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Drug Discovery for Human African Trypanosomiasis: Identification of Novel Scaffolds by the Newly Developed HTS SYBR Green Assay for *Trypanosoma brucei*

Joana Faria[1,3,§], Carolina B. Moraes[1,4,§], Rita Song[2], Bruno S. Pascoalino[4], Nakyung Lee[1], Jair L. Siqueira-Neto[1,¶], Deu John M. Cruz[1], Tanya Parkinson[5], Jean-Robert Ioset[5], Anabela Cordeiro-da-Silva[3,6], and Lucio H. Freitas-Junior[1,4]

Abstract

Human African trypanosomiasis (HAT) is a vector-transmitted tropical disease caused by the protozoan parasite *Trypanosoma brucei*. High-throughput screening (HTS) of small-molecule libraries in whole-cell assays is one of the most frequently used approaches in drug discovery for infectious diseases. To aid in drug discovery efforts for HAT, the SYBR Green assay was developed for *T. brucei* in a 384-well format. This semi-automated assay is cost- and time-effective, robust, and reproducible. The SYBR Green assay was compared to the resazurin assay by screening a library of 4000 putative kinase inhibitors, revealing a superior performance in terms of assay time, sensitivity, simplicity, and reproducibility, and resulting in a higher hit confirmation rate. Although the resazurin assay allows for comparatively improved detection of slow-killing compounds, it also has higher false-positive rates that are likely to arise from the assay experimental conditions. The compounds with the most potent antitrypanosomal activity were selected in both screens and grouped into 13 structural clusters, with 11 new scaffolds as antitrypanosomal agents. Several of the identified compounds had IC_{50} <1 μM coupled with high selectivity toward the parasite. The core structures of the scaffolds are shown, providing promising new starting points for drug discovery for HAT.

Keywords

anti-infective drugs, cell-based assays, ultrahigh-throughput screening, fluorescence methods

Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a vector-transmitted disease caused by 2 subspecies of the digenetic protozoan parasite *Trypanosoma brucei*, namely, *T. b. gambiense* and *T. b. rhodesiense*. The disease is endemic in 36 Sub-Saharan Africa countries where the vector, the parasite, and the animal reservoir coexist. HAT has a fatal outcome if left untreated. Although the number of reported cases dropped from 37,385 in 1998 to 9689 in 2009, many remain unreported—and therefore untreated—due to the lack of specificity of the clinical diagnostic and the limited access to the infected populations.[1] In addition, *T. brucei* has an economic importance, because some subspecies are pathogenic to cattle, causing a wasting disease known as *nagana* and resulting in annual losses of US$1.5 billion in agricultural incomes.[2]

There are 2 *T. brucei* subspecies responsible for the disease in humans: *T. b. gambiense*, which is found in West Africa and responsible for more than 95% of the cases of the disease, and *T. b. rhodesiense* in East Africa, which is

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responsible for the other 5% of the cases.[3] A third subspecies, *T. brucei brucei*, is unable to infect primates, although it is genotypically very similar to the 2 pathogenic subspecies, making it a good experimental model.

Because there are no vaccines available, drugs remain the main control strategy for HAT. There are 4 approved drugs for chemotherapy. Suramin, pentamidine, and melarsoprol are the most widely used and were developed before 1950. Eflornithine was approved in 1990, and since then advances in HAT treatment have been slow.[4] Furthermore, current treatments possess several limitations, such as limited efficacy and severe side effects due to toxicity, including mortality due to treatment.[1,5–7] Nifurtimox, a drug used for the treatment of Chagas disease, was introduced in 2009 in the World Health Organization's (WHO) List of Essential Medicines to be used as part of the nifurtimox–eflornithine combination therapy (NECT), providing a treatment less favorable toward the development of drug resistance and simpler to administer than the eflornithine monotherapy.[2,8]

High-throughput screening (HTS) of compound libraries using whole-cell assays is a well-established approach for drug discovery programs in neglected diseases. This kind of assay normally relies on simple pathogen viability readout, precluding the need for a validated target, which can be difficult to achieve; and, in contrast to a biochemical target-based assay, in this assay active compounds are discovered under physiologically relevant conditions. In addition, whole-cell-based assays have been quite successful in resulting in approved drugs for infectious diseases in general.[3,9,10] In spite of its compelling advantages, only recently were whole-cell-based assays reported in HTS format for *T. brucei*. Luciferase and resazurin-based cell viability assays were proposed in a 384-well format.[4,11–13] The latter was recently used to screen 87,296 compounds, resulting in 6 hits from 5 new chemical classes with activity confirmed against *T. b. rhodesiense*.[14]

In spite of its versatility and widespread use, the resazurin assay does possess some important limitations.[15] Resazurin accepts electrons from free radicals and from the electron transport chain within the inner mitochondrial membrane, generating ROS and interfering with energy homeostasis.[16,17] In addition, the resazurin screening assay for assessment of *T. brucei* viability in 384-well format assays has additional issues that might lead to misinterpretation of the results. Specifically, the assay requires long exposure of live parasites to resazurin at room temperature.[13] The long exposure to resazurin coupled with room temperature incubation could compromise *T. brucei* viability and could potentially interfere with compound activity during drug screening.

As an alternative to the resazurin assay, a SYBR Green–based whole-cell assay was developed for *T. brucei* in a 384-well format. SYBR Green is a cyanine dye that binds to nucleic acids, preferably to double-stranded DNA (dsDNA), thus providing an indirect assessment of cell number in a population. A SYBR Green–based whole-cell assay has been intensively used in antimalarial drug discovery.[18–22] Because *T. brucei* is an exclusively extracellular parasite, whole-cell assays based on quantitative detection of nucleic acids could be a feasible alternative.

Material and Methods

Cell culture: Bloodstream forms of *T. b. brucei* Lister 427 were cultured in HMI-9 medium as described previously.[23] The human acute leukemia monocyte cell line THP-1 (Korean Cell Line Bank No. 40202; Korean Cell Line Bank, Seoul, South Korea) was cultivated in RPMI medium (Welgene, Daegu, South Korea). Both culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). Cultures were kept in vented flasks in a humidified atmosphere of 5% CO_2 at 37 °C. Parasites were maintained in log-phase growth (between 1×10^5 and 1×10^6 parasites/mL) and subcultured every 24 h. THP-1 cultures were diluted every 3 or 4 days to maintain the cell density between 1×10^5 and 8×10^5 cells/mL. Both cells and parasites were kept for a maximum of 20 subcultured dilution cycles. All cultures were often tested for mycoplasma contamination, and only mycoplasma-negative cultures were used in this study.

Growth curves: For the determination of growth curves in 384-well plates, exponentially growing parasites were diluted in fresh HMI-9 complete media for the initial inoculums (0.5–676.7 × 10^3 trypanosomes/mL). Fifty microliters of each inoculum were added to 384 plates (Greiner, Frickenhausen, Germany), followed by the addition of 10 µL 3% DMSO in phosphate buffered saline (PBS). Several wells were prepared per inoculum. Plates were incubated at 37 °C for 5 days, and parasites from 3 different wells per initial inoculum were harvested and counted daily in a Neubauer chamber for the determination of growth curves. Three independent experiments were performed.

Reference and library compounds: Melarsoprol was kindly provided by Gilles Courtemanche (Sanofi, Paris, France); pentamidine and eflornithine [D, L-α-difluoromethylornithine (DFMO)] were purchased from Sigma-Aldrich (St. Louis, MO). A library comprised of 4000 synthetic, kinase inhibitor-like compounds was purchased from BioFocus (Livingstone, NJ).[24,25]

Compound preparation: Prior to the addition to assay plates, library and reference compounds in 100% DMSO stocks were diluted in PBS in intermediate 384-well plates at 1:33 (v/v) in the case of the SYBR Green assay, and 1:17 (v/v) in the case of the resazurin assay, using a 384-channel pipetting head and a CyBi-Well pipettor (CyBio, Jena, Germany). Compound dilutions for dose–response curves (DRCs) were carried out manually in 100% DMSO in

384-well plates, followed by the same transfer and dilution scheme of intermediate plates described above. Unless otherwise noted, final DMSO concentration was 0.5% for test and control wells in both the resazurin and SYBR Green assays.

SYBR Green assay: Unless otherwise noted, trypanosomes in log-phase growth were suspended at 6×10^3 trypanosomes/mL in complete HMI-9 medium and, under continuous agitation, dispensed into 384-well black plates (Greiner) at 50 µL/well using a Wellmate dispenser (Thermo Scientific, Waltham, MA), followed by transfer of 10 µL of compounds from the intermediate plates as described above. Plates were incubated for 72 h at 37 °C and 5% CO_2, at which point the trypanosomes were lysed by the addition of 15 µL of 5× SYBR Green I (10,000× in DMSO; Invitrogen, Carlsbad, CA) in lysis solution [30 mM Tris pH 7.5, 7.5 mM EDTA, 0.012% saponin, and 0.12% Triton X-100, modified from Co et al.;[20] saponin was obtained from Sigma-Aldrich (Cat. No. S4521)]. The plates were agitated at 1700 rpm for 45 s using the MixMate plate mixer (Eppendorf, Hamburg-Eppendorf, Germany) and incubated in the dark for 1 h at room temperature, followed by reading on a Wallac Victor 3 plate reader (PerkinElmer, Waltham, MA), with excitation at 485 nm/emission at 530 nm during 3 min per plate.

Resazurin assay: The resazurin-based viability assay was performed as described[13] with minor modifications. Resazurin is the dye present in alamar blue, and commercial resazurin powders offer a more affordable alternative to the alamar blue reagent. Exponentially growing trypanosomes were diluted to 2×10^3 trypanosomes/mL in complete HMI-9 medium, plated at 55 µL/well in 384-well black plates using a Wellmate dispenser, and incubated at 37 °C and 5% CO_2. After 24 h, 5 µL of compound solutions were transferred from intermediate plates to assay plates as described above. After an additional 48 h incubation at 37 °C, 10 µL of 140 µM resazurinin in PBS were added to assay plates. Unless otherwise noted, plates were further incubated for 2 h at 37 °C followed by 22 h in the dark at room temperature. Plates were read on a Wallac Victor 3 (PerkinElmer) plate reader with excitation at 535 nm/emission at 590 mm during 3 min.

Cell Titer Glo assay: Occasionally, plates prepared following either the resazurin assay protocol or the SYBR Green assay protocol were developed with the luminescence-based adenosine triphosphate (ATP) measurement reagent Cell Titer Glo (Promega, Fitchburg, WI). The protocol was adapted from Sykes and Avery.[12] Briefly, 30 µL of culture were transferred from assay plates to flat-bottom 384-well white plates (Corning, Corning, NY), mixed with 30 µL of HMI-9, followed by the addition of 15 µL of Cell Titer Glo reagent (Promega). Plates were shaken for 2 min at 500 rpm in a Mixmate plate mixer and incubated for 10 min in the dark at room temperature. The luminescence signal was measured on a Victor 3 (PerkinElmer) plate reader. When Cell Titer Glo was specifically used for the development of plates prepared following the resazurin assay protocol, plates were removed from the incubator 74 h after trypanosome plating and then maintained for 22 h at room temperature in the dark prior to plating of Cell Titer Glo reagent.

Signal stability: After the development of plates with either the SYBR Green or resazurin assay protocol, DMSO and reference-drugs DRC plates were kept in the dark at either room temperature or 4 °C, and the fluorescence was measured daily to determine signal stability. For this purpose, 3 DMSO and/or DRC plates that result from independent parasite cultures and that had been assayed on different days were monitored by both methods.

Time-kill assays: Evaluation of compound antiparasitic activity throughout time was adapted from Jacobs et al.[26] and assessed as follows. Test and reference compounds were twofold serially diluted in 100% DMSO and transferred to assay plates following the same scheme described above. For each compound, 10 concentration points were prepared, and the initial concentrations in the assay plate were 40 µM for test compounds, 200 nM melarsoprol, 120 nM pentamidine, and 900 µM eflornithine. Parasites were plated following the SYBR Green assay protocol described above, and at every 12 or 24 h as indicated, duplicate plates were developed either with SYBR Green or with Cell Titer Glo as described above.

Library screening against trypanosomes: The BioFocus library was screened against *T. b. brucei* at 10 µM with both the SYBR Green and resazurin assays, in duplicate. Compounds were plated immediately after the parasites for the SYBR Green assay, and 24 h after the parasites for the resazurin assay. The whole library was screened in a single day for each replicate. Compounds with normalized antiparasitic activity equal to or greater than 80% were considered hits. The primary screening hits that were selective toward *T. b. brucei* (i.e., not toxic for human cell lines at 10 µM) were "cherry-picked" for confirmatory screening and potency assessment in dose–response curves. Hits were twofold serially diluted to yield 10-point curves starting at 40 µM. For all plates, 200 nM melarsoprol and 0.5% DMSO were used as positive and negative controls, respectively.

Counter-screening: The 4000-compound library had been previously screened against a panel of human cell lines in our laboratory (data not shown), and the cytotoxicity properties toward these cell lines were determined by a viability index (VI), calculated as follows: $VI = (A \times B \times C \times D) / 10^8$, where A, B, C, and D correspond to the normalized viability (%) of cultures of, respectively, the HeLa, Huh7.5, THP-1, and U2OS cell lines when exposed for 60 h to 10 µM of test compound in 0.5% DMSO. The normalized viability (%) of each cell line after compound exposure was determined by the resazurin method. Briefly, 1×10^4 cells/well were plated in 384-well black plates followed by compound treatment in a total volume of 60 µL. After

48 h incubation at 37 °C, resazurin was added to a final concentration of 10 μM, followed by a further 12 h incubation and plate reading as described above.

In confirmatory counter-screening, hit compounds had their potency against THP-1 cells determined in dose–response curves as described above for trypanosomes. Briefly, compounds were diluted and plated as described above, followed by plating of 1×10^4 THP-1 cells/well in RPMI containing 10% FBS, totaling a final volume of 60 μL/well. Plates were incubated at 37 °C and 5% CO_2 for 60 h prior to the addition of 5 μL of 130 μM resazurin in PBS, and they were incubated further for 12 h before reading.

Data analysis: The activity of test compounds was normalized against controls from the same plate according to the following formula: Activity (%) = $[1 - (F_{Cpd} - F_{Pos}) / (F_{Neg} - F_{Pos})] \times 100$, where F_{Cpd} corresponds to the emitted fluorescent signal expressed in arbitrary fluorescence units for the test compound; and F_{Neg} and F_{Pos} correspond to the mean fluorescent signal of the negative and the positive control wells, respectively. In time-kill experiments and THP-1 counter-screening, the positive control was replaced by the blank (complete media containing 0.5% DMSO). For estimation of the hit confirmation rate, compounds were considered "confirmed" when the normalized antiparasitic activity at 10 μM was ≥80%. For screening quality control purposes, the IC_{50}s of reference drugs as well as the coefficient of variability (CV) of control wells and the Z'-factor of each plate were monitored. The CV was calculated by the following equation: CV = (SD / MS) × 100, where SD and MS are the standard deviation of the signal and mean signal of the control wells from the same plate. The Z'-factor was calculated according to Zhang et al.[27] The detection limit (DL), expressed in trypanosomes per milliliter, was calculated according to the following equation: DL = $(3 \times SD_{Blank}) / S$, where SD_{Blank} corresponds to the standard deviation of the blank (HMI-9 medium); and S corresponds to the sensitivity of the method, or the slope of the standard curve of parasite concentration per milliliter versus fluorescence signal for each method. Dose–response curves were prepared using the function log(inhibitor) versus normalized response–variable slope from the GraphPad Prism software, version 5.00 for Windows (www.graphpad.com).

Chemical-clustering analysis: Hit compounds were clustered based on chemical scaffold and activity using the Pipeline Pilot software, version 8.5 (Accelrys; www.accelrys.com). For clustering, extended-connectivity fingerprints (ECFP-4 and ECFP-6) were used, and the predefined average number of molecules per cluster was 10.

Results

SYBR Green Assay Development and Comparison to Resazurin Assay

The resazurin assay has been so far the simplest and most affordable assay for HTS using African trypanosomes. While performing this assay in 384-well plates, we observed that *T. brucei* bloodstream forms presented a strongly reduced motility at the assay endpoint, even in untreated controls (**Suppl. Video S1** and **Suppl. Fig. S3**). The reduced motility was suggestive of decreased trypanosome viability, and it prompted us to hypothesize whether during the course of the resazurin assay development the parasite population had its viability reduced and consequently would be sensitized to compound action; this could potentially result in the selection of false-positive hits during HTS. To address this issue and provide an alternative HTS method, the SYBR Green assay was developed for *T. brucei* in a 384-well format.

During assay development, experimental conditions such as the initial inoculum of trypanosomes in 384-well plates, the incubation time to achieve optimal trypanosome growth, and the volume and the composition of the lysis solution were optimized. Growth curves for different initial inoculums (several densities ranging from 0.5 to 676.6×10^3 trypanosomes/mL) were performed to determine the most suitable inoculum density to achieve optimal trypanosome growth in 384-well plates. **Supplemental Figure S1A** shows that an initial inoculum of 6000 trypanosomes/mL results in a population range of $2–3 \times 10^6$ trypanosomes/mL (equivalent to the maximum culture density observed for this strain—data not shown) in the well after 72 h of growth. This trypanosome density was chosen as the initial inoculum for the development of the SYBR Green assay protocol. Because compounds are normally solubilized in DMSO, the maximum concentration tolerated by trypanosomes in assay conditions was found to be 0.5% DMSO (data not shown).

To ensure the optimal conditions to expose dsDNA after cell lysis, 4 different formulations of lysis buffer were tested on several inoculums that simulate the maximal range of trypanosome densities found in the well at assay endpoint (**Suppl. Fig. S1B**). The best combination was 0.01% saponin and 0.1% Triton X-100 in 15 μL of lysis solution per well, with less or more concentrated solutions resulting in decreased signal when parasite density ranges from 3×10^6 to 4×10^6 trypanosomes/mL. The temperature (room temperature or 37 °C) and the incubation period (1, 4, or 24 h) for signal development were also taken into account to ensure the best relationship between the stability and intensity of the SYBR Green signal. No significant differences or gain in assay quality were found, however, between the tested conditions; and, to secure a simpler and time-effective method, 1 h incubation at room temperature was chosen as the final incubation condition for SYBR Green signal development. For such conditions, the detection limit of the method was calculated as 2.05×10^4 trypanosomes/mL, approximately 3.5 times lower than the mean detection limit of resazurin (**Table 1**).

SYBR Green Assay as an Alternative to Resazurin Assay

After establishing the final experimental parameters, the SYBR Green and resazurin assays were compared regarding the main parameters analyzed in HTS campaigns: the

Table 1. Overview on the Performance Parameters for Both SYBR Green and Resazurin Methods for Final Assay Conditions.

Parameter	SYBR Green	Resazurin
Detection limit ($\times 10^4$ parasites/mL)[a]	2.05 ± 0.31	7.77 ± 6.01
Coefficient of variation of negative control (%)[a]	4.32 ± 1.01	8.47 ± 2.17
Z'-factor[a]	0.76 ± 0.06	0.71 ± 0.10
IC_{50} Melarsoprol (nM)[a]	7.98 ± 0.88	10.59 ± 0.70
IC_{50} Pentamidine (nM)[a]	5.57 ± 1.06	5.97 ± 0.40
Stability at 4 °C[a]	20 days	4 days
Stability at RT[a]	20 days	Less than 24 h
Assay total time	73 h	96 h
Estimated detection reagent cost per plate (US dollars)[b]	1.62	1.53[c]

RT, room temperature.
[a]The values correspond to the mean ± standard deviation obtained from 3 independent experiments.
[b]Products supplied by Life Technologies (Carlsbad, CA).
[c]US$17.61/plate for alamar blue.

Z'-factor, the coefficient of variation, and the IC_{50}s of reference drugs—in this case, melarsoprol and pentamidine. The results are summarized in **Table 1**.

The SYBR Green assay presented lower variability, as indicated by the coefficient of variation of the negative control (4.32% for the SYBR Green assay vs. 8.47% for the resazurin assay) and a considerably more stable signal at both 4 °C and room temperature. In addition, the SYBR Green assay is faster, requiring a total of 73 h for completion—saving around 24 h in comparison with the resazurin assay. Both assays are comparable in terms of assay Z'-factor; IC_{50} values for melarsoprol and pentamidine, which are in accordance with the literature;[13,28] and estimated cost of detection reagent (dye only) per plate. When alamar blue is considered instead of resazurin, however, the reagent cost per plate increases approximately 10 times (**Table 1**).

The SYBR Green Assay Detects Activity of Both Fast- and Slow-Killing Compounds at the Assay Endpoint

One limiting factor for SYBR Green assay sensitivity in monitoring antitrypanosomal activity would be the required time for DNA degradation after cell death. Time-kill experiments for reference drugs were performed using the SYBR Green assay protocol, and developed with either SYBR Green or Cell Titer Glo as detection reagents to better understand the dynamics of the SYBR Green assay for the detection of fast- and slow-killing compounds and the sensitivity of the method when compared to Cell Titer Glo. The activity of melarsoprol and pentamidine, fast trypanocidal compounds, and eflornithine, a slow-killing compound, was monitored throughout time (**Suppl. Fig. S2**). Cell Titer Glo was able to detect cell death caused by all 3 drugs earlier than SYBR Green. At the HTS assay endpoint (72 h), SYBR Green could detect compound activity equally efficiently as Cell Titer Glo for 100% efficacious concentrations, or slightly less efficiently for subefficacious concentrations. Thus, the SYBR Green assay is sufficiently sensitive to detect the antitrypanosomal activity of fast-killing compounds at the assay endpoint, and also of slow-killing compounds to some extent.

Comparison of the SYBR Green and Resazurin Assays in HTS of a Kinase-Focused Library

The primary screen of a commercial kinase-focused library of 4000 compounds was performed at 10 µM and in duplicate (2 independent runs) to minimize variation and permit robust comparison of both assays. A summary of the results is shown in both **Figure 1** and **Table 2**. The SYBR Green assay screen had a slightly better performance in terms of quality control parameters (**Table 2**), and the IC_{50}s for melarsoprol and pentamidine were within the expected range (**Suppl. Table S1**).

The binned distribution of compounds and controls per mean value of normalized activity showed a markedly different distribution pattern between the resazurin and SYBR Green assay screens (**Fig. 1A**). The greater variability of the negative control population in the resazurin assay was clear, because it was also evident that, for this method, the activity of compounds was mostly distributed in extremes (i.e., most compounds displayed either very high or very low activity); fewer compounds displayed activity in the intermediate range when compared to SYBR Green assay screen compound distribution.

The selected hits were further filtered for the removal of compounds that showed promiscuous cytotoxicity against human cell lines. Because this library had been previously screened against a panel of human cell lines (data not shown), only compounds that had a human cell line

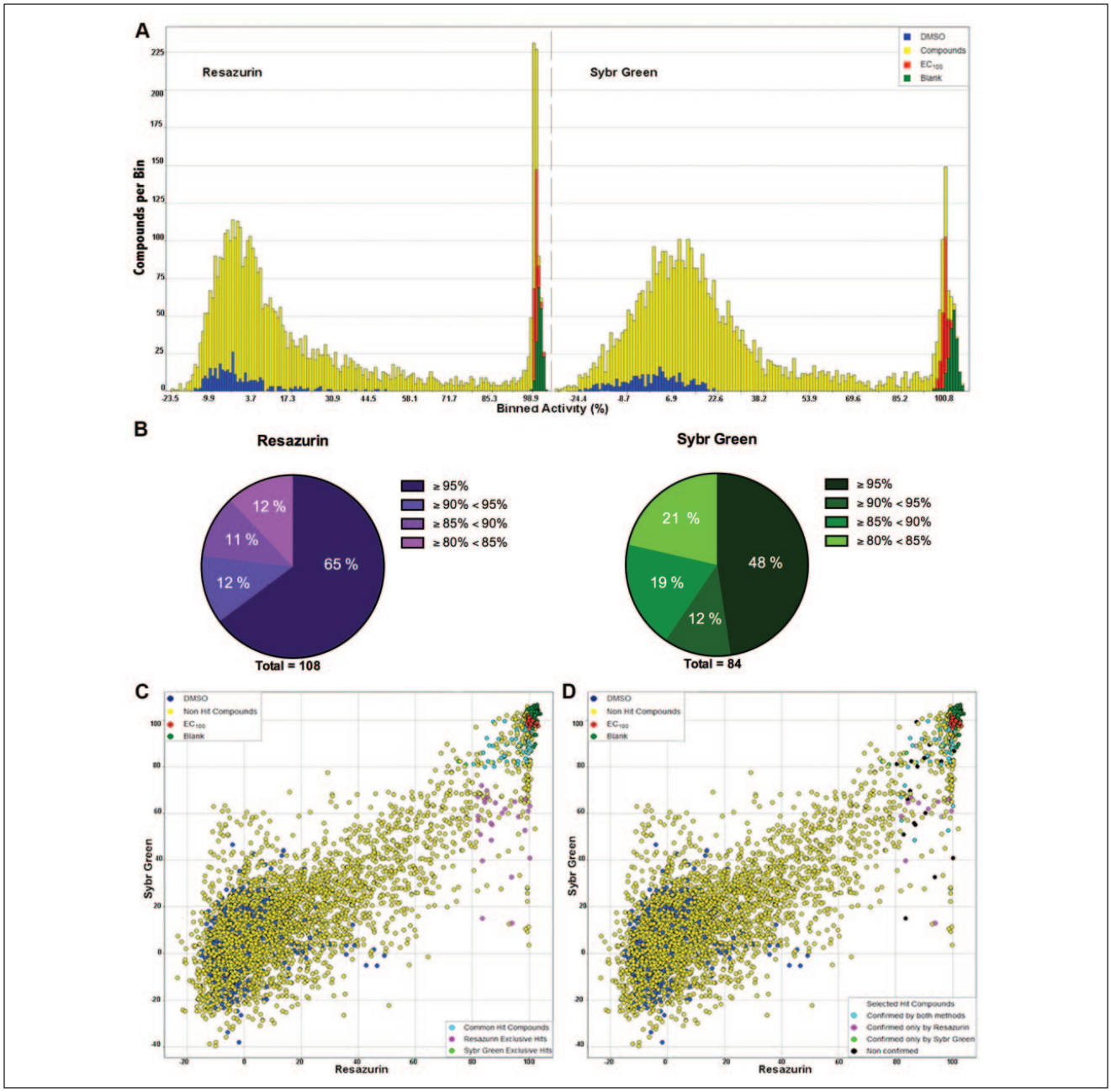


Figure 1. Comparison of antitrypanosomal activity profiles between the resazurin and SYBR Green assays. (**A**) Graphic representation of the library compounds distribution per normalized activity bin [Binned Activity (%), x-axis] for both resazurin and SYBR Green methods. The y-axis shows the number of compounds per bin. Blue, negative control wells (trypanosomes only); yellow, compound wells (trypanosomes and compound at 10 μM); red, positive control wells (trypanosomes and melarsoprol EC_{100} = 40 μM); and green, blank (complete media only). Two independent runs were undertaken for both methods. (**B**) Distribution of primary screening hits per activity bins as indicated in the legends for the resazurin assay screen (left) and SYBR Green assay screen (right) of 4000 compounds. The total number of hits selected is shown below the pie charts. (**C**) Whole screen compound and control activity correlation between the resazurin and SYBR Green methods, highlighting the selected hit compounds after primary screening. Data refer to each well-normalized activity (i.e., percent inhibition in relation to mean values of plate controls). Compounds with activity ≥80% and nontoxic to human cells (data not shown) were selected as hits. Dark blue: negative control wells; red, positive control wells; dark green, blank wells; yellow, nonhit compounds; light blue, nonexclusive (common to both screens) hit compounds; magenta, resazurin screen exclusive hit compounds; and light green, SYBR Green screen exclusive hit compounds. (**D**) Same as in (C), highlighting the results of activity confirmation screening. Selected hits were "cherry-picked" and tested in serial dilution in both assays, and they were considered "confirmed" when the normalized antitrypanosomal activity was ≥80% at 10 μM. The color code for wells is the same as in (C), except for the following: light blue, hits confirmed by both methods; magenta, hits confirmed only by the resazurin assay screen; light green, hits confirmed only by the SYBR Green assay screen; and black, hits that were not confirmed in either method. Among the primary screening selected hits, 5 compounds, which are pointed out in dark gray, were not available for hit confirmation. Data refer to 2 independent experiments.

Table 2. Comparison of SYBR Green and Resazurin Assays' Quality Control Parameters in the High-Throughput Screening of 4000 Compounds.

Parameter	SYBR Green	Resazurin
Z'-factor[a]	0.70 ± 0.04	0.67 ± 0.06
Coefficient of variation of negative control (%)[a]	2.32 ± 0.78	7.70 ± 1.94
Coefficient of variation of positive control (%)[a]	8.76 ± 2.00	6.32 ± 0.90
Coefficient of variation of blank (%)[a]	3.45 ± 2.20	6.06 ± 2.75

[a]Parameters were calculated for individual plates, and the values shown are the means ± standard deviations for all 26 compound plates run (2 runs of 13 plates each) with each assay.

viability index between 1.0 and 1.5 were further progressed for antitrypanosomal activity confirmatory screening.

The difference in activity pattern between the 2 assays reflected the number of hit compounds selected from both screens: 108 compounds with normalized activity ≥80% were selected as hits in the resazurin assay, whereas 84 compounds were selected as hits in the SYBR Green assay following the same selection criteria. This difference was also evidenced with hit compounds distribution per activity bins of 5% increments, showing the increased proportion (65%) of hits with very high activity (≥95%) in the resazurin assay screen, whereas the SYBR Green assay screen had 48% of the hits with activity ≥95% (**Fig. 1B**). Among the hit compounds, 82 were common to both resazurin and SYBR Green assays, 2 compounds were selected as exclusive hits of the SYBR Green assay, and 26 as exclusive hits of the resazurin assay (**Fig. 1C**).

Regarding the whole population of compounds and control wells, both assays were robust and resulted in reproducible data as shown in **Supplemental Table S2**. The correlation coefficient (*r*), determined for normalized activity between the first and second screening runs for each assay and including compounds and controls, was 0.94 for the resazurin assay and 0.82 for the SYBR Green assay, and the correlation was 0.92 between the resazurin assay runs and 0.73 between the SYBR Green assay runs. When only normalized activity of hit compounds was considered, the correlation between runs was 0.65 for the SYBR Green assay and 0.44 for the resazurin assay (**Suppl. Table S2**). This value was similar to the correlation between the resazurin and SYBR Green assay screens, considering the hit compounds' mean normalized activity of both runs (*r*, 0.48) (**Suppl. Table S2**). In summary, the correlation for the whole population of wells was higher in the resazurin assay screens; however, the SYBR Green assay had a better performance of correlation between runs when normalized activity of hit compounds was concerned.

Confirmatory and Counter-Screening for the Determination of Compounds With Selectivity Toward T. brucei

Among the 110 selected compounds, 3 compounds were not available for further studies: 2 hits were common to both assays, and 1 hit was exclusive of the resazurin assay. Thus, 79 compounds selected by both methods, 25 compounds selected only in the resazurin assay, and 2 selected only in the SYBR Green assay were tested for activity confirmation against *T. b. brucei* in dose–response curves to determine the compound potency. In parallel, the hits were also assayed against THP-1 cells to determine compound selectivity toward the parasites. A hit was considered confirmed when the normalized activity of the compound at 10 μM was ≥80% in the confirmatory screening.

Considering only the common hit compounds, the hit confirmation rate was 82.3% (65/79); when considering only the exclusive hits of this assay, the hit confirmation rate dropped to 64% (16/25). For the SYBR Green assay screen, the total hit confirmation rate was 86.4% (70/81) and thus higher than that of the resazurin assay screen. Both exclusive hit compounds for this assay confirmed, and regarding the common hits, the confirmation rate was 84.8% (67/79). One of the 2 SYBR Green exclusive hits was also confirmed by the resazurin assay, thus representing a missed hit or a false negative in the resazurin assay primary screening. From the 16 compounds selected as resazurin assay exclusive hits, 6 were also confirmed by SYBR Green assay. These compounds were also active in the SYBR Green assay primary screen, albeit with lower activity than in the resazurin assay primary screen, and therefore were excluded as hits in the SYBR Green assay screen due to the stringent activity cutoff applied in hit selection (**Fig. 1D**).

Potentiation of Compound Activity in the Resazurin Assay

The resazurin assay screen had a higher hit rate, and the hits had higher activity in comparison with the activity found in the SYBR Green assay screen. Furthermore, the resazurin assay screen had a higher rate of exclusive confirmed hits that could not be detected by the SYBR Green assay. These results could be explained by the fact that the SYBR Green assay may not detect slow-killing compounds within the assay time window; however, this claim is not supported by the time-kill assays performed with eflornithine (**Suppl.**

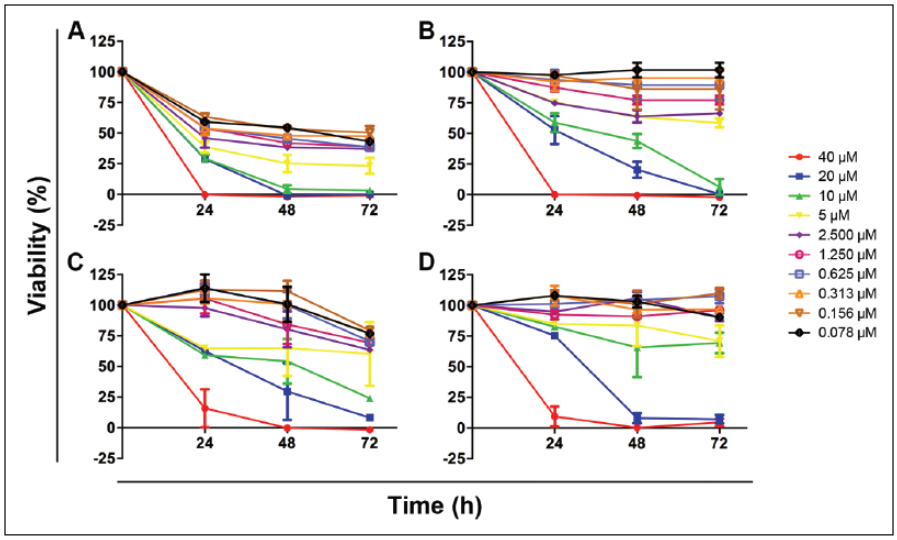


Figure 2. Some of the resazurin assay screen exclusive hits are slow-killing compounds, whereas others kill trypanosomes fast but have only minor activity at screening conditions with the SYBR Green assay. Nonexclusive (**A**) and resazurin exclusive hits (**B–D**) were tested in serial dilution in time-kill assays following the SYBR Green assay experimental conditions, except for the use of Cell Titer Glo as an alternative reagent for signal development and direct assessment of trypanosome viability. Representative compounds are shown. Two independent runs were undertaken.

Fig. S2). Alternatively, compound activity could potentially be exacerbated in the resazurin assay due to the possible reduction in parasite viability under the assay experimental conditions, as we first hypothesized. To further investigate why some compounds were exclusively selected and confirmed for antitrypanosomal activity by the resazurin assay only, and address the hypothesis of whether trypanosomes have reduced viability in the resazurin assay, some hit compounds were selected for further experiments: 9 compounds that were confirmed only by the resazurin assay, and 7 compounds that were confirmed by both assays. In the first set of experiments, parasites and compounds were plated and incubated following the SYBR Green assay protocol, and compound activity was measured every 24 h using either SYBR Green or Cell Titer Glo for signal development.

Representative examples of profiles obtained with hits common to both resazurin and SYBR Green assays (**Fig. 2A**), and hits exclusive to the resazurin assay (**Fig. 2B–D**), are shown. The plot for the common hit showed that at 10 μM (the concentration applied in the primary screening), this compound was able to kill 100% of the population with as little as 48 h of exposure, whereas 10 μM of resazurin assay screen exclusive hits plotted in **Figure 2B** could only reduce population viability to 0% after 72 h exposure, suggesting that indeed the SYBR Green assay screen preferentially detected fast-killing compounds. The other common hit compounds were also identified as fast-killing compounds, and in some cases they showed 100% antitrypanosomal efficacy after 24 h exposure, regardless of their potency (data not shown). Among the 7 compounds that had been confirmed only by resazurin assay, 2 other compounds presented the same pattern observed in **Figure 2B**, further suggesting that a potential limitation of SYBR Green assay may be less sensitivity toward slow-killing compounds. It is also important, however, to emphasize that these compounds can also be detected as active in the SYBR Green assay, albeit with reduced potency at the assay endpoint, presumably due to incomplete DNA degradation. The plots in **Figure 2C and 2D**, however, showed a different pattern: 10 μM of compound in **Figure 2C** had maximum activity of approximately 75% at 72 h exposure, demonstrating that although this compound can partially inhibit the growth of *T. brucei*, it could not be detected by the SYBR Green assay screen because the activity at 10 μM falls lower than the cutoff applied in the primary screening (normalized activity ≥80%). The compound in **Figure 2D**, however, showed a remarkably different activity pattern in the SYBR Green assay: at 20 μM, it reduced the population viability to nearly 5% at 48 h, in a concentration–time-to-kill kinetic similar to 20 μM of the common hit, shown in **Figure 2A**. At 10 μM, however, the compound in **Figure 2D** reduced population viability only to 69.49% (i.e., showed an activity of only approximately 30.51%, far lower than the 80% or more detected in the resazurin assay screening). Altogether, these results suggest that whereas the SYBR Green and resazurin assays may result in the detection of hits with different kinetics to kill trypanosomes, the resazurin assay is also selecting hits that are apparently very potent against trypanosomes under the resazurin assay conditions but only mildly potent under the SYBR Green assay conditions.

As a counterproof, all of the 16 confirmed hits were tested in a second set of experiments, but this time using the resazurin assay as the screening protocol and the Cell Titer Glo reagent as the reporter of cell viability.

The results summarized in **Figure 3** show the activity at 10 μM of these compounds obtained in both sets of experiments described above. Most nonexclusive hits had activity greater than 80% in all tested conditions, except for compound Hit ID 3, in which the activity at 10 μM was approximately 40% in the resazurin assay using resazurin for signal

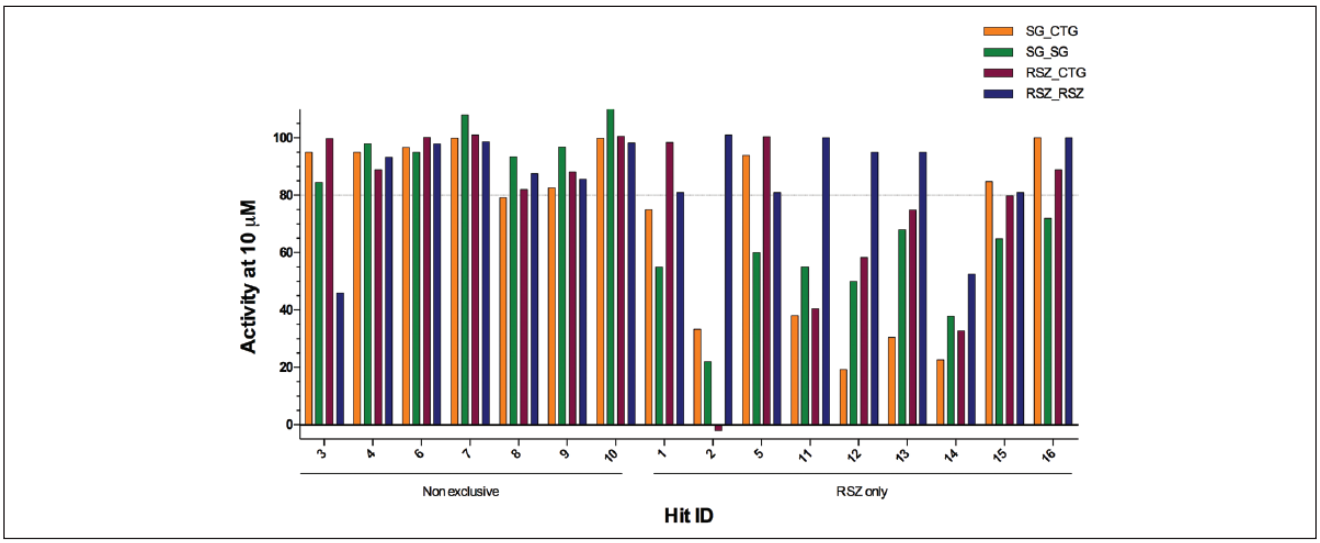


Figure 3. Some resazurin assay screen exclusive hits have pronounced activity in the presence of resazurin. Selected nonexclusive and resazurin assay screen exclusive hits (RSZ only) either were tested in serial dilution following the SYBR Green assay experimental conditions and developed with either Cell Titer Glo (SG_CTG, orange bars) or SYBR Green (SG_SG, green bars), or were tested following the resazurin assay experimental conditions and developed with either Cell Titer Glo (RSZ_CTG, purple bars) or resazurin (RSZ_RSZ, blue bars). A dotted line is shown at 80%, the activity cutoff applied in the selection of hits in the primary screening and used as activity confirmation criteria in the confirmatory dose response screening (shown here). Data refer to 2 independent replicates.

development (RSZ_RSZ). This can be attributed to the higher variation observed with the resazurin assay.

The resazurin assay screen exclusive hits can be classified in 2 major activity patterns: The first group of compounds (Hit ID 1, 5, 15, and 16) was active in all conditions, albeit moderately active (activity ranging from approximately 50% to 70%) only in the SYBR Green assay developed with SYBR Green (SG_SG) assay (**Fig. 3**), suggesting that they are slow-killing compounds, and in these cases there is a delay in the detection of cell death by the SYBR Green assay. A second group of compounds (Hit ID 2, 11, 12, and 13) were markedly highly active in the presence of resazurin only (RSZ_RSZ) (**Fig. 3**), regardless of the experimental protocol used in compound testing. Importantly, these compounds were only moderately active in the other assay setups tested, including the resazurin assay developed with Cell Titer Glo (RSZ_CTG), suggesting that, in these cases, compound activity was increased only in the presence of resazurin and differences in detection of antitrypanosomal activity between the resazurin and SYBR Green assays are not due to the speed with which the compound exerts its cidal effect.

Novel Scaffolds for Antitrypanosomal Drug Discovery

Despite the differences in the hit profile between the compound found in both assay protocols, the majority of the most potent hits were confirmed in both assays. The selectivity index toward trypanosomes (SI) varied from 3.9 to more than 512; indeed, 72% of the compounds presented an SI higher than 10 (data not shown).

The 72 confirmed compounds were analyzed for chemical clustering based on scaffold similarity, yielding 13 clusters. Among these, 11 clusters consisted of novel scaffolds with previously unknown antitrypanosomal activity (**Fig. 4**).

Discussion

The aim of this study was to develop a novel and improved drug-screening assay for drug discovery for HAT, and, in doing so, deliver new potential drug candidates. HTS of compound libraries using whole-cell assays is a simple and viable approach for early drug discovery programs in HAT; nevertheless, there are few reports based on whole-cell HTS assays for *T. brucei*. In the present work, we propose a novel, robust, suitable-to-automation, cost- and time-effective, simple, and reproducible 384-well-format HTS SYBR Green–based assay.

To validate the SYBR Green assay and, most importantly, discover new antitrypanosomal chemical entities, a kinase-focused library composed of 4000 compounds was screened against the *T. brucei* Lister 427 strain using both the SYBR Green and resazurin assays in HTS mode. The parallel screening of the same library in both assays allowed for comparison of the assays' performance in real HTS

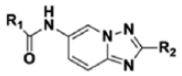
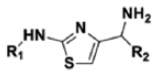
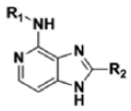
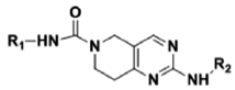
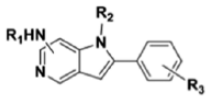
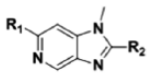
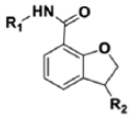
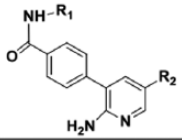
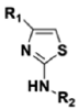
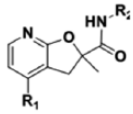
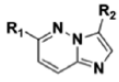
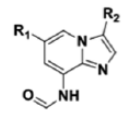
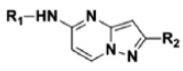
Group	Core structure	Scaffold name	Number of hit compounds	Number of compounds with IC_{50} < 1μM	Number of compounds with SI > 100	Substituents
1		triazolopyridine amide	13	9	8	− R_1: alkyl, substituted phenyl, substituted heterocycles − R_2: substituted or non-substituted phenyl, non-substituted heterocycles
2		aminoethyl thiazole	9	0	0	− R_1:substituted phenylamide, substituted phenylurea − R_2: substituted or non-substituted phenyl, non-substituted cyclohexyl
3		amino imidazopyridine	7	1	1	− R_1:alkyl, substituted and non-substituted cyclohexyl, substituted phenyl − R_2: substituted or non-substituted phenyl, non-substituted heterocycles
4		dihydropyrido pyrimidine carboxamide	2	0	0	− R_1, R_2: substituted phenyl
5		pyrrolo pyridine	19	6	6	− R_1: substituted aromatic amine groups − R_2: H, methyl − R_3: H, methoxy, amide, dioxane, halogen
6		N-methyl imidazopyridine	3	1	0	− R_1, R_2: substituted or non-substituted aromatic amine, substituted or non-substituted phenyl
7		hydrobenzofuran carboxamide	1	1	1	− R_1: alkyl group − R_2: substituted phenyl
8		aminopyridine	1	0	0	− R_1: alkyl containing tertiary amine − R_2: substituted phenyl
9		amino thiazole	10	3	1	− R_1: heterocycle or phenyl substituted alkyl containing primary amine − R_2: substituted phenyl, substituted heterocycles
10		dihydrofuropyridine carboxamide	1	1	0	− R_1, R_2: substituted phenyl
11		imidazopyridazine	3	0	0	− R_1, R_2: substituted phenyl
12		imidazopyridine formamide	2	0	0	− R_1, R_2:substituted phenyl
13		pyrazolo pyrimidine	1	0	0	− R_1: cyclohexyl − R_2: substituted phenyl

Figure 4. Scaffolds with potent antitrypanosomal activity discovered in the screening of a 4000-compound kinase-focused library against *T. brucei*. The core structure of hit chemical clusters is shown, together with the number of hit compounds that integrate each cluster, the number of hits with IC_{50} in the nanomolar range, and the number of compounds with a selectivity index (SI) greater than 100. The substituents for each scaffold are shown in the last column.

conditions and also whether the hit profiling obtained in both screens would be considerably different.

A thorough comparison demonstrated that the SYBR Green assay is more sensitive, less variable, and faster to develop than the resazurin assay, all features of major importance in HTS. SYBR Green also had a better confirmation rate (approximately 86%) than the resazurin assay (75%), and some of the exclusively identified hits identified in the resazurin assay could not be confirmed or presented lower activity when rescreening with SYBR Green or Cell Titer Glo. This fact, allied with the increased activity of the selected hits in the resazurin assay, raised the hypothesis that this assay could lead to an additive "intrinsic activity" that further debilitates trypanosomes, thus decreasing the population viability and artificially increasing hit rates. Resazurin has been extensively used for monitoring *T. brucei* viability in drug assays in 96-well plates; however, no data regarding toxicity to trypanosomes are available in the literature. We suppose that in the 384-well-plate format, a longer exposure to resazurin (24 h) in comparison to the 96-well-plate format (4 h) and/or a relatively long incubation period at room temperature (22 h) could account for the reduced parasite motility observed at the resazurin assay endpoint (**Suppl. Video S1**), suggesting that parasite viability is reduced in these experimental conditions.

The practical effects of using a resazurin assay for HTS for HAT drug discovery can be speculated. Besides offering no advantage in terms of cost and time, the resazurin assay resulted in a higher rate of false positives. The greater assay variability could also result in a higher rate of false negatives in the resazurin assay. The resazurin assay, however, seems to be more sensitive for the detection of compounds that kill trypanosomes slowly. This could be due to cell membrane permeability kinetics, features that can be improved by chemical optimization of scaffolds; alternatively, it could (also) be due to the compound mechanism of action.

The major novelty of this study was the discovery of novel scaffolds with potent antitrypanosomal activity (**Fig. 4**). Kinase targets have been addressed by the pharmaceutical industry and offer potential for the development of new drugs in several therapeutic areas.[29] The library screened in this study is a focused set of approximately 4000 drug-like small molecules of structural features that favor interactions with kinases and/or phosphatases. Knowledge of the crucial role of protein kinases in cell survival in multicellular organisms has directed attention toward this enzyme class in parasitic protozoa to search for alternative drug targets to treat tropical diseases.[30,31]

Most of the 72 hit compounds identified here against *T. b. brucei* presented a high selectivity index toward trypanosomes. Among those, triazolopyridine-based inhibitors (**Fig. 4**, group 1) have been described as atypical kinase inhibitors, applicable to p38 mitogen-activated protein kinase (MAPK).[32] There are 3 major classes of MAPKs: ERK, p38, and JNK. So far, 2 ERK homologues (KFR1 and TbMAPK2) were identified in *T. brucei* along with 2 kinases that share features of both ERK and cyclin-dependent kinases (TbECK1 and TbMAPK5).[30,33] TbMAPK5 is involved in the growth and differentiation of bloodstream forms, and knockdown studies showed a reduction of the peak parasitemia in mice.[33] Extensive studies are needed, however, to determine the targets of the compounds reported in this article. Although not mandatory, knowledge of the target and its structure would facilitate the development of highly antiparasitic selective drugs. And, although some hits already have good potency, further medicinal chemistry optimization is necessary to improve activity and/or pharmacological properties and establish the structure–activity relationship (SAR). In conclusion, the contribution of this study is not only a novel HTS *T. brucei* viability assay that overcomes the limitations of the standard method but also attractive chemical scaffolds that will be valuable starting points for HAT drug development.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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