Invited Article

Application of a resazurin-based high-throughput screening assay for the identification and progression of new treatments for human African trypanosomiasis

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ABSTRACT

Human African trypanosomiasis (HAT) is caused by the protozoan parasite Trypanosoma brucei, and the disease is fatal if untreated. There is an urgent need to develop new, safe and effective treatments for HAT because current drugs have extremely poor safety profiles and are difficult to administer. Here we report the development and application of a cell-based resazurin reduction assay for high throughput screening and identification of new inhibitors of T. b. brucei as starting points for the development of new treatments for human HAT. Active compounds identified in primary screening of ~48,000 compounds representing ~25 chemical classes were titrated to obtain IC_{50} values. Cytotoxicity against a mammalian cell line was determined to provide indications of parasite versus host cell selectivity. Examples from hit series that showed selectivity and evidence of preliminary SAR were re-synthesized to confirm trypanocidal activity prior to initiating hit-to-lead expansion efforts. Additional assays such as serum shift, time to kill and reversibility of compound effect were developed and applied to provide further criteria for advancing compounds through the hit-to-lead phase of the project. From this initial effort, six distinct chemical series were selected and hit-to-lead chemistry was initiated to synthesize several key analogs for evaluation of trypanocidal activity in the resazurin-reduction assay for parasite viability. From the hit-to-lead efforts, a series was identified that demonstrated efficacy in a mouse model for T. b. brucei infection and was progressed into the lead optimization stage. In summary, the present study demonstrates the successful and effective use of resazurin-reduction based assays as tools for primary and secondary screening of a new compound series to identify leads for the treatment of HAT.

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Human African trypanosomiasis (HAT) or sleeping sickness is caused by the trypanosomatid protozoan parasite Trypanosoma brucei. Trypanosome parasites are transmitted to humans through a bite from the tsetse fly insect vector. The two main trypanosome species that cause HAT are the West African variant (T. b. gambiense) and the East African variant (T. b. rhodesiense) (Mauldin, 2006). Without therapeutic intervention, trypanosomes migrate from the bloodstream into the central nervous system (CNS) leading to a second chronic stage of the disease that results in a coma and ultimately death of the patient. Approximately 50 million people in more than 20 sub-Saharan African countries are at risk of contracting HAT and the World Health Organization (WHO) estimates that 30,000 new cases of HAT occur each (Brun et al., 2010; WHO, 2012).

tion, a situation that presents significant challenges in endemic areas with limited public health facilities and resources. The non-CNS penetrating drugs pentamidine (T. b. gambiense) or suramin (T. b. rhodesiense) are effective for treatment of acute disease (Stage 1) when the parasites are limited to the hemolymphatic system. After parasites enter into the CNS (Stage 2 disease), patients are generally treated with either the arsenic derivative melarsoprol or the ornithine decarboxylase inhibitor eflornithine. Eflornithine is only effective against the subspecies T. b. gambiense (Burchmore et al., 2002) and is difficult to administer in the disease endemic region. Melarsoprol is particularly unsafe because it causes reactive encephalopathy in 5–10% of patients, and 50% of these patients die from this adverse reaction (Pépin and Milord, 1994; Brun et al., 2001). Of particular concern is the increased incidence of melarsoprol treatment failures of up to 25% that have been reported in endemic areas (Brun et al., 2001). Additionally, trypanosomes

change their main surface coat, the variant surface glycoprotein (VSG), to evade the host immune response, and this has severely limited options for vaccine development as a means to control HAT (Pays, 1995). Consequently, chemotherapy remains a major tool for HAT control efforts, and this clearly requires new, safe and effective drugs.

To accelerate the discovery of novel leads for the treatment of HAT, it is essential to have a simple, robust and inexpensive assay to identify and advance suitable chemical starting points targeting *T. b. brucei*. The assay should serve as a tool for high throughput screening (HTS) to assess large sets of chemical libraries and prioritize newly synthesized analogs in hit-to-lead and lead optimization phases of the discovery process. The establishment of a simple *in vitro* cell culture system for axenic growth of *T. b. brucei* (Baltz et al., 1985; Hirumi and Hirumi, 1989; Kaminsky and Zweygarth, 1989) has led to the exploration of a variety of whole cell assay formats (Muskavitch et al., 2008) for the identification of compounds with anti-trypanosomal properties. For evaluating viability following exposure to test compounds, the Alamar Blue™ (resazurin) (Räz et al., 1997; Sykes and Avery, 2009) and Cell-Titer-Glo™ luminescent cell viability assay (Mackey et al., 2006) methods have emerged as those most amenable to HTS because of their high signal-to-background ratio and reproducibility. The resazurin assay is preferred due to lower cost and has been used for quantitative evaluation of the proliferation of various cell types including human cell lines (Ahmed et al., 1994; O'Brien et al., 2000), fungi (Tiballi et al., 1995), bacteria (Baker and Tenover, 1996; Franzblau et al., 1998) and protozoan parasites (Räz et al., 1997; Shimony and Jaffe, 2008; Sykes and Avery, 2009; Nare et al., 2010). Resazurin is an oxidation–reduction sensitive dye that changes color and fluorescence properties upon reduction by living cells. Only viable cells generate a fluorescent signal because they are metabolically active. These properties of resazurin have been exploited to provide a quantitative measurement of parasite proliferation and viability to identify a variety of inhibitor compounds. The incorporation of such a robust and reliable assay into a drug discovery screening cascade could play a key role in advancement of *T. b. brucei* hits through hit-to-lead and lead optimization phases.

Here we report the development and application of a simple and inexpensive resazurin-reduction *T. b. brucei* cell-based HTS assay for primary screening and hit-to-lead progression of new lead chemical series for the treatment of HAT. The assay measures the ability of *T. b. brucei* parasites to metabolize resazurin after a 72 h incubation in the presence of test compounds. Using this assay, a library of ∼48,000 SCYNEXIS proprietary compounds representing 25 unique chemical classes was screened at a nominal concentration of 2 μg/mL. Compounds showing >75% inhibition were selected and their IC_{50} determined using a triplicate 10-point titration procedure. In parallel, these compounds were evaluated in a similar resazurin-based cytotoxicity assay in mouse L929 fibroblasts to facilitate identification of broadly biocidal compounds. Screening data from each series was examined for preliminary structure–activity relationships (SARs) for trypanocidal activity, and those showing selectivity for trypanocidal activity relative to cytotoxicity were progressed into a variety of secondary assays to select candidates for hit-to-lead optimization in an integrated drug discovery program. Key secondary assays included assessment of changes in potency in the presence increasing serum, time to kill measurements and determinations of reversibility of trypanocidal effects following transient exposure to test inhibitor. Based on profiling and prioritization of newly synthesized compounds from the hit-to-lead effort using the resazurin-based trypanocidal and cytotoxicity assays, a lead class with efficacy in the mouse model for HAT was identified.

2. Materials and methods

2.1. Parasite and cell culture

The bloodstream-form trypanosome *T. b. brucei* 427 strain was used in all assays for assessment of compound sensitivity *in vitro*. Parasites were routinely cultured in T-25 vented cap flasks (Corning Incorporated, NY) and kept in humidified incubators at 37 °C and 5% CO_2. The parasite culture media was complete HMI-9 medium (Hirumi and Hirumi, 1989) containing 10% FBS, 10% serum plus (JRH Biosciences Inc., Lenexa, KS) 100 units penicillin and 0.1 mg/mL streptomycin. To ensure log growth phase, trypanosomes were sub-cultured every 2–3 days at a dilution of 1:100 in fresh HMI-9 medium. L929 mouse fibroblast cells (ATCC, Manassas, VA) were maintained as adherent cultures in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 100 units penicillin and 0.1 mg/ml streptomycin.

2.2. Optimization of resazurin reduction to measure T. brucei viability

To determine the relationship between cell number and resazurin fluorescence signal, *T. b. brucei* parasites were serially diluted (100 μL) into 96-well tissue culture plates from a stock solution with 1×10^7/mL. A stock solution of resazurin was prepared in Dulbecco's phosphate buffered saline (D-PBS) at 12.5 mg/mL and various concentrations (5–20 μL/well) were tested by incubation with parasites at 37 °C. Fluorescence due to reduction of resazurin by viable parasites was read on the EnVision fluorometer (λ_{ex} = 544 nm; λ_{em} = 590 nm) (Perkin Elmer) following incubation periods of up to 8 h. For determination of ideal seeding density for compound evaluation, *T. b. brucei* parasites were plated into 96-well plates at various starting densities and incubated at 37 °C and 5% CO_2 for 72 h prior to addition of resazurin and assessment of viability.

2.3. In vitro compound sensitivity assay and high throughput screening

To prepare for assay, *T. b. brucei* parasites in the log phase of growth were diluted 1:10 in HMI-9 media, and 10 μL was counted using a hemocytometer. Parasites were diluted to 2×10^5/mL in HMI-9 to generate a $2\times$ working concentration for assay to evaluate trypanocidal activity. For HTS, compounds were added to test plates to achieve a final concentration of 2 μg/mL. For IC_{50} determination, compounds were serially diluted in dimethyl sulphoxide (DMSO) and 0.5 μL added to 50 μL HMI-9 into 96-well plates using Biomek NX liquid handler (Beckman Coulter, Fullerton, CA). Parasites from the diluted stock were added to each well (50 μL) using a Multidrop 384 dispenser (Thermo Electron Corporation) to give a final concentration of 1×10^5/mL parasites and a final DMSO concentration of 0.5%. After 72 h of incubation at 37 °C and 5% CO_2, resazurin (20 μL of 12.5 mg/mL stock in phosphate buffered saline) was added to each well using a Multidrop 384 dispenser (Fisher Scientific) and plates were incubated at 37 °C for 4–6 h or until a signal to background ratio of 8–10-fold was achieved. Fluorescence was read on an EnVision plate reader as described in the optimization protocol.

Compounds showing ⩾75% inhibition when tested at 2 μg/mL were evaluated in a 10-point dose response, 2-fold dilution experiment starting at 5 μg/mL. For calculation of IC_{50} values, sigmoidal dose response curves were generated using XLfit curve fitting software from IDBS (Guilford, UK). IC_{50} is defined as the amount of compound required to decrease cell viability of *T. brucei* by 50% compared to those grown in the absence of test compound.

2.4. Mammalian cell cytotoxicity assay

To prepare for assay, L929 cells in log growth phase were removed from the T-25 flask using 1 mL of 0.05% trypsin and a

10 μL aliquot was counted using a hemocytometer to determine cell concentration. Cells were diluted to 2×10^4/mL in DMEM to prepare for plating, and 100 μL of cells was added to a 96-well plate using a Multidrop 384 dispenser. Cells were allowed to adhere for 24 h prior to removal of plating media and replacement with 50 μL of fresh medium into each of the wells. Compounds to be tested were serially diluted in DMSO and 0.5 μL of the dilutions added to the 50 μL of DMEM already in the plates. An additional 50 μL of DMEM was added using a Multidrop 384 dispenser to ensure proper compound mixing within each well and to achieve the desired starting concentration. After 72 h of incubation at 37 °C and 5% CO_2, resazurin was added and the fluorescence signal allowed to develop for 3–4 h until a signal to background ratio of 10-fold was achieved prior to reading and data evaluation as described for the *T. b. brucei* assay.

2.5. Serum shift assay

To assess the potential effects of protein binding on trypanocidal activity, parasite viability assays were performed in the presence of increasing concentrations (2.5–20%) of FBS. Serum plus was not added to the HMI-9 media used in this assay because the supplement contains up to 12% bovine serum. *T. b. brucei* were conditioned to the test serum concentration for at least one round of passage prior to assay. Compound addition, incubation, signal detection and IC_{50} determinations were conducted as described in the general *T. b. brucei* screening assay. Compound activity was also evaluated on parasites in the presence of horse or mouse serum added to a final concentration of 10%.

2.6. Time to kill assay

Determination of compound-mediated killing of *T. b. brucei* in vitro over time (i.e. time to kill) was conducted using the CellTiter-Glo™ reagent (Promega Inc., Madison, WI) to measure parasite ATP content as a real time indicator of viability. Test compounds were serially diluted into white clear-bottom 96-well plates (Corning Inc. Life Sciences, Lowell, MA) containing HMI-9 media (50 μL/well), and parasites were added at a concentration of 2×10^5/mL (50 μL/well) to duplicate plates for each evaluation time point. At the selected time, 45 μL of CellTiter-Glo™ reagent was added to each set of duplicate plates. The contents of the wells were mixed to lyse the parasites, and plates were incubated in the dark for 10 min and read on the EnVision plate reader using a luminescence protocol.

2.7. Test for reversibility of trypanocidal effects

To establish the time required to cause persistent or irreversible effects by test compounds, *T. b. brucei* parasites were assessed for their ability to recover from transient exposure to test compounds. Parasites were seeded in 96-well plates at 1×10^6/mL (100 μL/well) and incubated with serially diluted test compound. One plate was prepared for each time point, and at the designated time, a plate was removed and centrifuged at 4,400 rpm for 5 min to sediment the parasites. The supernatant was aspirated and 100 μL of warmed HMI-9 was added to the plate. The plate was then returned to the centrifuge, and this process was repeated for a total of three washes. Parasites were re-suspended in 100 μL of warmed media and 20 μL of this suspension was added to 80 μL of HMI-9 media in triplicate plates. Following a 72 h incubation, resazurin was added and trypanocidal activity determined as described for the *in vitro* sensitivity assay.

2.8. Efficacy in the mouse model for acute HAT

For efficacy studies against acute infections, groups of three female Swiss Webster mice (Ace Animals, Boyertown, PA) were injected intraperitoneally (i.p.) with freshly drawn infected rat blood containing 500 trypanosomes (*T. b. brucei* EATRO 110 strain). Test compounds were formulated in 20% ethanol/50% polyethylene glycol 400% and 30% carboxymethylcellulose and given at 200 μL per 25 g animal orally or via i.p. injection. Animals were monitored daily for a period of 30 days for signs of compound toxicity and clinical disease. After the end of the each dosing period, parasitemia was checked twice weekly by microscopy using smears prepared from tail vein blood. Animals remaining parasite free for more than 30 days beyond the end of the treatment period were considered cured (Bacchi et al., 1996).

3. Results and discussion

The trypanosomatid protozoan parasites *T. b. brucei* grow quite robustly under axenic conditions *in vitro*, and they reach sufficient density in microtiter plates to produce a readily detectable fluorescence signal upon incubation with resazurin. When added at 20 μL/well, from a 25 mg/mL stock, resazurin is reduced linearly over a 3–4 h period after which there appears to be a saturation of the fluorescence signal, particularly at high parasite densities (Fig. 1A). The maximum density reached in the control wells is in the range of $2–5 \times 10^6$/mL and the signal to background (S/B) ratio is 8–10-fold following incubation with resazurin. The S/B for *T. b. brucei* reported in the current study is lower than that reported for *T. b. rhodesiense* (15:1) and much higher than the 3:1 obtained with *T. b. gambiense* (Räz et al., 1997). These differences can be attributed to either low dehydrogenase activity responsible for metabolizing resazurin or reduced uptake of the dye substrate. Differences in the composition of media used to culture trypanosomes could potentially account for some of the variation observed in studies from different laboratories.

For plate based assays, *T. b. brucei* parasites were seeded at 1×10^5/mL which resulted in a low level or non-detectable level of fluorescence at the start of the assay process and a 10-fold increase in signal after the 72 h assay period. From studies of DMSO amounts to be used in the assay, ~0.5% was the maximum concentration that did not result in negative effects on parasite growth during the assay (data not shown). This level of DMSO tolerance in the assay is consistent with that reported previously from other studies (Räz et al., 1997; Sykes and Avery, 2009). To assess plate uniformity and signal to noise, the maximum, median and minimum signal for developing the HTS assay was determined using 0.4% DMSO, 5 ng/mL and 2,000 ng/mL of pentamidine respectively (Fig. 1B). The typical Z′ for the assay was 0.7–0.9 and this parameter is used to accept results from screening activities. Results from this experiment suggest that the resazurin assay is capable of discriminating among compounds with different levels of inhibition (0–100%) in the *T. b. brucei* viability assay during the screening process. In addition to primary screening, the resazurin-reduction assay was evaluated for its suitability for the assessment of IC_{50} values to allow for comparison of compounds during the screening and hit progression efforts. Previous studies have determined that IC_{50}s from the resazurin-based assay are comparable to those obtained using other fluorophores, e.g. BCECF-AM (Räz et al., 1997) to assess parasite viability. In this study, we found that measurement of *T. b. brucei* IC_{50} values using resazurin assay or the more expensive Cell-Titer-GLO assay (Mackey et al., 2006) yielded very comparable results (data not shown). Dose response curves and IC_{50} values for the reference compounds pentamidine and suramin
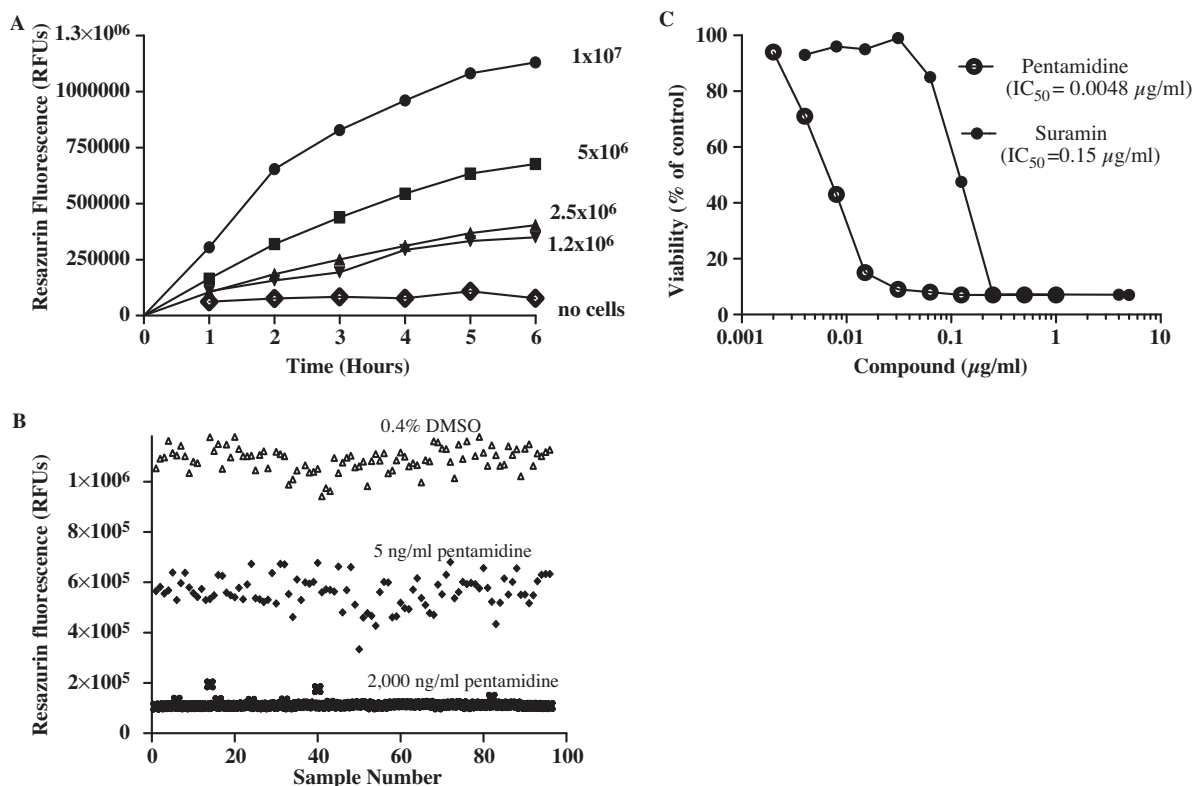
Fig. 1. (A) Correlation between the numbers of *T. b. brucei* parasites and resazurin reduction signal. Serial two-fold dilutions of parasites starting at 1×10^7/mL were prepared in 100 μL followed by addition of resazurin and fluorescence measurement to determine parasite viability. (B) Validation of *T. b. brucei* automated screening assay. *T. b. brucei* parasites were treated with 0.4% DMSO, 5 ng/mL pentamidine (50% inhibition) or 100 ng/mL pentamidine (100% inhibition) to produce maximum, median and minimum fluorescence signal upon addition of resazurin. (C) Dose response of pentamidine and suramin on *T. b. brucei* growth following 72 h incubation in the presence of inhibitors. Data represents averages of triplicate determinations measured as a percentage of the resazurin signal in the absence of compound.

from the resazurin based assay are shown in Fig. 1C, and these values are consistent with those reported in the literature (Räz et al., 1997; Sykes and Avery, 2009).

Using conditions established in the optimization phase, the resazurin-based assay was used to screen a ~48,000 small molecule library in which each compound was added at a final concentration of 2 μg/mL. The library consisted of approximately 25 distinct structural classes with approximately 2,000 compounds per chemical class. Each screening plate contained 88 test compounds, pentamidine (wells A1 and B1), suramin (wells C1 and D1) and DMSO in wells E1-H1 to enable calculation of% inhibition of parasite viability using maximum (DMSO) and minimum (pentamidine and suramin) signals. Using an inhibition cut-off of ⩾75%, which corresponds to the mean + 3 standard deviations of all the screen values, approximately 1,106 compounds (2.3% hit rate) were selected for further evaluation (Fig. 2A and B). In order to confirm trypanocidal activities observed in the primary screen, all actives were subjected to dose response analysis starting at 5 μg/mL and compound series with representative analogs which exhibited $IC_{50}s \leqslant 1$ μg/mL were advanced into the next phase of testing. Additionally, to progress into the next phase of testing, actives from the primary HTS campaign were required to show selectivity for parasites versus mammalian cells grown under similar conditions. Therefore, all HTS hits were tested for cytotoxicity against the L929 mouse fibroblast cell line. In cases where the majority of *T. b. brucei* active members of a chemotype showed cytotoxicity towards mammalian cells, the entire series was deprioritized for progression into hit-to-lead phase. The result of this evaluation led to the selection of 150 compounds from six chemical classes which exhibited the desired combination of *in vitro* potency against *T. b. brucei*, low cytotoxicity towards mammalian cells and some evidence of preliminary SAR

(Fig. 2B). The six distinct chemical series with the total numbers screened per series and selections for dose response analysis are shown in Table 1.

After preliminary screening and hit confirmation, a hit-to-lead effort was initiated on six selected chemotypes (Table 1). Newly synthesized compounds were evaluated in dose response with *T. b. brucei* and cytotoxicity testing with L929 cells. Representative compounds from the synthetic efforts were progressed into a variety of secondary biological assays to assess serum shift (protein binding), time to kill and reversibility of compounds effects (Fig. 4). The serum shift assays measured changes in IC_{50} values by using the resazurin-based *T. b. brucei* viability assay in the presence of increasing concentration of serum (2.5–20%). This process serves as an early indicator of potential impact of compound binding to serum protein on potency against *T. b. brucei*. Serum shift analysis on hit-to-lead compounds in this study is exemplified using representatives from three different hit series (Fig. 3). Sulfanilides and amidoximes show a significant shift in $IC_{50}s$ when serum is increased from 2.5% to 20% (20–30-fold shift) while arylazoles are not affected (<3-fold shift). Based on these findings, part of the early synthetic effort on sulfanilide and amidoxime optimization focused on reducing lipophilicity which appears to be a major contributor to serum protein binding. The chemical stability of sulfanilides was found to vary widely under aqueous conditions in the presence or absence of serum and this series was discontinued. For arylazoles, the key liability was the narrow selectivity for parasite vs. mammalian cells and the series was ultimately dropped for this reason (Table 2).

Because our compound progression criteria require demonstration of efficacy in the mouse model to be characterized as leads (Fig. 4), we profiled the potency of various newly synthesized
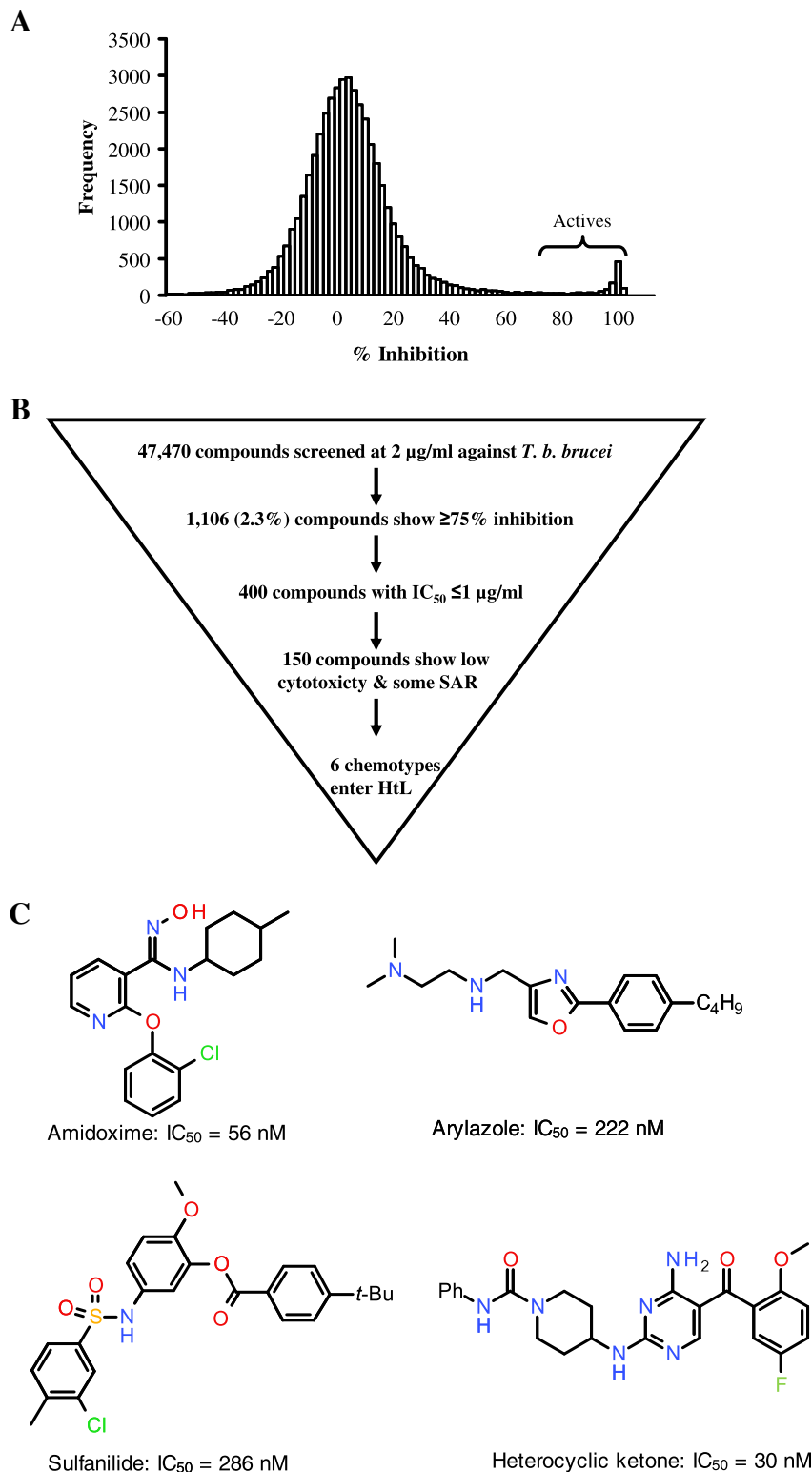
Fig. 2. (A) Frequency distribution of the 47,470 compounds based on the resazurin viability signal after 72 h incubation of *T. b. brucei* in the presence of 2 μg/mL compound. Based on these data, a cut-off of ⩾75% inhibition (3 SD) was used to selective actives for progression in the next step of the screening cascade. (B) HTS and follow-up strategy for trypanocidal screening using the resazurin assay. Funneling of the 47,470 compounds through a variety of assays and selection of ideal lead series to enter hit-to-lead optimization phase is shown. (C) Structures and IC₅₀ for representative compounds from the hit series that entered hit-to-lead activities.

compounds against *T. b. brucei* grown in the presence mouse serum. To do this, *T. b. brucei* parasites were adapted to grow in the presence of 10% mouse serum prior to determination of IC₅₀s on representative compounds from the six chemotypes. Compounds from the amidoxime chemotype were found to be completely

inactive in the presence of mouse serum. All the other series displayed essentially equivalent potency in both mouse and bovine serum (data not shown). While the amidoxime hit to lead effort identified compounds with sub-micromolar activity against *T. b. brucei in vitro*, further exploitation of this chemotype was limited

Table 1
Hit classes identified from primary screening of *T. b. brucei* using the resazurin-reduction HTS assay and selected for progression into hit-to-lead phase.

| Compound class[a]	Screened[b]	Hits[c]	Hit rate (%)
Aminoketones	779	61	8
Pyramidoximes	2151	240	11
Arylazoles	2239	52	2
Aminooxazoles	2339	159	7
Sulfanilides	1130	23	2
Heterocyclic ketones	3985	302	8

[a] Chemical series that were selected after HTS for further investigation.

[b] Total number of analogs screened from each of the libraries that showed selectivity and evidence of SAR following analysis of HTS using cell-based *T. b. brucei* assay.

[c] Number of compounds in each library that show ⩾75% inhibition of *T. b. brucei* when screened at a nominal concentration of 2 μg/mL in the HTS assay.

by complete loss of potency in the presence of mouse serum. An understanding of the mechanisms that limit efficacy of amidoximes in the presence of mouse serum is required before this series could be developed any further as a potential drug candidate for the treatment of HAT. Interestingly, compounds from this class were also inactive in the presence of horse serum, which is often used in place of bovine serum in *T. b. brucei* culture (Kaminsky and Brun, 1998). Amidoxime compounds with reasonable *in vitro* pharmacokinetic properties were found to be inactive in the *in vivo* mouse model for HAT, consistent with lack of trypanocidal activity against the parasite in vitro when assays are conducted in the presence of mouse serum.

To initiate a preliminary understanding of the pharmacodynamics of the early hit-to-lead compounds, time to kill studies were conducted in which the ATP-dependent luciferase assay was used
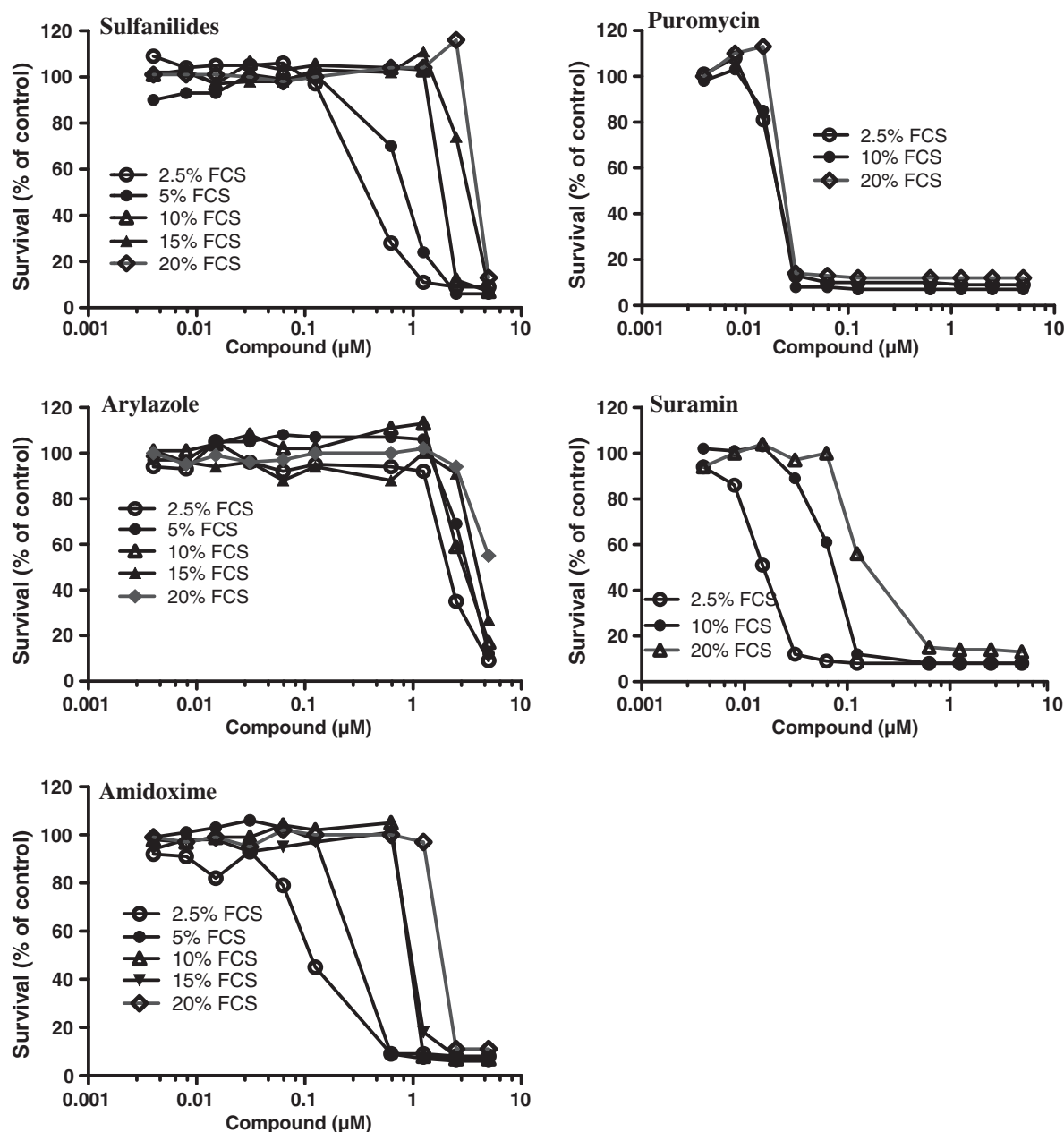
Fig. 3. Shift in trypanocidal activity of compounds in the presence of different concentrations of serum. *T. b. brucei* parasites were incubated with different concentrations of test compound in the presence of increasing concentrations (2.5–20%) of bovine serum. Parasite viability was determined after 72 h using resazurin reduction assay.
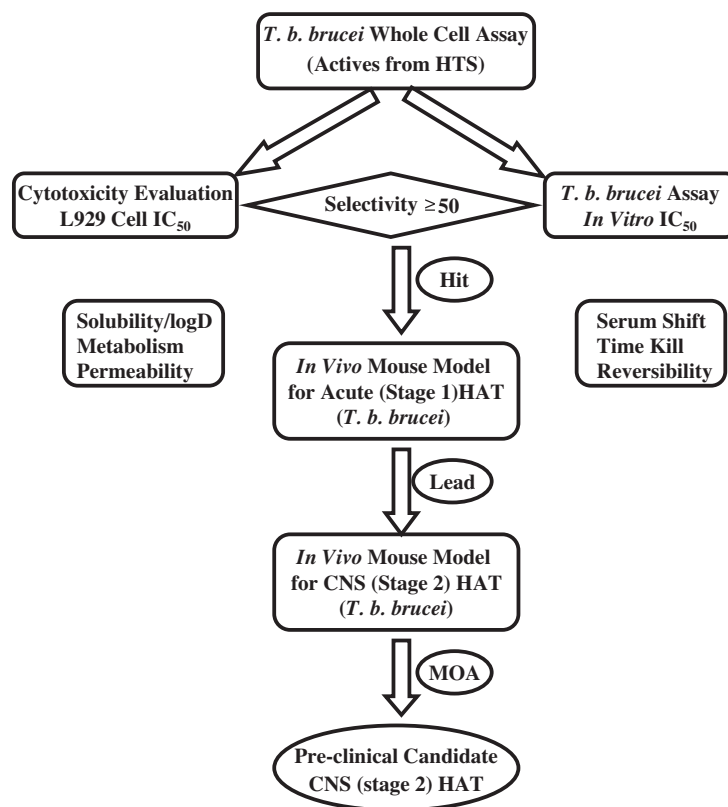
Fig. 4. Overall strategy and screening cascade for the discovery and progression of new treatment for human African trypanosomiasis. Actives from high throughput screening with the resazurin-based whole cell *T. b. brucei* assay where evaluated against mammalian cells to determine parasite versus host cell selectivity. Hits from this process were re-synthesized to confirm activity prior to initiation of hit-to-lead expansion. Compounds from the hit-to-lead process were evaluated in a series of assays to determine physico-chemical properties, metabolic stability, permeability, changes in potency in the presence of serum, time to kill kinetics and reversibility of trypanocidal effects. Representative analogs from each of the hit-to-lead series were evaluated for efficacy in a mouse model for acute HAT prior to initiation of lead optimization process to develop compounds with the ability to cure Stage 2 disease.

in place of the resazurin assay to determine *T. b. brucei* viability. Fluorescence signal development in the resazurin assay requires as least 3–4 h to assess parasite viability and is therefore not appropriate for time to kill assays where instant measurement of viability is essential. Representative compounds from the amidoxime and arylazole chemotypes displayed rapid parasite killing with significant reduction in parasite viability in the first 3 h of incubation with inhibitors. Maximum IC_{50} values achieved within 24 h (Table 3) were similar to those observed when parasites where incubated for 72 h in the presence of inhibitors, suggesting rapid cidal action. In contrast, aminooxazoles and heterocyclic ketones showed very limited effects on parasites in the first 3–6 h, and IC_{50}s on representative compounds from this class were significantly higher at 24 h than at 72 h, suggesting slower onset

of trypanocidal effects (Table 3). Control compounds suramin and pentamidine displayed time to kill kinetics intermediate between these two groups of newly synthesized hit-to-lead compounds. Time to kill kinetic studies have been used effectively to correlate *T. b. brucei* parasite killing *in vitro* with that observed *in vivo* and thus predict dose regimens required to achieve efficacy *in vivo* (Jacobs et al., 2011). Although fast killing mechanisms could potentially arise from non-specific effects on parasites, the amidoxime class of compounds displayed good SAR and selectivity for parasite versus mammalian host cell killing. In contrast, the arylazole class was ultimately dropped for poor or flat SAR and lack of selectivity for mammalian cell versus parasite killing.

Representatives from hit-to-lead series were also evaluated *in vitro* to determine the reversibility of trypanocidal effects using

Table 2
Summary of hit-to-lead activities on select HTS leads from *T. b. brucei* screening.

Compound class	HtL[a]	Best active[b]	Selectivity[c]	Series outcome[d]
Aminoketones	31	15 μM	47	Insufficient potency
Pyramidoximes	161	70 nM	>143	Inactive in mouse serum
Arylazoles	278	200 nM	20	Limited selectivity
Aminooxazoles	53	3.8 μM	>6	Insufficient potency
Sulfanilides	122	205 nM	>500	Poor properties
Heterocyclic ketones	301	5 nM	>2000	Active in mouse model[e]

[a] Total number of analogs synthesized to explore SAR around the original hits.
[b] Most potent compound synthesized during the hit to lead phase and results are IC_{50} values for killing *T. b. brucei* parasites as measured by the resazurin reduction assay.
[c] Denotes a measure of the difference in IC_{50} for parasite versus cytotoxicity toward the L929 fibroblast cells.
[d] Final decision made on each of the HtL series based on profiling newly synthesized analogs through a variety of biological assays.
[e] Mice with an acute *T. b. brucei* infection were cured with a 40 mg/kg daily dose for 4 days of representative compounds from this class (Mercer et al., 2011).

Table 3
Time-dose response (time to kill) IC_{50} values for exposure of *T. b. brucei* to representative series synthesized in the hit-to-lead phase.

Compound Class	3 h	6 h	24 h	72 h
Pyramidoximes	0.450	0.141	0.098	0.07
Arylazoles	1.004	0.130	0.234	0.21
Aminooxazoles	>10	>10	2.310	1.64
Heterocyclic ketones	>10	>10	1.453	0.04
Pentamidine[a]	>2.94	>2.94	0.015	0.0065
Suramin[a]	>7	>7	0.19	0.1

Assays were conducted starting at 10 μM and IC_{50} values were determined from 10-point dose response curves using Cell-Titer Glo reagent following compound exposure for the indicated times.

[a] Indicated values (μM) are IC_{50}s for *T. b. brucei* following compound exposure.

a pulse exposure followed by compound washout and incubation in the absence of inhibitor compound for up to 72 h. Because this assay involves removal or wash-out of compounds at various points after treatment (Nare et al., 2010; Jacobs et al., 2011), followed by incubation for up to 72 h, we were able to use the resazurin assay to measure parasite viability as an indicator of the ability of the parasites to recover from compound effects. In these experiments (Table 4), we demonstrate that a short exposure (1–3 h) to arylazole or amidoxime compounds is sufficient to produce irreversible effects on the survival of trypanosome cultures *in vitro*. The inability of *T. b. brucei* to recover from transient exposure suggests that enough arylazole or amidoxime compound is retained within the parasites or that they exert irreversible effects on the appropriate parasite biology during this limited time frame. Implications for compounds with fast and irreversible mechanism of killing are that high serum concentration is likely to be required for a short period of time to achieve *in vivo* efficacy. In contrast, parasites exposed to heterocyclic ketones and aminooxazoles demonstrated essentially full recovery following short exposure (1–2 h) but significant non-reversible effects were evident within increased exposure time. This suggests that the latter classes of compounds are either slow acting or that there is reversible interaction with the target site of action within the parasites. Suramin and pentamidine are known to accumulate in trypanosomes through endocytosis (Fairlamb and Bowman, 1980) and the P2 transporter (Mathis et al., 2006) respectively and their effects following short exposure (3–6 h) suggest that sufficient compound is accumulated and retained after the wash-out procedure.

Representative compounds from each of the key hit-to-lead series were evaluated in a mouse model for acute HAT. Compounds were administered twice daily by the intraperitoneal route to maximize exposure, as full pharmacokinetic analysis was not available in this early phase. As shown in Table 2, only representative compounds derived from the heterocyclic ketone synthetic effort demonstrated activity when given at 20 mg/kg twice daily for 4 days. Properties of this class of compounds, including the poten-

tial molecular target that is the basis for the anti-parasitic activity have been reported in recent publications (Mercer et al., 2011; Perales et al., 2011). The progression of this series of compounds for use in the treatment of Stage 2 HAT in a mouse model was limited by our inability to balance the permeability across the blood–brain barrier with retention of sufficient potency against *T. b. brucei* to achieve efficacy.

In summary, we have developed and deployed a resazurin-reduction based whole cell *T. b. brucei* assay to screen a proprietary library of ~48,000 compounds representing 25 different structural classes. Representative compounds from six chemotypes were selected for further characterization because they exhibited the desired combination of *in vitro* potency, low cytotoxicity to mammalian cells, i.e. selectivity and some evidence of preliminary SAR. A hit-to-lead program was initiated to follow-up actives from five chemical series and newly synthesized analogs were evaluated through a variety of secondary assays, most of which were dependent on measurement of parasite viability using the resazurin-reduction signal measurement. *In vivo* efficacy was achieved with one of the five hit-to-lead series, thus validating the assays and screening strategy described in this work.

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Table 4
Evaluation of reversibility of compound trypanocidal effect *in vitro*.

Compound Class	1 h	3 h	6 h	72 h
Pyramidoximes	3.50	0.82	0.54	0.07
Arylazoles	20.18	3.81	1.80	0.21
Aminooxazoles	>23.06	>23.06	>2.06	3.78
Heterocyclic ketones	>22.40	>22.40	0.51	0.04
Pentamidine	>2.94	1.2	0.28	0.004
Suramin	>7	2.8	0.86	0.05

To establish time or concentration required to cause irreversible effects, *T. b. brucei* parasites were incubated with increasing concentration of test compounds. At indicated times samples were withdrawn and compounds were washed out through centrifugation. Viability was assessed after incubation in compound-free medium for 72 h and IC_{50}s were determined and reported in μM.

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