions of the HeterocyclicN-oxide Resazurin with NADH, GSH, and Dopa. *Archives of Biochemistry and Biophysics*, *327*(2), 239–248. https://doi.org/10.1006/ABBI.1996.0116

Quijada, L., Guerra-Giraldez, C., Drozdz, M., Hartmann, C., Irmer, H., Ben-Dov, C., … Clayton, C. (2002). Expression of the human RNA-binding protein HuR in Trypanosoma brucei increases the abundance of mRNAs containing AU-rich regulatory elements. *Nucleic Acids Research*, *30*(20), 4414–4424. https://doi.org/10.1093/nar/gkf577

Räz, B., Iten, M., Grether-Bühler, Y., Kaminsky, R., & Brun, R. (1997). The Alamar blue® assay to determine drug sensitivity of African trypanosomes (T.b. rhodesiense and T.b. gambiense) *in vitro*. *Acta Tropica*, *68*(2), 139–147. https://doi.org/10.1016/S0001-706X(97)00079-X

Shaw, A. P. M., Cecchi, G., Wint, G. R. W., Mattioli, R. C., & Robinson, T. P. (2014). Mapping the economic benefits to livestock keepers from intervening against bovine trypanosomosis in eastern Africa. *Preventive Veterinary Medicine*, *113*(2), 197–210. https://doi.org/10.1016/J.PREVETMED.2013.10.024

Simarro, P. P., Cecchi, G., Franco, J. R., Paone, M., Diarra, A., Ruiz-postigo, J. A., … Jannin, J. G. (2012). Estimating and mapping the population at risk of sleeping sickness. *PLoS Neglected Tropical Diseases*, *6*(10), e1859. https://doi.org/10.1371/journal.pntd.0001859

Sokolova, A. Y., Wyllie, S., Patterson, S., Oza, S. L., Read, K. D., & Fairlamb, A. H. (2010). Cross-resistance to nitro drugs and implications for treatment of human African trypanosomiasis. *Antimicrobial Agents and Chemotherapy*, *54*(7), 2893–2900. https://doi.org/10.1128/AAC.00332-10

Stijlemans, B., Caljon, G., Van Den Abbeele, J., Van Ginderachter, J. A., Magez, S., & De Trez, C. (2016). Immune evasion strategies of Trypanosoma brucei within the mammalian host: Progression to pathogenicity. *Frontiers in Immunology*, *7*(233), 1–14. https://doi.org/10.3389/fimmu.2016.00233

Sui, Y., & Wu, Z. (2007). Alternative statistical parameter for high-throughput screening assay quality assessment. *Journal of Biomolecular Screening*, *12*(2), 229–234. https://doi.org/10.1177/1087057106296498

Suganuma, K., Allamanda, P., Hakimi, H., Zhou, M., & Angeles, J. M. (2014). Establishment of ATP-based luciferase viability assay in 96-well plate for Trypanosoma congolense. *The Journal of Veterinary Medical Science*, *76*(11), 1437–1441. https://doi.org/10.1292/jvms.14-0273

Suganuma, K., Molefe, N. I., & Inoue, N. (2017). In D. F. Gilbert & O. Friedrich (Eds.), *An ATP-based luciferase viability assay for animal African trypanosomes using a 96-well plate BT - cell viability assays: Methods and protocols* (pp. 89–95). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4939-6960-9_8

Sykes, M. L., & Avery, V. M. (2009a). A luciferase based viability assay for ATP detection in 384-well format for high throughput whole cell screening of Trypanosoma brucei brucei bloodstream form strain 427. *Parasites & Vectors*, *2*(54), 1–12. https://doi.org/10.1186/1756-3305-2-54

Sykes, M. L., & Avery, V. M. (2009b). Development of an Alamar blue ™ viability assay in 384-well format for high throughput whole cell screening of Trypanosoma brucei brucei bloodstream form strain 427. *The American Journal of Tropical Medicine and Hygiene*, *81*(4), 665–674. https://doi.org/10.4269/ajtmh.2009.09-0015

Sykes, M. L., Baell, J. B., Kaiser, M., Chatelain, E., Moawad, S. R., Ganame, D., … Avery, V. M. (2012). Identification of compounds with anti-proliferative activity against Trypanosoma brucei brucei strain 427 by a whole cell viability based HTS campaign. *PLoS Neglected Tropical Diseases*, *6*(11), e1896. https://doi.org/10.1371/journal.pntd.0001896

Van Reet, N., Pyana, P., Rogé, S., Claes, F., & Büscher, P. (2013). Luminescent multiplex viability assay for Trypanosoma brucei gambiense. *Parasites & Vectors*, *6*(207), 1–9.

Van Reet, N., Van de Vyver, H., Pyana, P. P., Van der Linden, A. M., & Büscher, P. (2014). A panel of Trypanosoma brucei strains tagged with blue and red-shifted luciferases for bioluminescent imaging in murine infection models. *PLoS Neglected Tropical Diseases*, *8*(8), e3054.

Voyton, C. M., Morris, M. T., Ackroyd, P. C., Morris, J. C., & Christensen, K. A. (2018). FRET flow Cytometry-based high throughput screening assay to identify disrupters of glucose levels in Trypanosoma brucei. *ACS Infectious Diseases*, *4*(7), 1058–1066. https://doi.org/10.1021/acsinfecdis.8b00058

Zhang, J. H., Chung, T. D. Y., & Oldenburg, K. R., Oldenburg, K. R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *Journal of Biomolecular Screening*, *4*(2), 67–73. https://doi.org/10.1177/108705719900400206

Zomerdijk, J. C. B. M., Kieft, R., & Borst, P. (1991). Efficient production of functional mRNA mediated by RNA polymerase I in Trypanosoma brucei. *Nature*, *353*(6346), 772–775. https://doi.org/10.1038/353772a0

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