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Image-Based High-Throughput Drug Screening Targeting the Intracellular Stage of *Trypanosoma cruzi*, the Agent of Chagas' Disease▽‡

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Chagas' disease, caused by infection with the parasite *Trypanosoma cruzi*, is the major cause of heart failure in Latin America. Classic clinical manifestations result from the infection of heart muscle cells leading to progressive cardiomyopathy. To ameliorate disease, chemotherapy must eradicate the parasite. Current drugs are ineffective and toxic, and new therapy is a critical need. To expedite drug screening for this neglected disease, we have developed and validated a cell-based, high-throughput assay that can be used with a variety of untransfected *T. cruzi* isolates and host cells and that simultaneously measures efficacy against the intracellular amastigote stage and toxicity to host cells. *T. cruzi*-infected muscle cells were incubated in 96-well plates with test compounds. Assay plates were automatically imaged and analyzed based on size differences between the DAPI (4′,6-diamidino-2-phenylindole)-stained host cell nuclei and parasite kinetoplasts. A reduction in the ratio of *T. cruzi* per host cell provided a quantitative measure of parasite growth inhibition, while a decrease in count of the host nuclei indicated compound toxicity. The assay was used to screen a library of clinically approved drugs and identified 55 compounds with activity against *T. cruzi*. The flexible assay design allows the use of various parasite strains, including clinical isolates with different biological characteristics (e.g., tissue tropism and drug sensitivity), and a broad range of host cells and may even be adapted to screen for inhibitors against other intracellular pathogens. This high-throughput assay will have an important impact in antiparasitic drug discovery.

Trypanosoma cruzi is the etiological agent of American trypanosomiasis, or Chagas' disease, a chronic infection affecting around 12 million people in Latin America (46). In areas of endemicity, reduviid insects are responsible for the natural transmission of *T. cruzi* to humans. Infection can also be transmitted through contaminated blood transfusion, organ transplant, contaminated food or drink, and via a transplacental route (35, 40, 47, 50, 57, 60). In addition, Chagas' disease has become an important opportunistic infection among patients with HIV infection and other types of immunosuppression (e.g., organ transplantation and cancer), which reactivate *T. cruzi* infection (4, 9, 12, 18, 29). More recently, due to immigrant carriers, Chagas' disease has been reported in areas where Chagas' disease is not endemic (40; http://www.treatchagas.org). Classic clinical manifestations of Chagas' disease derive from infection of heart muscle cells leading to progressive cardiomyopathy (28, 32, 51, 53, 54, 56). Chagasic cardiopathy is the major cause of heart failure in Latin America. Sudden cardiac death accounts for 55 to 65% of deaths in Chagas' disease (52). In Brazil, development of megasyndromes is also common (41).

Chagas' chemotherapy is directed at eradicating the parasite and ameliorating clinical manifestations (2, 17, 30, 31, 48, 61, 62,). There are no vaccines and very limited drug options. Benznidazole and nifurtimox are used during the acute phase and when reactivation under immunosuppressive conditions occurs. Treatment of chronically infected patients improves cardiac status, but both compounds have significant toxicity, leading to severe adverse effects that require medical supervision (13). Retrospective studies have revealed that neither benznidazole nor nifurtimox completely clears parasitemia, and drug resistance is common (30, 62). New therapy for Chagas' disease is a critical need (21, 43).

T. cruzi is a genetically heterogeneous group of organisms (23, 24). Development of *T. cruzi* in mammalian hosts is characterized by an obligate intracellular cycle initiated by the entry of trypomastigotes into host cells. The infectious form rapidly transforms to a proliferating amastigote stage that remains free within the host cell cytoplasm. After a quiescent prereplicative lag period, amastigotes divide for 7 to 9 generations before transforming back to trypomastigotes that lyse the host cell and are released to the bloodstream. The length of the intracellular cycle is characteristic of each clonal *T. cruzi* population and may vary from 4 days to several weeks (23; J. C. Engel, unpublished data).

The intracellular life cycle of *T. cruzi* can be reproduced in cell culture and is used as a model for studying host-parasite interactions and for drug screening. We had previously designed a low-throughput, multidose *T. cruzi* drug screening method that can distinguish compounds with either trypano-

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cidal or trypanostatic activity (26). This assay has been shown to predict antiparasitic activity in animal models of infection. With an automated fluorescence microscope system that allows the screening of large sets of compounds, we have now developed a high-throughput screening (HTS) assay in a microtiter plate format that measures inhibition of parasite proliferation. This assay can then be used to screen large numbers of potential antiparasitic agents prior to testing them with the more laborious, but more precise, trypanocidal assay.

Pharmaceutical companies have extensively used HTS technology to identify and characterize bioactive molecules for a number of human diseases, ranging from cancer to osteoporosis (3, 59). Large libraries of compounds have also been screened to identify hits for parasites (1, 33, 63). To assay diverse chemical compounds, a luciferase HTS assay was developed for *Trypanosoma brucei*, the bloodstream parasite that causes African sleeping sickness (42) and fluorescence-activated cell sorter (FACS) analysis HTS was recently used for the erythrocytic stages of malaria (63). An HTS drug screening method using *T. cruzi* parasites that express the *Escherichia coli* β-galactosidase gene has been described. This assay requires the use of transfected parasites that catalyze a colorimetric reaction with chlorophenol red β-D-galactosidase as a substrate (7, 11). The requirement for transfection of the reporter gene is a significant limitation because of the huge natural diversity of *T. cruzi* (23) and the difficulty in transfecting some *T. cruzi* populations (J. C. Engel, unpublished data).

We have now developed, optimized, and validated a cell-based HTS assay that can be used with a variety of untransfected *T. cruzi* isolates and host cells and that can simultaneously measure efficacy against the parasite and host cell toxicity. The flexibility of this HTS assay allows the use of any parasite strains, including recent clinical isolates that may have different biological characteristics (e.g., tissue tropism and drug sensitivity). In addition, the assay can accommodate a broad range of host cells ranging from primary cultures to established cell lines with defined biological, biochemical, and metabolic pathways. This flexibility can help better define diverse aspects of drug-parasite-host interactions (e.g., drug metabolism, targeting, or toxicity in a particular cell type). To validate our HTS assay, we have screened a library of 909 bioactive compounds (64) and identified 55 hits.

MATERIALS AND METHODS

Cell culture. Bovine embryo skeletal muscle (BESM) (25) and human hepatoma Huh-7 (44) cells were routinely cultured in RPMI 1640 medium supplemented with 5 to 10% heat-inactivated fetal calf serum (FCS) at 37°C in 5% CO_2. The *T. cruzi* CA-I/72 clone (25) and PSD-1 strain come from chronic chagasic patients. The Sylvio-X10/7 clone and its parental strain were derived from an insect used in xenodiagnosis of an acute case in Brazil (49). *T. cruzi* stocks were maintained by weekly passage in BESM and Huh-7 cells. Infectious trypomastigotes were collected from culture supernatants.

Compound library. A vinyl sulfone cysteine protease inhibitor, K11777 (also known as K777; *N*-methyl-piperazine-Phe-homoPhe-vinylsulfonyl-phenyl), a trypanocidal compound that has passed through rodent, dog, and primate safety studies (20, 26, 43) (SRI International Project 10043–202, 2001), was used as a positive control to standardize the protocol. Posaconazole was purchased at a pharmacy and purified (22). Nifurtimox was a kind gift from Bayer, Germany. A small set of 10 compounds, cherry-picked from the Small Molecule Discovery Center (SMDC) medicinal chemistry library, was used for assay validation versus our established, in-house trypanocidal assay (26). These compounds were dissolved in dimethyl sulfoxide (DMSO) at 2 mM. A library of 909 clinical compounds donated by Iconix Biosciences and consisting largely of FDA-approved drugs was screened in a 96-well plate format. The Iconix compounds were dissolved in DMSO at 1 mM.

HTS assay and EC_{50}. Sterile, black 96-well plates with clear-bottom wells (Greiner Bio-One) were seeded with mammalian cells in 150 μl culture medium. BESM (2,000/well) and Huh-7 (1,000/well) cells were then infected with 10^3 and 5×10^3 CA-I/72 trypomastigotes, respectively, in 50 μl of culture medium/well. Sixteen hours postinfection, culture medium (200 μl/well) was replaced and test compounds (10 μM) were added as indicated. Positive control wells (100% *T. cruzi* growth) contained 1% (vol/vol) DMSO, while negative control wells (100% *T. cruzi* growth inhibition, or 0% growth) were treated with 20 μM K11777 (20, 26). Culture plates were incubated for an additional 72 h at 37°C with 5% CO_2. Cells were then washed once with phosphate-buffered saline (PBS), fixed for 2 h with 4% paraformaldehyde, and rinsed again with PBS to remove the fixative. Fifty microliters/well of Vectashield mounting medium (Vector Labs) containing the DNA fluorescent dye DAPI (4′,6-diamidino-2-phenylindole) was added. Plates were kept in the dark at 4°C until image acquisition was performed. For 50% effective concentration (EC_{50}) determinations, compounds were serially diluted 2-fold in DMSO, with final assay concentrations ranging from 0.4 to 50 μM. All other assay procedures were performed as described above.

Image acquisition and data analyses. Plates were screened in an IN Cell Analyzer 1000 (GE Healthcare). The excitation and emission filters used to detect DAPI were 350/50 nm and 460/40 nm, respectively. Seven image fields (10×) were acquired per well, each with an exposure time of 150 ms. The IN Cell Workstation 3.5 multitarget analysis module was used for image analyses. Top-hat segmentation parameters were set to identify host "nuclei" with a minimum area of 125 μm^2, while intracellular parasite kinetoplasts were defined as "organelles" using a segmentation range of 1 to 2 μm^2. The ratio of parasite kinetoplast DNA (kDNA) to host nuclei was selected as the measurement output.

For assessment of the assay window, the untreated/treated ratio (signal/background [S/B] ratio) was calculated using the equation $S/B = \mu_{c+}/\mu_{c-}$, where μ_{c+} and μ_{c-} are the mean values of positive and negative controls, respectively. As large screens are expensive in time and resources, the Z' value is used to quantify the suitability of a particular high-throughput screen and as a measure of statistical effect size. The assay performance was then assessed using the Z' value, defined by the equation $Z' = 1 - [(3\sigma_{c+} + 3\sigma_{c-})/(\mu_{c+} - \mu_{c-})]$, where σ_{x+} and σ_{x-} are the standard deviations of positive and negative controls, respectively (65). EC_{50} curve fitting was carried out using GraphPad Prism 4 Software (GraphPad Software, Inc., San Diego, CA). Selectivity windows (cell toxicity EC_{50}/*T. cruzi* EC_{50}) and percentage of inhibition were calculated for each compound.

RESULTS

Development of an HTS assay. To identify bioactive compounds against pathogenic *T. cruzi* amastigotes, we developed a 96-well-plate, cell-based HTS assay suitable for screening compound libraries. The assay was standardized for BESM and Huh-7 cells using a cell/trypomastigote ratio that yielded an infection of approximately 30% of cells. Intracellular *T. cruzi* amastigotes may be visualized by fluorescence microscopy (Fig. 1A). In the HTS assay, both host cell nuclei and *T. cruzi* kinetoplast DNA (kDNA) were labeled with DAPI, which stains kDNA more strongly than parasite nuclei (Fig. 1 and 2). No significant differences in kDNA and nucleic DNA (N-DNA) staining that might interfere with HTS data collection were observed between various *T. cruzi* strains with the exposures used to collect images (Fig. 2) (data not shown). To determine the assay window and assay performance, we collected seven image fields per well with an IN Cell microscope (10×). Typical BESM nuclei measure over 10 μm in diameter, while amastigote kinetoplasts are 1 to 2 μm in diameter (Fig. 1 and 2). These significant size differences were exploited for image segmentation (Fig. 1B).

To evaluate compound bioactivity, we calculated kDNA/host cell nucleus (parasite/host) ratios. A significant reduction in the parasite/host ratio in treated cultures, as compared to

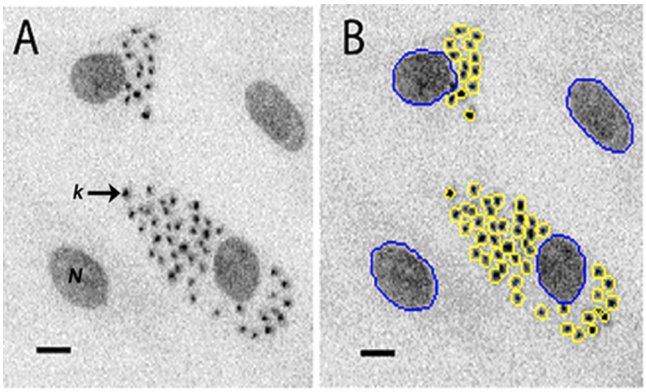


FIG. 1. (A) Amplified fluorescence image obtained in the IN Cell Analyzer 1000 (10×) of BESM nuclei (N) and *T. cruzi* kDNA (k) stained with DAPI. (B) Image segmentation of BESM nuclei (blue outline) and *T. cruzi* kDNA (yellow outline) using the Developer Toolbox software. Each size bar is 10 μm.

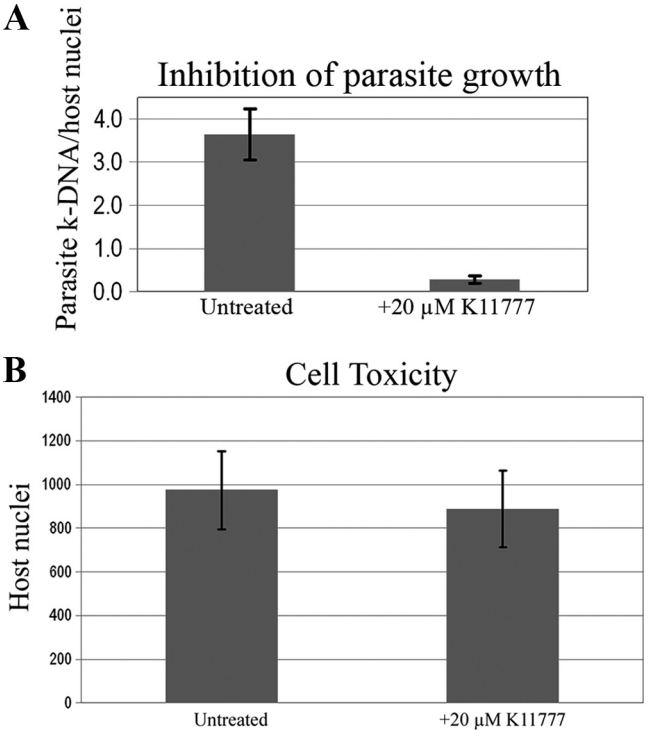


FIG. 3. (A) K11777 prevents *T. cruzi* development in BESM cells. Mean numbers of parasites/cell are higher in untreated controls than in cultures treated with 20 μM K11777. Standard deviations are shown as error bars. (B) K11777 does not induce cell toxicity, as evidenced by similar counts of BESM cell nuclei of untreated and treated controls. Standard deviations are shown as error bars.

untreated controls, provided a quantitative measure of parasite growth inhibition after 72 h of treatment. Images from the IN Cell Analyzer 1000 showed a marked reduction of parasites in cells treated with the trypanocidal drug K11777 as compared to untreated controls (Fig. 2). The mean number of parasites/cell for infected untreated (S) cells was 3.64 ± 0.59 in 16 different optical fields (n = 16 wells), and for infected cells treated with 20 μM K11777 (B), the mean value was 0.27 ± 0.04 (n = 16 wells) (Fig. 3A). The untreated/treated (S/B) ratio was 13.44, and the Z' value was 0.45. The mean for noninfected host cells (0.01 ± 0.0008) (background and nonspecific counts) (n = 16) was negligible and, consequently, was not used to correct parasite counts. *T. cruzi* replication did not cause significant host cell lysis within the 4-day screening period used.

Counts of host nuclei were used as a quantitative measure of cell toxicity induced by compounds. A reduction in mammalian host nuclei for a test compound was indicative of compound toxicity. No statistically significant differences were found between the mean count of host nuclei for untreated control cells (973 ± 178) (0% growth inhibition) and that for K11777-treated cells (887 ± 174) (n = 16) at a concentration that induced 100% *T. cruzi* growth inhibition, confirming the absence of *in vitro* drug toxicity for K11777 (Fig. 3B).

Our HTS assay was designed to evaluate the effect of a single drug dose on parasite growth at a fixed time point postinfection (88 h). In contrast, our more laborious trypanocidal assay is a multidose treatment method that uses nonproliferating, irradiated host cells and can differentiate between trypanostatic

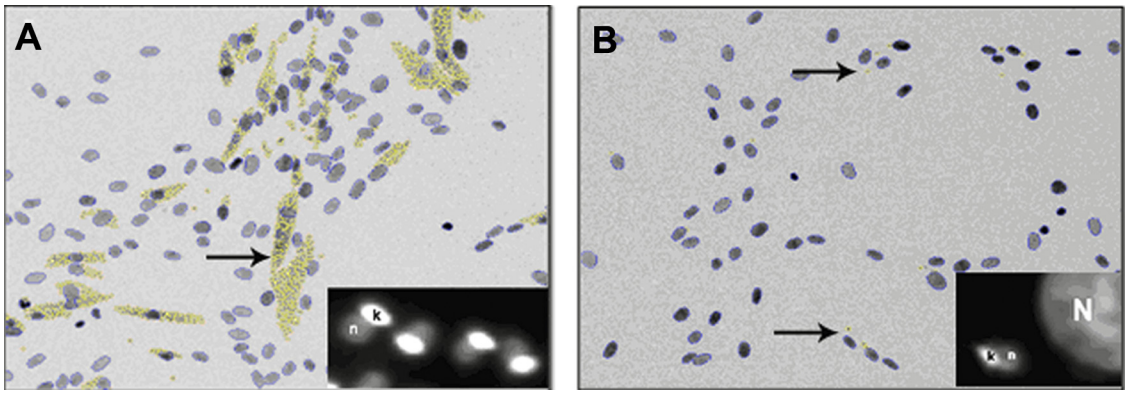


FIG. 2. Representative images collected with the IN Cell 1000 microscope of BESM cells infected with *T. cruzi* for 88 h (10×). (A) Untreated control. Similar *T. cruzi*-infected cells are amplified in Fig. 1. (B) Culture treated with 20 μM K11777 for 72 h. Arrows indicate intracellular parasites also highlighted in yellow by IN Cell software. Inserts show the relative fluorescence of DAPI-stained parasite kDNA (k) and nucleic (n) DNA and host cell nucleus (N) (40×).

TABLE 1. Comparison of HTS and trypanocidal assays

Parameter	Characteristic	
	HTS assay	Trypanocidal assay
Host cells	All mammalian cell lines	Irradiated J774 macrophages
T. cruzi	All strains	All strains
Method	96-well plates Single dose of compound Time point fixed at 88 h postinfection	12- to 24-well plates Multiple doses of compound Time point not fixed
Readout	All compounds evaluated at 88 h Automated (IN Cell)	Requires phase-contrast microscopy by observer Requires completion of the intracellular life cycle Drugs verified as ineffective at 5 days Compounds verified as trypanostatic between 6 and 40 days Compounds verified as effective and trypanocidal at 40 days
Information provided	Growth inhibition	Cure vs no cure

and trypanocidal compounds. Differences between both assays are summarized in Table 1.

To validate the *T. cruzi* HTS imaging assay, we double tested 10 compounds that exhibited various degrees of inhibition in our well-established trypanocidal assay (26). Results summarized in Table 2 indicate a good correlation of compound activity between both assays. Compounds showing selectivity windows of >3 (cell toxicity EC_{50}/*T. cruzi* EC_{50}) in the 4-day HTS assay correlated well with the prolonged survival (>40 days) in the trypanocidal assay using *T. cruzi*-infected macrophages. Compounds with lower selectivity indexes (<3) had lower or negligible activity in the long-term assay. In one instance, SMDC compound 256064 with a selectivity window value of >3, was toxic to macrophage cells in the long-term trypanocidal assay. For further validation, we determined EC_{50}s for K11777, posaconazole, and nifurtinox using three different *T. cruzi* stocks (Table 3). These EC_{50} determinations confirm the efficacy and sensitivity of the HTS assay with various *T. cruzi* isolates. These results also show a higher sensitivity of Sylvio-X-10/7 to posaconazole and nifurtimox than the other parasite strains, while PSD-1 showed higher resistance to nifurtimox.

TABLE 3. EC_{50} values for different *T. cruzi* isolates

T. cruzi strain	EC_{50} (μM)[a]		
	K11777	Posaconazole	Nifurtimox
CA-I/72	4.2 ± 0.1	0.2 ± 0.03	1.2 ± 0.4
Sylvio-X10/7	3.0 ± 0.5	0.08 ± 0.02	0.7 ± 0.2
PSD-I	3.0 ± 0.2	0.13 ± 0.02	2.1 ± 0.3
BESM (host cell)	>20.0	>2.0	>10.0

[a] EC_{50}s are means ± standard errors from two independent experiments each run in duplicate.

Drug library screens. As a validation of the assay, a library of 909 drugs mainly approved by FDA was screened in singlicate ($n = 1$) at 10 μM. Compounds identified as "primary hits" (161 [17.7%]) were arbitrarily selected based on a cutoff of ≥53% *T. cruzi* growth inhibition calculated from the parasite/host ratio and ≤32% cell toxicity as calculated from comparative counts of host nuclei between treated and untreated cultures. This low threshold was purposely selected to evaluate sensitivity of the HTS assay. Cutoff values were selected as 1 standard deviation away from the mean value of all compounds tested. Using the primary hit criteria, 63 compounds were selected, giving a screening hit rate of 6.9% based on the cell toxicity inhibition cutoff of ≤32%. Of these, 55 compounds (6%) were confirmed as "hits" in a dose-response study with determinations of *T. cruzi* EC_{50} of ≤50 μM (confirmed hit rate of 87% and overall library hit rate of 6%). The identity and efficacy of the confirmed hits are summarized in Table 4. The chemical structures and therapeutic use of clinical compounds with a >5-fold selectivity window against *T. cruzi* are shown in Table 5. Typical dose-response analyses for each hit are exemplified in Fig. 4, which shows EC_{50} calculations for Iconix compound 130071 (fluphenazine dihydrochloride). The EC_{50}s obtained for 130071 were 4 μM for *T. cruzi* amastigotes and 21 μM for BESM cells, with a selectivity window of 5.25.

TABLE 2. Validation of the HTS assay

SMDC compound no.	Chemotype	Trypanocidal assay result (40 days of incubation at 10 μM)	HTS assay (4 days)		
			T. cruzi EC_{50} (μM)	Cell toxicity EC_{50} (μM)	Selectivity window[a]
256122	Vinylsulfone	>40 days of survival (trypanocidal)	1	>20	>20
256123	Vinylsulfone	>40 days of survival (trypanocidal)	1	7	7
256162	Vinylsulfone	>40 days of survival (trypanocidal)	4	15	3.8
256157	Vinylsulfone	>40 days of survival (trypanocidal)	6	>20	>3.3
256171	Oxadiazole	~19 days of survival (trypanostatic)	10	>20	>2
256064	Vinylsulfone	Toxic to macrophages	3	11	3.7
256037	Vinylsulfone	Toxic to macrophages	8	18	2.3
256002	Vinylsulfone	Toxic to macrophages	11	17	1.5
256189	Oxadiazole	5 days of survival (not active)	13	>20	>1.5
281566	Oxadiazole	5 days of survival (not active)	>20	>20	1

[a] Cell toxicity EC_{50}/*T. cruzi* EC_{50}.

TABLE 4. Identity and efficacy of confirmed hits

Compound	*T. cruzi* EC_{50} (μM)	Cell toxicity EC_{50} (μM)	Selectivity window[a]
Furazolidone	<0.4	>50	>125
Cycloheximide	<0.4	>50	>125
Terconazole	<0.4	24	>60
Docetaxel	<0.8	44	>56
Azelastine	1	49	49
Amiodarone	0.8	36	45
RWJ-68354	5	>50	>10
Dihydroergocristine mesylate	5	47	9.4
Mycophenolate mofetil	7	>50	>7.1
Clomipramine	8	>50	>6.3
Nitrofurazone	8	>50	>6.3
Fluphenazine	4	21	5.3
Vinorelbine	10	>50	>5.0
Haloperidol	7	33	4.7
Metergoline	4	18	4.5
Kainic acid	3	13	4.3
Phenothiazine	6	26	4.3
Loperamide	3	12	4.0
Carvedilol	8	31	3.9
Imatinib	9	32	3.6
Hydroxyprogesterone	5	17	3.4
Nifursol	15	>50	>3.3
3,3′,4′,5-Tetrachlorosalicylanilide	8	26	3.3
Abamectin	4	12	3.0
U-78517f	17	>50	>2.9
Genaconazole	18	>50	>2.8
Benztropine	17	46	2.7
Nelfinavir	12	32	2.7
Perphenazine	5	13	2.6
Promazine	13	33	2.5
Desloratadine	12	30	2.5
Lomerizine	9	22	2.4
Dyclonine hydrochloride	15	36	2.4
Ergocornine	18	43	2.4
Fluoxetine	7	16	2.3
Doxazosin	12	27	2.3
Raloxifene	10	22	2.2
Tacrine	24	>50	>2.1
Amitraz	16	33	2.1
Amitriptyline	12	23	1.9
Loratadine	9	17	1.9
Nefazodone	8	15	1.9
Tirilazad	28	>50	>1.8
Myrtecaine	14	24	1.7
Cyproterone acetate	32	>50	>1.6
Norethindrone acetate	17	28	1.6
Clobetasol propionate	25	41	1.6
Naftopidil	11	18	1.6
1-Benzylimidazole	35	>50	>1.4
Bopindolol	40	>50	>1.3
Prazosin	12	16	1.3
Sibutramine	8	10	1.3
Homochlorcyclizine	12	13	1.1
Naloxonazine	17	16	0.9
C8 ceramide	13	11	0.8

[a] Cell toxicity EC_{50}/*T. cruzi* EC_{50}.

DISCUSSION

High-content analysis is a powerful tool for drug discovery, as the technique combines both the biological relevance of cell-based assays with image data sets that can be queried for multiple measurements. In the *T. cruzi* HTS assay herein reported, we strived for simplicity and cost effectiveness by using a single dye, DAPI, to stain both host nuclei and parasite kDNA. The HTS assay allowed differentiation of host cells and intracellular parasites based on significant size differences between nuclei and kinetoplasts, respectively (Fig. 1 and 2). Such discrimination is not possible using conventional HTS microplate readers that can only measure total fluorescent signals.

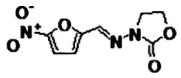
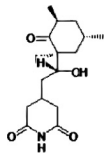
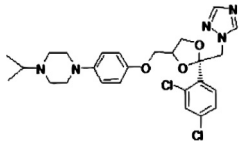
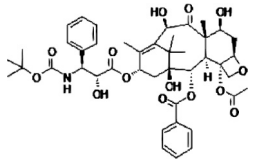
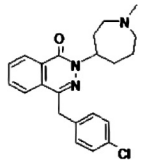
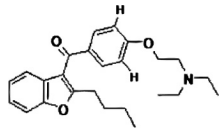
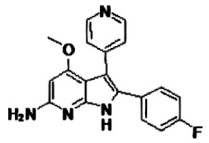
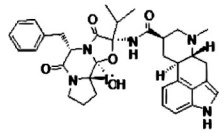
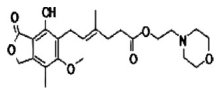
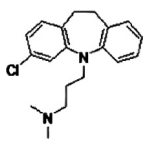
The HTS assay is not constrained to a specific strain of *T. cruzi* or host cell. In fact, the one-stain-for-two-species imaging method can be applied to assays involving other intracellular stages of parasites such as *Leishmania* and *Toxoplasma gondii*. In addition to screening libraries for drug leads, the HTS assay may well be used for the identification of factors that modulate the intracellular life cycle of *T. cruzi* or other intracellular pathogens.

The imaging HTS assay was timed so that the *T. cruzi* strain used had not completed its intracellular life cycle (88 h postinfection). Consequently, no significant host cell lysis was observed and parasites remained within the cytoplasm. Imaging prior to host cell lysis allowed us to perform all necessary culture steps and the fixation process without parasite loss, thus avoiding underestimation of parasite counts. To validate the HTS imaging assay, results from 10 compounds were evaluated comparatively with our nonautomated, multidose trypanocidal assay (26). Although the HTS assay was not designed to identify trypanocidal compounds but only compounds that hinder parasite growth, the results correlated well with those of the trypanocidal assay (Table 2). The HTS assay is therefore a useful tool to first identify bioactive compound hits from large libraries. The trypanocidal assay can be time-consuming and has low throughput due to the need for long-term incubation (40 days) and manual microscopic inspection of the cultures. In the HTS assay, seven image fields per well in a 96-well plate were captured within 17 min. The fluorescent signal from DAPI-stained nuclei and kinetoplasts remained stable for days, thus enabling a substantial gain in throughput by processing at least 25 plates together, corresponding to 2,000 compounds for a primary screening or 250 compounds for EC_{50} determinations, within a workday. Plates were screened in the IN Cell Analyzer 1000 imager fitted with an automated robotic arm. Image data sets queried with one algorithm could be revisited to quantify additional features. Thus, simple reanalysis of the existing data set could provide a higher content of information.

HTS and medium-throughput assays have been developed for other parasitic diseases (1, 42, 63). Constraints in their use for *T. cruzi* are a consequence of the particular biological characteristics of the life cycle of this parasite. The only multiplying stage of the parasite in the mammalian host is intracellular, preventing the use of bioluminescence measurements to quantify metabolic activity like ATP production (42). Genetically modified parasites that express transfected foreign proteins have been engineered and used successfully to screen chemical libraries (7, 11). However, the use of different populations of *T. cruzi* including clinical isolates in such assays is constrained by the difficulty in establishing stable transfectants of many parasite strains.

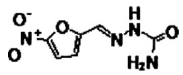
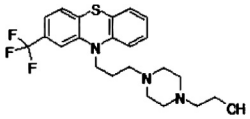
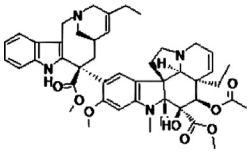
To address enormous unmet medical needs, there have been recent efforts in "repurposing" drugs (14, 15, 45, 55) from other therapies even for neglected parasitic diseases (5, 8, 19, 27, 34, 38, 39). The current therapeutic drugs for Chagas'

TABLE 5. Chemical structures and therapeutic use of clinical compounds with a >5-fold selectivity window against *T. cruzi*

Compound	Structure	Selectivity window	Therapeutic use[a]	FDA approved[a]	Reported trypanosome-related effect
Furazolidone		>125	Antibacterial	Yes	Trypanocidal
Cycloheximide		>125	Antifungal Antibacterial		Trypanocidal
Terconazole		>60	Antifungal	Yes	
Docetaxel		>56	Oncology	Yes	Trypanocidal
Azelastine		49	Allergy	Yes	
Amiodarone		45	Antianginal Antiarrhythmic	Yes	Trypanocidal
RWJ-68354		>10	Antiinflammatory		
Dihydro-ergocristine mesylate		9.4	Vascular dementia (vasodilator agent)	Yes	
Mycophenolate mofetil		>7.1	Antiinflammatory	Yes	
Clomipramine		>6.3	Depression	Yes	Trypanocidal

Continued on following page

TABLE 5—*Continued*

Compound	Structure	Selectivity window	Therapeutic use[a]	FDA approved[a]	Reported trypanosome-related effect
Nitrofurazone		>6.3	Antibacterial	Yes	Trypanocidal
Fluphenazine		5.3	Psychosis	Yes	Trypanocidal (*T. brucei*)
Vinorelbine		>5.0	Oncology	Yes	

[a] As described in reference 64.

disease were developed more than 40 years ago and have severe side effects (13). In addition, they are not efficient in clearing parasite infections in the chronic stage of the disease and there are naturally drug-resistant populations of *T. cruzi* (30). In an effort to repurpose clinical drugs from other therapies for neglected tropical diseases, we screened a small library of FDA-approved drugs and identified 17 compounds that showed at least 5-fold selectivity between the inhibition of *T. cruzi* and host cell toxicity (Table 4). Of these, five compounds (cycloheximide, nitrofurazone, furazolidone, terconazole, and nelfinavir) are anti-infective drugs against bacteria, fungi, or viruses (e.g., HIV), while the other 12 are used in various medical therapies. Among the hits identified, the topical antibiotic nitrofurazone has been reported to have *in vitro* activity against *T. cruzi* (16) and has been administered orally in the treatment of *Trypanosoma brucei* subsp. *gambiense* sleeping sickness (27). Another nitrofuran, furazolidone, is active against *T. cruzi* in mice (8). Trypanocidal activity has been reported for docetaxel (39), cycloheximide (34), and amiodarone (5). Nelfinavir, an HIV protease inhibitor, is active against *Toxoplasma gondii* (19). Haloperidol, an antipsychotic drug associated with the blockade of postsynaptic dopamine D2 receptors, is also active against *Toxoplasma gondii* (38) and was previously reported as inactive against the trypomastigote form of *T. cruzi* (37). Fluphenazine is a member of the phenothiazines that has been shown to inhibit the growth of *T. brucei in vitro* (58) but was inactive (36) or weakly active (6) against the *T. cruzi* enzyme trypanothione reductase. Carvedilol, a β-adrenergic receptor blocker, was demonstrated to improve Chagas' cardiomyopathy in patients when added to treatment with renin-angiotensin system (RAS) inhibitors (10). It will be interesting to reexamine the relationships between the therapeutic mode of action of these clinical compounds and their trypanocidal activity.

Taken together, our results indicate that the cell-based imaging HTS assay can be conveniently deployed as a primary screen in the drug discovery pipeline for Chagas' disease.

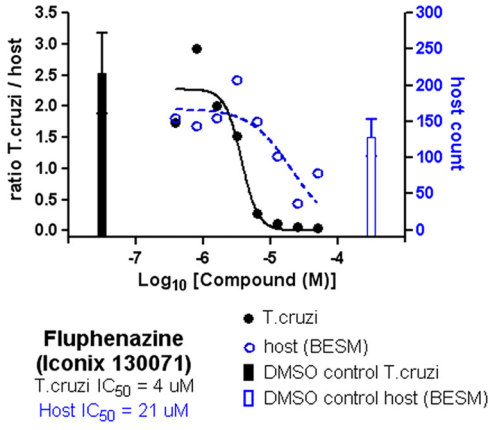


FIG. 4. Titration of fluphenazine and Iconix compound 130071 and EC_{50} values for *T. cruzi* and BESM cells. IC_{50}, 50% inhibitory concentration.

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