FULL PAPER *Parasitology*

Establishment of ATP-Based Luciferase Viability Assay in 96-Well Plate for *Trypanosoma congolense*

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ABSTRACT. Animal African trypanosomosis (AAT), caused by *Trypanosoma congolense,* is widespread throughout sub-Saharan Africa. There are significant concerns related to the current drugs available for the treatment of AAT due to their limited effectiveness across species and their adverse effects. Moreover, drug resistant trypanosomes have recently been reported in the field. High throughput screening (HTS) of large chemical compound library collections is a promising approach for identifying novel drug candidates. While HTS for *Trypanozoon* trypanosomes, *T. brucei* sspp. and *T. evansi* is well established, no assays have been developed for *T. congolense*. In the present study, the authors developed an ATP-based luciferase viability assay for *T. congolense* in a 96-well plate format. The calculated 50% inhibitory concentration (IC_{50}) values for pentamidine and diminazene were 10–100 times higher in *T. congolense* than in *T. brucei.* This result suggests that the transporters for the 2 tested compounds differ between *T. congolense* and *T. brucei.* This assay could further be applied to screen novel chemical compounds for the treatment of AAT caused by *T. congolense*.

KEY WORDS: Animal African trypanosomosis, drug screening, luciferase assay, *Trypanosoma congolense*

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Animal African trypanosomosis (AAT) in domesticated and wild animals, known as Nagana, is mainly caused by *Trypanosoma congolense, T. vivax* and *T. brucei brucei* [9]. The most consistent clinical features of Nagana in livestock are intermittent fever and anemia, although general leukopenia, splenomegaly and hepatomegaly, and weight loss are also present in some cases. *T. congolense* and *T. vivax* only proliferate in blood circulation, whereas *T. b. brucei* has also been found to infect the central nervous system [2, 14, 16, 21]. The FAO estimates that AAT causes a combined economic loss of agricultural gross domestic product of US$ 4.75 billion per year (http://www.fao.org/ag/againfo/programmes/en/paat/disease.html). Currently, 3 drugs are commercially available for the treatment of AAT, namely: diminazene, isometamidium and homidium. The latter 2 drugs can also be used for chemoprophylaxis [9]. In the case of human African trypanosomosis (HAT) treatment, pentamidine and suramine are the first choice of drugs for the treatment of *T. b. rhodesiense* and *T. b. gambiense,* respectively [2]. However, drug resistant trypanosomes and drug refractory trypanosomosis have been reported from the field for both HAT and AAT [1, 6, 7, 19]. Novel trypanocidal compounds are therefore needed for the treatment of drug resistant trypanosomosis.

High throughput screening (HTS) assay of large compound libraries has been undertaken to discover novel therapeutic candidates for several parasites [18]. This has been performed for a number of parasitic diseases, such as American trypanosomosis (Chagas' disease), leishmaniosis and malaria [8, 13, 22, 25]. The HTS approach has been proven to have the potential to identify new drugs with novel modes of action. HTS approaches with specific targets have been well established and reported for *Trypanozoon* trypanosomes, *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense* and *T. evansi* [10, 20, 23, 24, 26]. A viability assay, utilizing Alamar Blue™ dye, has been developed for all *T. brucei* sspp. [20, 23]. However, the Alamar Blue™ dye assay has some problems in that it is time-consuming to perform and its sensitivity is low. Recently, a luciferase viability assay for measuring the ATP concentration of cells was adapted to HTS to evaluate viable *T. b. brucei* and *T. b. gambiense* cell numbers [24, 26]. In comparison, the luciferase assay method offers greater sensitivity and is less time-consuming than the Alamar Blue™ assay system [23, 24]. This assay could therefore be used in HTS for important animal parasites, such as *T. congolense*. However, no HTS assay for *T. congolense* has previously been developed or reported.

In the present study, we initially established an ATP-based luciferase viability assay for *T. congolense* using the 2 available drugs against trypanosomosis: diminazene and pentamidine. We applied this assay in evaluating ATP congruent to the *T. congolense* cell number in culture to develop an HTS system for this trypanosome.

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MATERIALS AND METHODS

Parasites and in vitro culture: Trypanosoma: *Trypanosoma congolense* IL3000, a savannah type strain isolated near the Kenya/Tanzania border in 1966, was used in this study. The blood stream form (BSF) of *T. congolense* IL3000 was propagated at 33°C in air using Hirum's modified Iscove's medium (HMI)-9 composed of Iscove's modified Dulbecco's medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 20% heat inactivated-fetal bovine serum (HI-FBS), 60 mM HEPES (Sigma-Aldrich), 1 mM pyruvic acid sodium salt (Sigma-Aldrich), 0.1 mM bathocuproine (Sigma-Aldrich), 1 mM hypoxanthine and 16 μM thymidine (HT supplement: Invitrogen, Tokyo, Japan), 10 μg/*l* insulin, 5.5 μg/*l* transferrin and 6.7 *n*g/*l* sodium selenite (ITS-X: Invitrogen), 0.0001% 2-β-mercaptoethanol (Sigma-Aldrich), 0.4 g/*l* BSA (Sigma-Aldrich) and 2 mM L-cysteine (Sigma-Aldrich), as previously reported [12]. The BSF cultures were maintained by replacing the entire culture supernatant with fresh medium every other day.

Optimization of cell density and calculation of doubling time: A growth curve was plotted to estimate the maximum number of cells and calculate the doubling time. One hundred μl of *T. congolense* (at a density of 1×10^5, 5×10^4 and 2.5×10^4 cells/m*l*) was incubated in a 96-well plate at 33°C. Trypanosomes were counted every 24 hr post-inoculation until day 6 using a counting chamber after appropriate dilution by phosphate buffered saline with glucose (PSG). Doubling time (Td) was calculated by counting the cells in the log phase of growth and using the equation: $Td=(t2 - t1) \times \log(2)/\log(q2/q1)$. Two measurements were made: the initial count (q1) at time (t1) and the resultant density following 24 hr incubation (q2, t2) [23, 24].

Optimization of dimethylsufoxide concentration: Fifty μl of different concentrations of dimethylsufoxide (DMSO; Wako Pure Chemical Industries, Ltd., Osaka, Japan) diluted in HMI-9 medium were added into a 96-well plate containing 50 μl of *T. congolense* at a density of 2×10^5 cells/m*l*. The plate was incubated for a further 72 hr, and the inhibition rate was calculated. The optimized luciferase assay protocol was then performed (see next section).

Finally optimized HTS assay conditions: After optimization, the final conditions for the assay were established for conducting the tests. Fifty μl of various concentrations of reference compounds in HMI-9 with 0.5% DMSO were added to a Nunc® MicroWell 96-well optical bottom plate (Thermo Fisher Scientific K.K., Yokohama, Japan) containing 50 μl of *T. congolense* at a density of 2×10^5 cells/m*l*. The final concentration of the parasite cell used in the assay was 1×10^5 cells/m*l*, and the DMSO was at a final concentration of 0.25% in 100 μl of HMI-9 per well. The cells were incubated for 72 hr, and subsequently, 50 μl of CellTiter-Glo™ Luminescent Cell Viability Assay reagent (Promega Japan, Tokyo, Japan) was added to evaluate intracellular ATP concentration. The plate was shaken for 500 shakes/min by MS3 basic plate shaker (IKA® JAPAN K.K., Osaka, Japan) in 2 min to facilitate cell lysis and release intracellular ATP, and then incubated for another 10 min at room temperature. The plate was read using a GloMax®-Multi+ Detection System plate reader (Promega Japan).

Statistical analysis of samples: The quality of this assay was evaluated by Z'-factor [28] with the following formula: Z'-factor $=1-\{(3\times SD\ A + 3\times SD\ B)/(mean\ A - mean\ B)\}$; where A=the mean signal of each assay (cell growth detected in wells containing *T. congolense* in HMI-9 without compounds) and B=the signal of 500 *n*g/m*l* of reference compounds to the cells to cause 100% culture death. The coefficient of the determination value (R^2) was calculated using GraphPad PRISM 5 (GraphPad Software Inc., CA, U.S.A.).

Determination of reference compounds 50% inhibitory concentration (IC_{50}): Diminazene aceturate (Sigma-Aldrich) and pentamidine (Sigma-Aldrich) were used for estimating drug sensitivity in this assay. Both compounds were stored as 10 mg/m*l* stock solutions in DMSO. Two-fold drug dilutions were made in triplicate from 500–1.95 *n*g/m*l* in HMI-9 with 0.5% DMSO. The IC_{50} value of each reference compound was calculated by plotting the% inhibition (0% inhibition=the luminescence of trypanosome culture well without any chemicals) against log in GraphPad PRISM 5 software (GraphPad Software Inc.).

RESULTS

Establishment of HTS assay: For establishment of ATP-based luciferase viability assay for HTS in *T. congolense*, the maximum cell concentration linearly correlated with luminescence was used to optimize the test. The luminescence signal was well correlated, in a linear fashion, with trypanosome cell concentrations of up to 1×10^6 cells/m*l* in 100 μl per well ($R^2 \geq 0.99$) (Fig. 1A). However, this linear correlation was not seen when a higher concentration was used (Data not shown). The condition of the cell culture that leads to a final concentration of 1×10^6 cells/m*l* in day 3 was optimized using different concentrations as shown in Fig. 1B. In our *in vitro* study, a start of 1×10^5 cells/m*l* culture reached nearly 1×10^6 cells/m*l* after 72 hr incubation (Fig. 1B). This condition was therefore utilized in the present study. Finally, the DMSO concentration needed for the assay was optimized by testing the effect of 0–4% DMSO on trypanosome growth. The results showed that 4% DMSO completely inhibited cell proliferation, whereas concentrations of ≤0.25% inhibited cell proliferation by <20% (Fig. 1C). In addition, using the information on the *T. congolense* culture day 1 to day 5 log phase proliferation, the doubling time was calculated to be 15 ± 2 hr (Fig. 1B). HMI-9 containing 0.25% DMSO was therefore utilized for the chemical compound dilution.

Evaluation of the HTS assay: To evaluate the established assay system, the authors initially evaluated pentamidine and diminazene aceturate, calculating its IC_{50} values (Fig. 2). The IC_{50} values of pentamidine and diminazene were calculated as 100.45 ± 26.08 *n*g/m*l* (169.48 ± 44.00 nM) and 55.98 ± 13.15 *n*g/m*l* (108.65 ± 25.25 nM), respectively (Table 1). In addition, the Z'-factor values of pentamidine and diminazene were calculated as 0.86 ± 0.06 and 0.94 ± 0.03, respectively (Table 1). These 2 compounds were also tested against

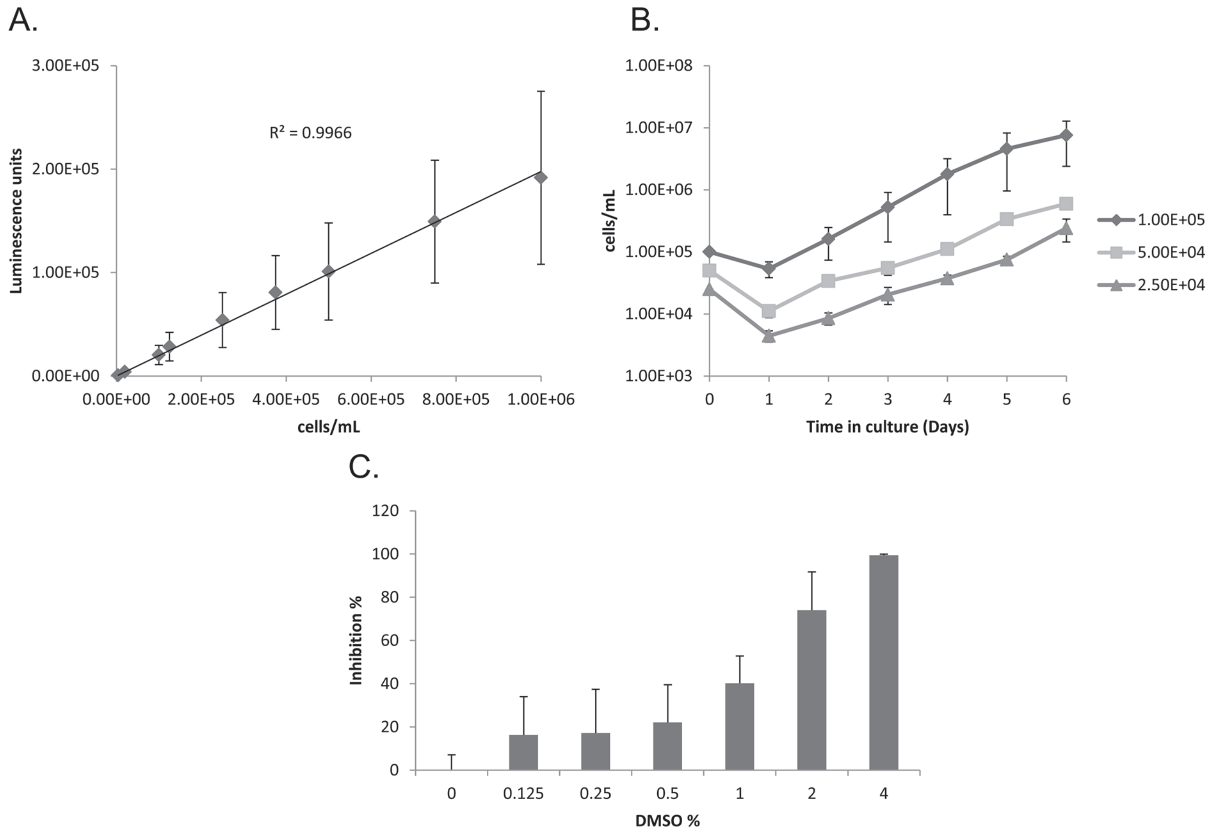

Fig. 1. Optimization of 96-well based screening assay conditions. (A) Correlation of cell number and luminescence. Cell concentration of *T. congolense* was evaluated using the ATP-based luciferase viability assay in 100 *µl* assay volume per well. Fifty *µl* of luciferase reagent was added to 50 *µl* of the cell culture for evaluation. All plots were calculated from 5 independent experiments and shown as the mean luminescence signal ± standard deviation. (B) Optimization of cell culture condition. *T. congolense* in 1×10^5, 5×10^4 and 2.5×10^4 cells/m*l* as the initial cell densities was cultured in a 96-well plate for 6 days. Trypanosome number was counted microscopically every day using a cell-counting chamber and plotted onto the graph. Each plot shows the mean number of trypanosomes ± standard deviation. (C) Estimation of trypanocidal effect of dimethylsufoxide (DMSO). Trypanocidal effect of DMSO was estimated by the ATP-based luciferase viability assay in the 96-well plate. Inhibition rate (%) was calculated from 4 independent experiments. Each column shows the mean inhibition rate (%) ± standard deviation.

T. b. brucei GUTat3.1 strain and *T. evansi* Tansui strain using this assay system (Supplemental Fig. 1). The IC_{50} values of pentamidine and diminazene in *T. b. brucei* were 0.64 ± 0.30 *ng*/m*l* (1.09 ± 0.50 nM) and 6.75 ± 0.40 *ng*/m*l* (13.09 ± 2.94 nM), respectively (Supplemental Table 1). In addition, those of *T. evansi* were 0.68 ± 0.40 *ng*/m*l* (1.15 ± 0.67 nM) and 8.15 ± 2.84 *ng*/m*l* (13.09 ± 2.94 nM), respectively (Supplemental Table 1). The results showed that the IC_{50} values of these compounds for *T. b. brucei* and *T. evansi* were 6–100 times lower than those of *T. congolense*.

DISCUSSION

In this study, the authors established and evaluated the ATP-based luciferase viability assay in 96-well plate for *T. congolense.* Because of the intracellular ATP levels detected in viable cells, the luminescence levels and the numbers of trypanosomes were directory proportional (Fig. 1A). To optimize the cell culture with a final concentration of 1×10^6 cells/m*l* on day 3, *T. congolense* was cultivated with different starting concentrations in 96-well plate (Fig. 1B). The concentration of *T. congolense* on day 1 was shown lower than that on day 0 (Fig. 1B). *T. congolense* BSF proliferates in a cell adhesion dependent manner at the vascular endothelium *in vivo* or at the bottom of the culture flask *in vitro* [11, 12]. Therefore, BSF that failed to adhere will eventually die on the first day of culture, resulting in a lower number of trypanosomes on day 1 than on day 0. To evaluate this assay, the Z'-factor was calculated. The Z'-factor values of pentamidine and diminazene for *T. congolense* were calculated as 0.86 ± 0.06 and 0.94 ± 0.03, respectively (Table 1). In addition, the Z'-factors for *T. b. brucei* and *T. evansi* were also >0.5 (Supplemental Table 1). These results suggested that the assay system could be applied to the HTS system for trypanosome, since it showed a remarkable Z'-factor value of 1> Z'-factor ≥0.5 [28].

Previous reports showed that the IC_{50} values of pentamidine and diminazene for *T. b. brucei* and *T. evansi* were

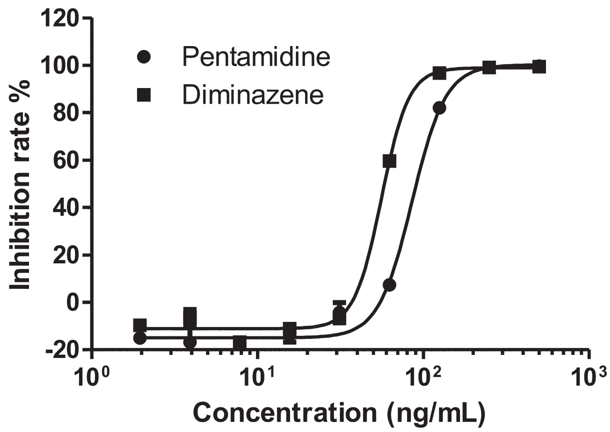

Fig. 2. The IC_{50} values of pentamidine and diminazene for *T. congolense.* The IC_{50} values of diminazene and pentamidine were evaluated for *T. congolense* using the ATP-based luciferase viability assay. The percentage of inhibition was relative to that of the control well (where no chemical compounds were added). The sigmoidal dose response curve graphs were plotted by GraphPad PRISM5 software.

2–100 times lower than our results with *T. congolense* [10, 20, 23, 24, 26]. These results suggest that pentamidine and diminazene transporter of *T. congolense* could differ to that of the *T. brucei*. It was previously reported that *T. brucei* adenosine transporter 1 (TbAT1), identified as a P2 type aminopurine transporter of *T. brucei*, is responsible for the diminazene and pentamidine uptake [17]. The orthologue of this gene exists in *Trypanozoon* trypanosomes, *T. brucei* sspp., *T. evansi* and *T. equiperdum* [3, 4, 15, 27]. *TbAT1* orthologous gene has recently been identified in *T. congolense* and was named as *T. congolense* adenosine transporter 1 (*TcoAT1*) [5]. However, sequencing analysis suggested that *TcoAT1* was not a true orthologous gene of P2-type purine transporter, *TbAT1*, but rather that it seemed to be an orthologous gene of P1-type purine transporter, *TbNT10* [17]. In addition, it was reported that the IC_{50} value of pentamidine and diminazene became >200 μM when TcoAT1 was overexpressed in diminazene resistant *T. b. brucei* (B48 strain) [17]. The IC_{50} values of *T. congolense* in our study were more than 1,000 times lower than in the aforementioned report, suggesting that in *T. congolense,* pentamidine and diminazene uptake likely occurs via an unknown aminopurine transporter.

HTS systems are very well established for HAT and leishmaniosis, where they are utilized for screening chemical compound libraries to facilitate the development of therapeutic medicine. In the case of *T. congolense*, which causes a neglected tropical parasitic animal disease, this report is useful for the development of a more sensitive assay, which is necessary for establishing HTS. This will therefore offer a great advantage and be an efficient tool for screening and determining novel animal trypanocidal drugs in future studies.

Table 1. Evaluation of the ATP-based luciferase viability assay for *T. congolense* using reference compounds

	Pentamidine	Diminazene
IC_{50} (*ng*/*ml*)	100.45 ± 26.08	55.98 ± 13.15
IC_{50} (nM)	169.48 ± 44.00	108.65 ± 25.51
Z'-factor	0.86 ± 0.06	0.94 ± 0.03
R^2	0.98 ± 0.02	0.99 ± 0.01

All values were calculated from 6 independent experiments and shown as the mean value ± standard deviation. IC_{50}: 50% inhibitory concentration, R^2: coefficient of determination.

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