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Research Article

Discovery of Trypanocidal Compounds by Whole Cell HTS of *Trypanosoma brucei*

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Chemotherapy against human African trypanosomiasis relies on four drugs that cause frequent and occasionally severe side-effects. Because human African trypanosomiasis is a disease of poor people in Africa, the traditional market-driven pathways to drug development are not available. One potentially rapid and cost-effective approach to identifying and developing new trypanocidal drugs would be high throughput-screening of existing drugs already approved for other uses, as well as clinical candidates in late development. We have developed an ATP-bioluminescence assay that could be used to rapidly and efficiently screen compound libraries against trypanosomes in a high throughput-screening format to validate this notion. We screened a collection of 2160 FDA-approved drugs, bioactive compounds and natural products to identify hits that were cytotoxic to cultured *Trypanosoma brucei* at a concentration of 1 μM or less. This meant that any hit identified would be effective at a concentration readily achievable by standard drug dosing in humans. From the screen, 35 hits from seven different drug categories were identified. These included the two approved trypanocidal drugs, suramin and pentamidine, several other drugs suspected but never validated as trypanocidal, and 17 novel trypanocidal drugs.

Key words: FDA, *trypanosoma brucei*, natural products, biological screening

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'Sleeping sickness' or human African trypanosomiasis (HAT) is caused by two protozoan parasites from the genus *Trypanosoma* and is a major cause of morbidity and mortality in sub-Saharan Africa. HAT poses a major health and economic burden in these regions accounting for about 1.5 million Disability-adjusted Life Years (DALYs), a unit measuring the loss of 1 year of healthy and productive life because of disease (1,2). In West Africa, sleeping sickness is characterized by a slow chronic disease caused by *Trypanosoma brucei gambiense*. East African sleeping sickness is an acute illness caused by *Trypanosoma brucei rhodesiense*. Both diseases are fatal if left untreated. The rate of re-emergence and the need for intervention against HAT has led to it being classified as a category 1 disease by the Special Program for Research and Training in Tropical Diseases (TDR) of the World Health Organization (2).

There are currently only four drugs (suramin developed in 1921; pentamidine developed in 1941; melarsoprol developed in 1949 and eflornithine developed in 1990) approved to treat HAT (3). However, eflornithine and pentamidine are ineffective against sleeping sickness caused by *T. b. rhodesiense*. Treatment with melarsoprol, the only generally effective first-line drug, can result in up to 10% mortality. Additionally, there has been an upsurge in the number of patients failing to respond to melarsoprol because of drug resistance (4). This rise in drug resistance coupled with antiquated and toxic drugs available to treat the disease, underscores the need for discovery of new chemotherapeutic agents against HAT.

Screening large chemical libraries to identify compounds with trypanocidal activity has been hindered in the past by the lack of efficient screening assays with available assays being labour intensive, relying on expensive instrumentation, or requiring radioisotopes (5). The process by which large number of compounds can be rapidly screened against *T. brucei* was facilitated by the introduction of the alamarBlue™ assay, which became a method of choice for carrying out *in vitro* drug screens (6,7). Here, we describe an alternative to the alamarBlue™ assay to screen inhibitors for their cytotoxicity to *T. brucei*. This assay measures ATP-bioluminescence generated when luciferase catalyses the transformation of luciferin into oxyluciferin, yielding PP_i, AMP, and light in the presence of cellular ATP and oxygen (8). The intensity of light in the reaction is proportional to the amount of ATP released from trypanosomes and correlates with the number of viable parasites (9). The assay can be easily adapted to automated high throughput-screening protocols and represents a rapid, sensitive and more efficient assay for screening large libraries to identify trypanocidal compounds. Using this assay

to screen a 2160 compound library of FDA-approved drugs, bioactive compounds and natural products revealed 35 hits that had trypanocidal activity at 1 μM or less. Because many of these hits are drugs already in human use for other diseases, they represent an exploitable shortcut to new chemotherapy for a neglected disease exacting a great human toll.

Materials and Methods

Maintenance of trypanosomes

For routine maintenance of tissue culture-adapted bloodstream form *T. brucei* strain 427, trypanosomes were cultured in T-75 vented cap culture-flasks (Greiner, Kremsmuenster, Austria) and maintained in humidified incubators under the cell culture conditions at 37 °C and 5% CO_2. The trypanosomes were grown in complete HMI-9 medium containing 10% FBS, 10% Serum Plus medium (JRH Inc., Lenexa, KS, USA) and 1X penicillin/streptomycin (10). The trypanosomes were subcultured in flasks every 48 h to maintain them in log growth phase.

Luciferase assay

Trypanosomes were diluted to 1.0×10^5 per mL in complete HMI-9 medium. For larger screens, the diluted trypanosomes were aliquoted in Greiner sterile 96-well flat white opaque culture plates using a WellMate cell dispenser from Matrix Tech. (Hudson, NH, USA). For smaller scale screens, diluted trypanosomes were dispensed manually using a multichannel pipette from Rainin Inc. (Oakland, CA, USA). Compounds to be tested were serially diluted in dimethyl sulphoxide (DMSO) by a MultiMek dispenser from Beckman Inc. (Fullerton, CA, USA) for large screens requiring automation, or by a multichannel pipette for smaller screens conducted manually. In both cases, the trypanosomes were incubated with compounds for 48 h at 37 °C with 5% CO_2 before monitoring viability. The trypanosomes were then lysed in the wells by adding 50 μL of CellTiter-Glo™ purchased from Promega Inc. (Madison, WI, USA). The lysed trypanosomes were placed on an orbital shaker at room temperature for 2 min. After lysis, the resulting ATP-bioluminescence of the trypanosomes in the 96-well plates was measured at room temperature using an Analyst HT plate reader purchased from Molecular Devices (Sunnyvale, CA, USA).

Compounds used for initial optimization of assay

Nine thiosemicarbazone derivatives (JM129, JM131, JM132, JM135, JM137, JM139, JM142, JM145, JM147) were synthesized as described by Fujii *et al.* (11). Z-Phe-Ala-CHN_2 was obtained from Bachem Inc. (Torrance, CA, USA). GW5074, LY294002, Rottlerin (Mallotoxin), Go6983, Staurosporine, and (-)Epigallocatechin gallate (EGCG) were purchased from Sigma (St Louis, MO, USA). TNP-470 was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Diagnosis, NCI, NIH (Bethesda, MA, USA). Arteether was obtained from Walter Reed Army Institute for Research (Bethesda, MA, USA). Mefloquine was obtained from Roche (Nutley, NJ, USA). ABT-751 was obtained from Abbott Laboratories (Abbott Park, IL, USA). Each of the compounds was diluted in DMSO and stored at a stock concentration of 10 mM at −80 °C.

Screening of an FDA-approved drug and natural product library

To test the assay in HTS format, a 2160 compound library (MicroSource Spectrum and Killer Collection) composed of drugs approved for human use; compounds with known biological activities and natural products were obtained from MicroSource Discovery Systems, Inc. (Gaylordsville, CT, USA). The screen was carried out at the Bay Area Screening Center (BASC) in the California Institute for Quantitative Biology (QB3), University of California, San Francisco. The library was screened in 96-well plate format. The composition of the library can be obtained from the company website (http://www.msdiscovery.com). Each of the compounds was diluted to 20 μM in 20% DMSO. Five microlitres of the diluted compounds was transferred by Multimek from the dilution plate into 95 μL of trypanosomes to give a final assay concentration of 1 μM in 1% DMSO by multichannel pipette (Santa Clara, CA, USA) Cultures were then assayed as discussed above. Data acquisition and analysis were carried out using PIPELINE PILOT 4.5.2 from SciTegic, Inc. (San Diego, CA, USA). The quality of the HTS assay was determined by calculating the Z_{prime} and $Z_{factors}$ using eqns 1 and 2 (12). In order to assess biological variability, the screen was run in duplicate on two separate days. Compounds having statistically significant parasite inhibition (Z_{score}: >1.96, $p < 0.05$) in both screens were declared as hits (12). The Z_{score} was calculated using eqn 3.

$$Z_{prime} = 1 - \frac{(3 \times SD_{highs} + 3 \times SD_{lows})}{(mean_{highs} - mean_{lows})} \quad (1)$$

$$Z_{factor} = 1 - \frac{(3 \times SD_{compound} + 3 \times SD_{lows})}{(mean_{compound} - mean_{lows})} \quad (2)$$

$$Z_{score} = \frac{(X - X_{mean})}{(SD_X)} \quad (3)$$

Results

T. brucei ATP-bioluminescence correlates with the number of viable trypanosomes

Proliferation or metabolic activity of cells in response to experimental conditions can be quantified by measuring ATP generation (13). ATP-bioluminescence has been established as an alternative to tritiated thymidine for measuring proliferation of mammalian cells (14). As ATP is an essential cofactor for biogenesis in *T. brucei*, we used the luciferase-based assay to validate the correlation between the number of viable trypanosomes and their ATP levels. A hemocytometer and Olympus CK40 microscope (Tokyo, Japan) were used to directly assess the number of *T. brucei* that seeded the wells of a 96-well plate. After trypanosomes were cultured in 96-well plates at 37 °C for 48 h, they were lysed directly in the wells by adding the luciferase reagent to release their intracellular ATP. The amount of ATP-bioluminescence was measured with a luminometer. The relationship between the numbers of viable *T. brucei* counted by hemocytometer was compared with the Relative Light Units (RLU) obtained by the luminometer using the Pearson *r*-coefficient. The calculated *r*-value was 0.9962 confirming a strong linear correlation between the number of viable trypanosomes and the RLU value (Figure 1).

Minimizing solvent effects and validating assay with Z-Phe-Ala-CHN_2

To optimize solvent formulation, the effects of DMSO on both assay reagents, and the cultured trypanosomes, were determined at 48 h by measuring ATP-bioluminescence using the luciferase-based assay. In medium containing 1% DMSO, the RLU values of the trypanosomes were reduced by 14% compared with control trypanosomes. At 2% DMSO, the RLU values were reduced by more than 60%, and above 2%, the RLU values were reduced by >90% (Figure 2). Increasing the concentration of DMSO did not have any effect on the luciferase assay reagents in the absence of trypanosomes even at 3% final assay concentration, a concentration that inhibited the trypanosomes by >90% (data not shown). Therefore, subsequent assays were carried out such that the DMSO concentrations did not exceed 1%.

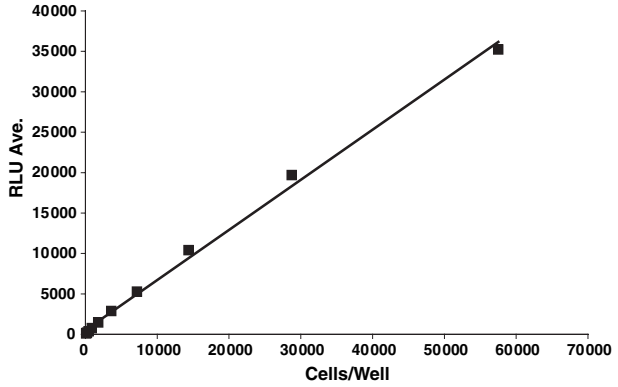


Figure 1: Correlation between the numbers of viable *Trypanosoma brucei* and ATP-bioluminescence. Log-phase *T. brucei* were cultured in HMI medium. The trypanosomes were pelleted by centrifugation and resuspended in 10% of the original volume. The density of the trypanosomes was determined by hemocytometer. Serial dilutions of the trypanosomes were carried out in 100 μL and placed in the wells of a 96-well plate. The trypanosomes were lysed by luciferase reagent and ATP-bioluminescence was determined by luminometer. Viable trypanosomes were counted in parallel by microscopic examination. The values represent the averages of three independent experiments.

The cysteine protease inhibitor Z-Phe-Ala-CHN_2 is an irreversible inhibitor of parasite cathepsin-L- and cathepsin-B-like proteases and has trypanocidal activity against *T. brucei* (15,16). The trypanosomal IC_{50} of this compound was calculated to ensure that the assay could be standardized with a known trypanocidal agent. RLU values were measured at 48 h after the trypanosomes were incubated with varying amounts of Z-Phe-Ala-CHN_2. The calculated trypanosomal IC_{50} of Z-Phe-Ala-CHN_2 was 0.6 μM (Figure 3). As there was a reproducible dose-dependent trypanocidal effect of *T. brucei* with Z-Phe-Ala-CHN_2, the compound was used as a positive control in subsequent assays.

Pilot compound screen

Thiosemicarbazones are a class of small-molecule inhibitors that have been evaluated against cancer, viral and parasitic disease models (17–19). Thiosemicarbazones can act as inhibitors to cysteine proteases and several have been shown to be trypanocidal (11,20). Nine thiosemicarbazones having different chemical modifications at the ethyl side chain were tested for structure–activity relationship (SAR) and their cytotoxicity against cultured *T. brucei*. Five of the modifications (JM132, JM135, JM139, JM142 and JM147) lowered the IC_{50} values of the thiosemicarbazones versus the present ethyl, (Table 1, Figure 4). However, none of the thiosemicarbazone inhibitors had trypanocidal IC_{50} values lower than the control.

To confirm the utility of this assay in a broader chemical space, compounds representing 10 diverse chemical scaffolds were tested including: the kinase inhibitors GW5074, LY294002, Rottlerin, Go6983, Staurosporine and EGCG; the metAP2 inhibitor TNP-470; the microtubulin inhibitor ABT-751; and the antimalarials mefloquin and arteether. The trypanosomal IC_{50} for these compounds were also readily calculated and several of the kinase inhibitors were notably active (Table 2).

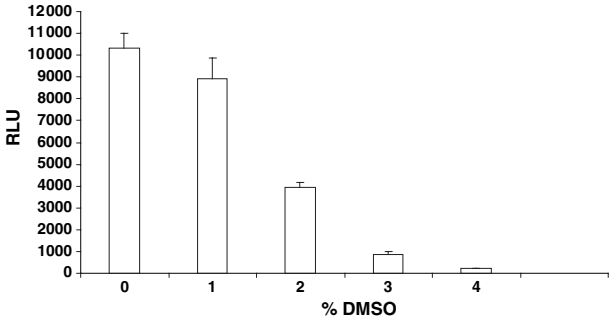


Figure 2: Effects of dimethyl sulphoxide (DMSO) on *Trypanosoma brucei*. Ten thousand *T. brucei* per well were incubated with increasing concentrations of DMSO for 48 h in 100 μL. After 48 h ATP-bioluminescence was determined by luminometer. The values represent the averages of three independent experiments.

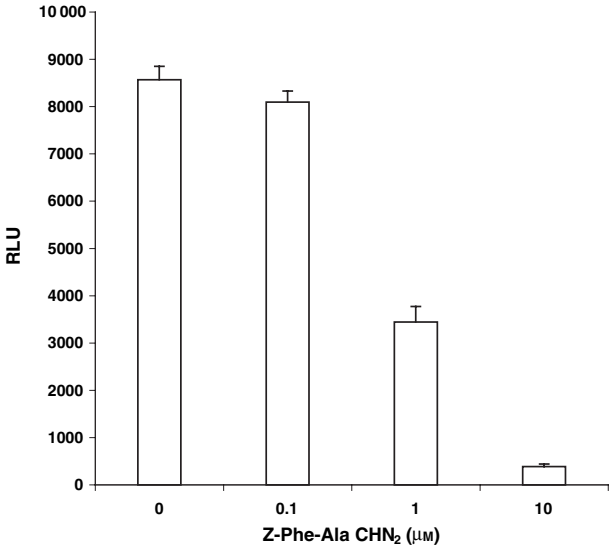


Figure 3: Concentration-dependent killing of *Trypanosoma brucei* by Z-Phe-Ala-CHN_2. Ten thousand *T. brucei* per well were exposed to different concentrations of Z-Phe-Ala-CHN_2 in 96-well plate format and then incubated at 37 °C for 48 h. The effects of this compound on *T. brucei* were determined by ATP-bioluminescence assay. The values represent the averages of three independent experiments.

Identifying known drugs or natural products as potential trypanocidal agents

To test the efficacy of the luciferase assay for use in large-scale automated screens and to identify new antitrypanosomal agents, a library (MicroSource Spectrum and Killer Collection) consisting of 2160 FDA-approved drugs, natural products and biologically active compounds was screened against *T. brucei* at a final concentration of 1 μM of test compound. The assay performed well physically, allowing for rapid and easily automated execution. Importantly, no significant time dependence was observed for the assay. In general, the assay provided very good quality, with average Z_{prime} values better than 0.6. Compounds from this library screen were ranked according to their Z_{factor} (12). The screen was run in duplicate on

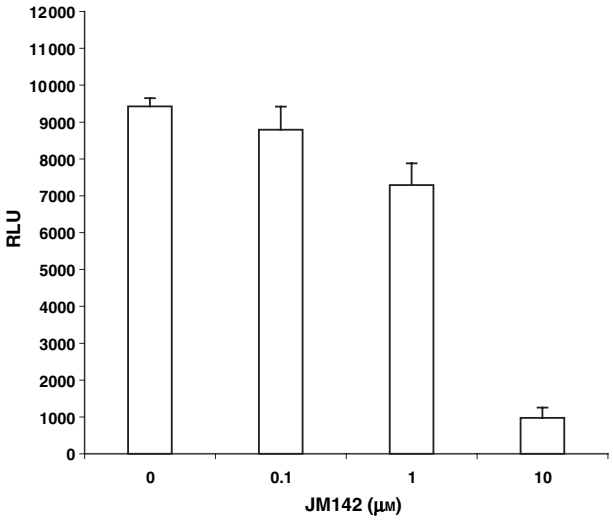


Figure 4: Representative data for thiosemicarbazones screened by the ATP-bioluminescence assay. One representative compound in the series from Table 1 is shown. The compounds were diluted in dimethyl sulphoxide (DMSO) and added to *Trypanosoma brucei* in 96-well plates at concentrations from 10 to 0.1 μM. The values represent the average values of duplicated assays to show dose dependence and reproducibility.

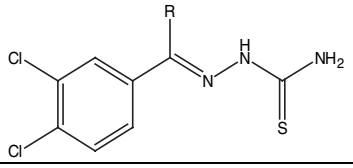
two separate days and the results were in good agreement. Any compound having a reproducible Z_{score} >1.96 ($p < 0.05$) in both screens was considered a hit. Using these criteria, we were able to identify 35 hits having 26 unique scaffolds. Detailed heat map data and statistics can be found at: http://druid.ucsf.edu/smdc/Protocol3B/Diagnostics.html and http://druid.ucsf.edu/smdc/Protocol3C/Diagnostics.html. The hits obtained from the screen of the MS library containing the 2160 compounds could be subdivided into several informative categories based on their modes of action (Table 3).

Identification of two approved trypanocidal drugs from the screen, suramin and pentamidine, served to validate the screen itself. Sixteen other hits were mentioned in published reports on *T. brucei* or related organisms in the past 40 years but had never been validated as antitrypanocidal (http://www.ucsf.edu/mckerrow/fruit.html).

The screen identified seven topical antiseptic/disinfectants including the known trypanocidal agent, gentian violet. In general, these are of less interest than the other hits as it is doubtful that they would be useful as leads for further development. However, they do provide potential decontamination procedures for use in handling trypanosomes.

Four hits were clustered as a group of potent eukaryotic protein synthesis inhibitors. Two of the protein synthesis inhibitors, emetine and lycorine, are classed as alkaloids and two, puromycin and anisomycin, are classed as antibiotics. These compounds are probably not useful for further development as such inhibitors tend to be cytotoxic and would most likely have side-effect profiles that would limit their use to a supervised hospital setting.

Table 1: Trypanosomal IC_{50} of nine mechanism-based thiosemicarbazone inhibitors



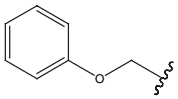
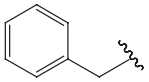
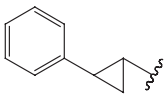
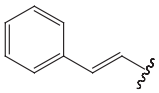
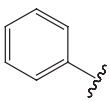
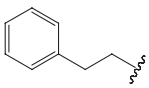
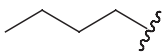
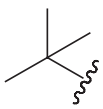
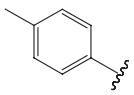
R-group	IC_{50} (μM)
 JM129	>10
 JM131	>10
 JM132	5.1
 JM135	6.5
 JM137	>10
 JM139	3.4
 JM142	3.0
 JM145	>10
 JM147	8.5
Z-Phe-Ala-CHN_2	0.60

Table 2: Trypanosomal IC_{50} of compounds representing 10 diverse chemical scaffolds

Antimalarial		Kinase inhibitors		Methionylaminopeptidase inhibitors		Tubulin polymerization inhibitors	
Inhibitor	IC_{50} (μM)	Inhibitor	IC_{50} (μM)	Inhibitor	IC_{50} (μM)	Inhibitor	IC_{50} (μM)
Arteether	76	EGCG	25	TNP-470	6.9	ABT-51	40
Mefloquine	249	GW 5074	8				
		LY294002	5				
		Rottlerin	5				
		Go 6983	5				
		Staurosporine	0.007				

Table 3: Hits obtained from MicroSource screen

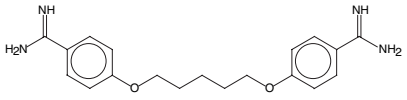
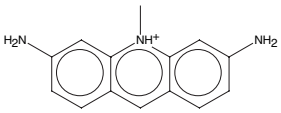
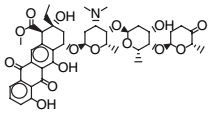
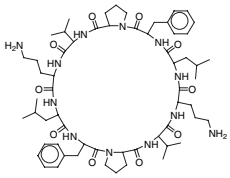
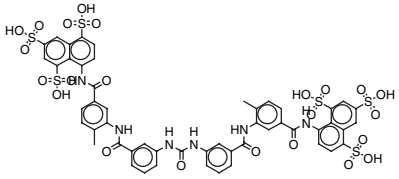
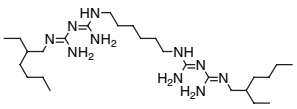
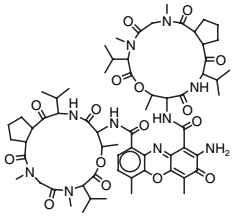
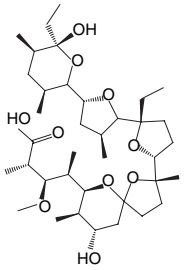
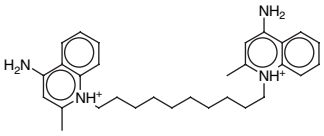
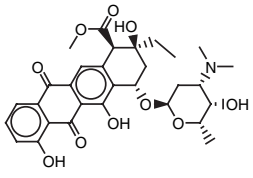
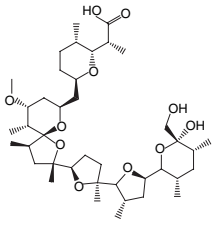
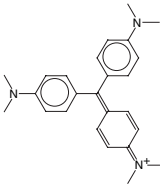
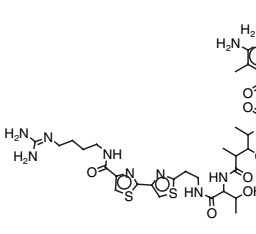
		Antibiotic	
Gold standards	Antiseptic disinfectant	Antineoplastic	Membrane disruptor
			
Pentamidine Isethionate	Acrifla Vinium HCl	Aclacinomycin A1	Gramicidin
			
Suramin	Alexidine HCl[a]	Actinomycin D	Monensin Sodium
			
	Dequalinium HCl[a]	Aklavine HC1[a]	Nigericin Sodium
			
	Gentian Violet	Bleomycin	

Table 3: (Continued.)

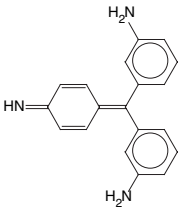
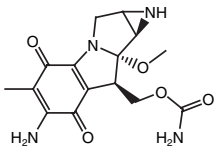
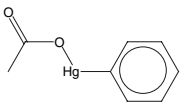
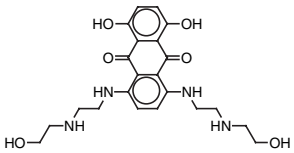
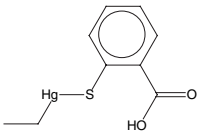
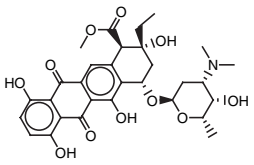
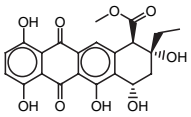
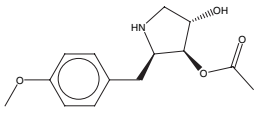
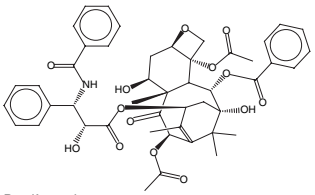
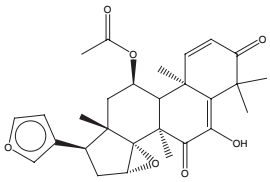
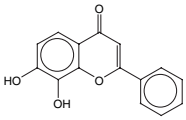
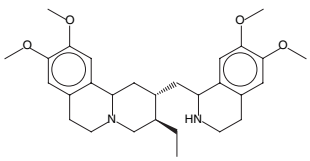
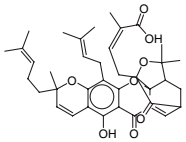
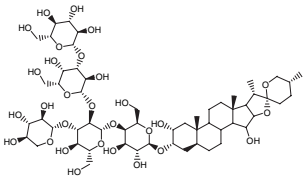
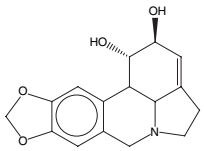
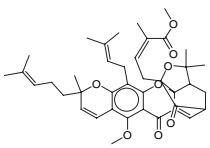
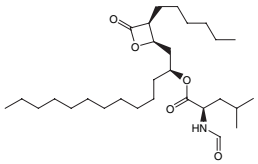
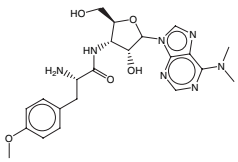
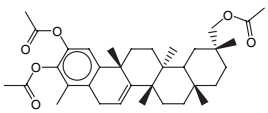
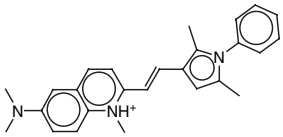
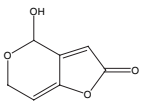
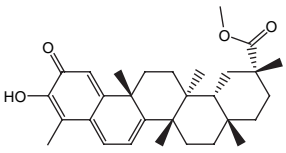
		Antibiotic	
	Antiseptic disinfectant	Antineoplastic	
	 Pararosaniline Pamoate	 Mitomycin C	
	 Phenylmercuric Acetate[a]	 Mitoxanthrone HCl	
	 Thimerosala[a]	 Rutilantin[a]	
		 Rutilantinone[a]	
Protein synthesis inhibitor	Antimitotic	Natural products (antitumour)	Miscellaneous
 Anisomycin	 Paclitaxel	 Anthothecol[a]	 7,8-Dihydroxyflavone[a]
 Emetine HCL		 Gambogic acid[a]	 Digitonin

Table 3: (Continued.)

Protein synthesis inhibitor	Natural products (antitumour)	Miscellaneous
		
Lycorine[a]	Dimethyl Gambogic acid[a]	Orlistat[a]
		
Puromycin	2,3,29-Triacetoxy-24-Nor-1,3,5,7-Friedelatetraenea[a]	Pyrvinium pamoate[a]
		
	Patulin[a]	
		
	Pristimerin[a]	

A hit was defined as giving >52% inhibition with Z_{factor} >1.96 ($p < 0.05$) at 1 μM drug concentration.
[a]Bioactivity against *Trypanosoma brucei* not described in previous literature.

Eleven of the hits were grouped as antibiotics. These had two different modes of action. Eight interfere with cell replication at some level and not surprisingly are also reported to function as antineoplastics. The remaining three antibiotics function as membrane disruptors.

Among the natural products represented in the MS library, seven that have been used as cancer chemotherapeutic agents had trypanocidal activity. The final trypanocidal group included four compounds whose modes of action are known, but which did not fall into similar functional or structural categories.

Discussion

Luciferase-based assays have been used extensively in high throughput screens to measure proliferation, metabolic activity and cytotoxicity in mammalian cells and mycobacteria (21,22). In this study, an ATP-bioluminescence assay was used to determine the effects of diverse chemical compounds on the survival and proliferation of cultured *T. brucei*.

Several other assays, originally designed to measure proliferation in mammalian cells, have been used to measure proliferation in *T. brucei*. The chemical reduction of alamarBlue™ by metabolically active cells is a widely used method for measuring proliferation and cytotoxicity in *T. brucei* (7). Like the ATP-bioluminescent assay, the alamarBlue™ assay requires only a single step of preparation. Although lysis of the parasite is not required in the alamarBlue™ assay, several hours are needed for the trypanosomes to chemically reduce the dye. The number of hours that the cultured trypanosomes require to reduce alamarBlue™ must be optimized for each strain before reliable results can be obtained with fluorescence or colorimetric instruments (7,23). By eliminating this incubation, the luciferase assay can increase the rate of throughput by several fold over the alamarBlue™ assay.

We validated the assay by observing a dose-dependent effect of Z-Phe-Ala-CHN_2 a protease inhibitor known to be cytotoxic to *T. brucei* (15,16,24). The IC_{50} of Z-Phe-Ala-CHN_2 observed against *T. brucei* in this study was consistent with that reported previously (16). Further validation was obtained by testing a panel of potential trypanocidal compounds representing diverse chemical scaffolds. The initial screen was conducted on a series of newly synthesized thiosemicarbazones that have been reported to inhibit the cysteine proteases of clinically important parasites (11). Chemical modification of the ethyl side chain revealed new SAR data for the

Table 4: IC_{50} of FDA-approved agents with previously unreported bioactivity against *Trypanosoma brucei*

Compound	Pharmacological action	Literature describing bioactivity against *T. brucei*	IC_{50} (μM)
Alexidine	Disinfectant and topical anti-infective agent also used as mouthwash to prevent oral plaque. Anti-infective agent, local disinfectants in mouthwashes	This study	3.0
Aklavine	Antineoplastic	This study	1.8
Anthothecol	Plant derivative. Not much information available. Similar compounds are being tested as antineoplastics	This study	1.7
Dequalinium Chloride	Topical bacteriostat that is available as various salts. It is used in wound dressings and mouth infections and may also have antifungal action, but may cause skin ulceration	This study	1.0
7,8-Dihydroxyflavone	Flavonoid (plant compound)	This study	2.3
2,3,29-Triacetoxy-24-nor-1,3,5,7-friedelatetraene	Celastrol derivative	This study	1.5
Gambogic acid	Principal pigment of gamboge resin which is an exudate of several *Garcinia* species Gamboge has a long history of medicinal uses in Southeast Asia	This study	1.8
Dimethyl gambogic acid		This study	1.3
Lycorine (Narcissine)	Toxic crystalline Amaryllidaceae alkaloid found in the bulbs of species of *Lycoris* and *Narcissus*; it causes vomiting, diarrhoea, convulsions, and sometimes death in humans and other animals. It is a powerful herbicide and algacide	This study	3.0
Orlistat	Lipid inhibitor	This study	0.23
Patulin	Mycotoxin produced by several species of *Aspergillus* and *Penicillium*. Has antibiotic properties, but shown to be carcinogenic and mutagenic causing chromosome damage in biological systems	This study	1.0
Pristimerin	Triterpenoid from the *Celastrae* family (plant derivative). Used in Chinese herbals	This study	1.5
Pyrvinium	An antihelmintic that acts by preventing the uptake of exogenous glucose and is administered orally in the treatment of enterobiasis (pinworm infections)	This study	3.0
Rutilantin	An antibiotic substance with antiphage activity	This study	0.2
Rutilantinone	Rutilantin derivative	This study	3.0
Thimerosal	A preservative in some vaccines and other products	This study	0.2

thiosemicarbazones. We also identified several compounds worthy of further exploration. These include the methionine aminopeptidase inhibitor TNP-470, and several kinase inhibitors (Table 2).

The hits obtained from screening the 2160 drugs and natural product library were most noteworthy, because such a pilot screen can be expanded to the larger and more complex library containing all of the FDA-approved drugs. Several of the compounds from the pilot screen shown in Table 3 are approved drugs with good safety profiles and oral bioavailability. They were trypanocidal at concentrations of 1 μM or less, which is easily achievable as a plasma concentration using published standard drug dosing. Identifying trypanocidal activity among these 2160 FDA-approved drugs represents exactly the type of development 'shortcut' international agencies-like the WHO and DNDi are seeking for quickly getting new compounds into clinical trials (25). For example, 7,8-dihydroxyflavone, an aromatase inhibitor, and the weight reduction drug, Orlistat, are orally bioavailable and known to be well tolerated in patients. Furthermore, the relatively low cellular IC_{50} for both of these drugs (2.3 and 0.23 μM respectively) make them ideal candidates to follow up in animal or clinical studies. There are also a number of antibiotics worth pursuing, and several intriguing natural products. While the antineoplastics seem less appealing because of known side-effects, they should not be ignored because paclitaxel, Taxol®, has a much better safety profile than melarsoprol, the only drug currently used for late-stage trypanosomiasis. Finally, it is noteworthy that the initial FDA screening experiment identified two known trypanocidal agents, pentamidine and suramin. Of the 33 other compounds identified in the FDA screen, 16 of them appear in published reports on assays with trypanosomes. The other 17 compounds identified from the screen have not appeared in literature as having trypanocidal activity. We have determined a cellular IC_{50} for each of the novel trypanocidal compounds as listed in Table 4.

We have posted a public website in which the results of this and later screens will be deposited (http://itsa.ucsf.edu/~schisto/fruit.html). The main goal of this website was to alert international agencies, non-profit organizations and the parasitology community to drugs already approved for human use that have significant trypanocidal activity at concentrations achievable through currently approved and safe dosing regimens. Development of the assay itself provides a foundation to screen other compound libraries in a similar manner, and the strategy of the assay for screening FDA libraries should be applicable to other parasitic organisms that are agents of major global health problems.

Acknowledgments

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References

1. Gross C.P., Anderson G.F., Powe N.R. (1999) The relation between funding by the National Institutes of Health and the burden of disease. N Engl J Med;340:1881–1887.
2. Remme J.H., Blas E., Chitsulo L., Desjeux P.M., Engers H.D., Kanyok T.P., Kayondo J.F. et al. (2002) Strategic emphases for tropical diseases research: a TDR perspective. Trends Microbiol;10:435–440.
3. Docampo R., Moreno S.N. (2003) Current chemotherapy of human African trypanosomiasis. Parasitol Res;90(Suppl. 1):S10–13.
4. Pepin J., Milord F. (1994) The treatment of human African trypanosomiasis. Adv Parasitol;33:1–47.
5. Kaminsky R., Brun R. (1993) In vitro assays to determine drug sensitivities of African trypanosomes: a review. Acta Trop;54:279–289.
6. Ahmed S.A., Gogal R.M. Jr, Walsh J.E. (1994) A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. J Immunol Methods;170:211–224.
7. Raz B., Iten M., Grether-Buhler Y., Kaminsky R., Brun R. (1997) The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T. b. rhodesiense* and *T. b. gambiense*) in vitro. Acta Trop;68:139–147.
8. DeLuca M., Dempsey M.E. (1970) Mechanism of oxidation in firefly luminescence. Biochem Biophys Res Commun;40:117–122.
9. Kangas L., Gronroos M., Nieminen A.L. (1984) Bioluminescence of cellular ATP: a new method for evaluating cytotoxic agents in vitro. Med Biol;62:338–343.
10. Hirumi H., Hirumi K. (1989) Continuous cultivation of *Trypanosoma brucei* blood stream forms in a medium containing a low concentration of serum protein without feeder cell layers. J Parasitol;75:985–989.
11. Fujii N., Mallari J.P., Hansell E.J., Mackey Z., Doyle P., Zhou Y.M., Gut J. et al. (2005) Discovery of potent thiosemicarbazone inhibitors of rhodesain and cruzain. Bioorg Med Chem Lett;15:121–123.
12. Zhang J.-H., Chung T.D.Y., Oldenburg K.R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J Biomol Screen;4:67–73.
13. Lundin A., Hasenson M., Persson J., Pousette A. (1986) Estimation of biomass in growing cell lines by adenosine triphosphate assay. Methods Enzymol;133:27–42.
14. Crouch S.P., Kozlowski R., Slater K.J., Fletcher J. (1993) The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Methods;160:81–88.
15. Shaw E. (1988) Peptidyl sulfonium salts. A new class of protease inhibitors. J Biol Chem;263:2768–2772.
16. Scory S., Caffrey C.R., Stierhof Y.D., Ruppel A., Steverding D. (1999) *Trypanosoma brucei*: killing of bloodstream forms in vitro and in vivo by the cysteine proteinase inhibitor Z-phe-ala-CHN2. Exp Parasitol;91:327–333.
17. Mishra V., Pandeya S.N., Pannecouque C., Witvrouw M., De Clercq E. (2002) Anti-HIV activity of thiosemicarbazone and semicarbazone derivatives of (+/–)-3-menthone. Arch Pharm (Weinheim);335:183–186.
18. Finch R.A., Liu M., Grill S.P., Rose W.C., Loomis R., Vasquez K.M., Cheng Y. et al. (2000) Triapine (3-aminopyridine-2-carboxaldehyde-thiosemicarbazone): a potent inhibitor of ribonucleotide reductase activity with broad spectrum antitumor activity. Biochem Pharmacol;59:983–991.
19. Condit R.C., Easterly R., Pacha R.F., Fathi Z., Meis R.J. (1991) A vaccinia virus isatin-beta-thiosemicarbazone resistance mutation maps in the viral gene encoding the 132-kDa subunit of RNA polymerase. Virology;185:857–861.
20. Du X., Guo C., Hansell E., Doyle P.S., Caffrey C.R., Holler T.P., McKerrow J.H. et al. (2002) Synthesis and structure-activity relationship study of potent trypanocidal thio semicarbazone inhibitors of the trypanosomal cysteine protease cruzain. J Med Chem;45:2695–2707.
21. Cooksey R.C., Crawford J.T., Jacobs W.R. Jr, Shinnick T.M. (1993) A rapid method for screening antimicrobial agents for activities against a strain of *Mycobacterium tuberculosis* expressing firefly luciferase. Antimicrob Agents Chemother;37:1348–1352.
22. Maffia A.M. III, Kariv I.I., Oldenburg K.R. (1999) Miniaturization of a mammalian cell-based assay: luciferase reporter gene readout in a 3 microliter 1536-well plate. J Biomol Screen;4:137–142.
23. Shawar R.M., Humble D.J., Van Dalfsen J.M., Stover C.K., Hickey M.J., Steele S., Mitscher L.A. et al. (1997) Rapid screening of natural products for antimycobacterial activity by using luciferase-expressing strains of *Mycobacterium bovis* BCG and *Mycobacterium intracellulare*. Antimicrob Agents Chemother;41:570–574.
24. Mackey Z.B., O'Brien T.C., Greenbaum D.C., Blank R.B., McKerrow J.H. (2004) A cathepsin B-like protease is required for host protein degradation in *Trypanosoma brucei*. J Biol Chem;279:48426–48433.
25. McKerrow J.H. (2005) Designing drugs for parasitic diseases of the developing world. PLoS Med;2:e210.