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Identification of growth inhibiting compounds in a *Giardia lamblia* high-throughput screen

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ABSTRACT

Giardia lamblia is one of the most common eukaryotic pathogens and is classified by the CDC as a category B agent of bioterrorism. In a departure from more traditional research focused on specific pathways or molecules, we have developed a high-throughput assay for screening libraries of small compounds for inhibitors and enhancers of trophozoite multiplication. Following a 24-h period of culture in 384-well plates in the presence of compounds, trophozoites were fixed, stained and enumerated. Quadruplicate screening of 1520 compounds from two libraries of known bioactives detected numerous inhibitory compounds. Based on a stringent cut-off of 5 standard deviations from the plate mean, 50 compounds (3.3%) were inhibitory. The activity of 3 compounds was confirmed in conventional culture. Although not meeting the threshold, one compound (indirubin) was identified as an agonist of trophozoite proliferation. Demonstrating the potential of high-throughput screening for rapidly finding new compounds which perturb *G. lamblia* multiplication, most of the hits identified by high-throughput screening do not appear to have been tested previously for their ability to affect *G. lamblia* trophozoites. High-throughput screening of bioactive compounds will open new avenues to a system-wide analysis of pathways affecting *G. lamblia* proliferation, and eventually to other phases of the life cycle.

1. Introduction

Research on *Giardia lamblia* is driven not only by the parasite's public health significance, but also by unusual morphological and metabolic features. The life cycle of this diplomonatid protozoan is direct, alternating between the dividing trophozoite stage and the cyst stage. Trophozoite multiplication presents many features not seen in a typical eukaryotic mitotic cycle, such as the presence of two nuclei and the alternation between tetraploid and octaploid sets of chromosomes [1]. Trophozoite division involves the duplication of a complex cytoskeleton and the inheritance by each daughter cell of a pair of nuclei [2]. In spite of its public health importance, our understanding of these processes, and of the regulation of the *G. lamblia* life cycle in general, is superficial and is mostly based on microscopic observations [3].

As with many pathogens, reductionist methods focused on specific molecules, pathways, or sub-cellular structures continues to drive research on *G. lamblia*. The sensitivity of *G. lamblia* trophozoites to oxygen has led to the perception that trophozoites cultures require sealed glass tubes [4–6]. This may have discouraged the exploration of alternative culture formats, in particular multi-well plates, which are needed for high-throughput screening (HTS). Our motivation to explore HTS in the context of *G. lamblia* trophozoite multiplication, is the potential of assumption-free methods to rapidly advance our understanding of regulatory networks and other complex biological systems. Particularly in a phylogenetically divergent organism as *G. lamblia*, an approach free of assumptions originating from more traditional biological systems has the advantage of being free of bias. The trophozoite HTS assay described here was designed to detect not only antagonists, but also agonists of trophozoite division. Whereas antagonists are of obvious relevance to the development of new therapeutics, both activities are of interest to more basic research questions, and to the application of chemical genetics to explore trophozoite multiplication.

Here, we describe a HTS for small molecules which perturb *G. lamblia* trophozoite proliferation in culture. We then re-screened a small number of inhibitory compounds to discriminate between

Abbreviations: HTS, high-throuput screening; DPI, diphenylenieiodonium chloride; NINDS, National Institute of Neurological Diseases and Stroke.

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toxic compounds which kill the parasite, and compounds which reversibly arrest mitosis. Future improvements to the assay will incorporate vital dyes to directly differentiate between toxic and reversible inhibitors.

2. Materials and methods

2.1. High-throughput screens

A stock culture of *G. lamblia* (WB strain, ATCC 50583 [7]) was maintained by serial passage in 20 ml screw-cap glass tubes in TYI-S-33 medium [4]. A volume of 45 μl TYI-S-33 medium was dispensed into each well of flat-bottom black 384-well plates with clear bottom (cat. no. # 3712, Corning, New York) with a Matrix WellMate liquid handling robot (Thermo Fisher Scientific, Hudson, New Hampshire). Portions of 100 nl of compounds (typical stock concentration is 5 mM) were pin-transferred to this plate and 45 μl of a suspension of 2×10^4 trophozoites/ml added to each well using the WellMate dispenser. Trophozoites were thus exposed to compounds for the entire duration of the experiment. Plates were incubated in a humidified 37 °C/5% CO_2 cell culture incubator for 24 h. The same set of six plates was screened with two duplicate screens.

Following incubation of cultures with 5 μg/ml compound, trophozoites were fixed in situ in 8% glutaraldehyde. To avoid dislodging the trophozoites, one half of the medium was aspirated from each well using a 16-channel wand (V&P Scientific Inc., San Diego, CA) without disturbing the trophozoite layer, and the same volume replaced with 8% glutaraldehyde, giving a final 4% glutaraldehyde concentration. Following a 10-min incubation, the glutaraldehyde/medium mixture was completely removed and replaced with 8% glutaraldehyde. Following a 5-min fixation period, the glutaraldehyde was removed and the wells gently washed with water. A volume of 90 μl of 20 μg/ml propidium iodide was then added to each well to stain the trophozoites. After a 30-min staining period, the propidium iodide solution was removed and the plates air dried in a desiccator.

Stained plates were imaged using an ImageXpress Micro imaging system (Molecular Devices, Sunnyvale, CA) fitted with a propidium iodide filter cube. Four non-overlapping images covering approximately 90% of each well's surface were acquired at 100× magnification and stored as 16-bit TIF files. The number of trophozoites in each image was determined using CellProfiler. CellProfiler is an open-source software package which can enumerate objects based on size, shape, or fluorescence intensity [8]. Trophozoites counts were acquired by counting any object within a range of 3–19 pixels.

2.2. Secondary screens

Trophozoites were inoculated at an initial concentration of 1.2×10^4 ml^{-1} into TYI-S-33 medium in 2 or 4 ml screw-cap glass vials. The following compounds were prepared at a concentration of 5 mg/ml in DMSO, except nocodazole which was dissolved at a concentration of 1 mg/ml: indirubin (BIOMOL cat. no. CC-206), diphenylenieiodonium chloride (DPI, BIOMOL cat. no. CN-240), gliotoxin (BIOMOL cat. no. PI-129), and nocodazole (Sigma cat. no. M1404). Compounds were added to each culture to a final concentration of 5 μg/ml (nocodazole at 1 μg/ml) at time 0, and the closed vials incubated at 37 °C. To measure the concentration of trophozoites at subsequent time points, vials were chilled on ice to release adherent trophozoites, the cultures inverted several times to suspend the parasites, 50 μl of culture removed, and trophozoites immobilized with the addition of an equal volume of a 1:1 dilution of 5% bleach, giving a final concentration of approximately 1.5% sodium hypochloride. Cultures were returned to 37 °C and the trophozoites in the sample counted in a hemocytometer.

Table 1
Summary statistics of high-throughput screen of 1520 compounds

	Cut-off (S.D. from mean)		
	−3	−5	−6
Number of hits	65	50	48
Empty well hits[a]	3	0	0
false Positive proportion	0.0038	0	0

[a] Number of wells without compound is 784.

2.3. Data analysis and definition of hits

For each of four replicated screens we first adjusted for positional effects within each plate using the median polish method [9,10]. This was necessary because trophozoites growth in edge rows and columns was inferior to that observed in interior wells. The corrected values were centered and standardized using the median and scaled median absolute deviation. The standardized values from four screenings were then averaged to obtain a summary score. Hits were then defined as normalized counts deviating from 0 by 3, 5 and 6 standard deviations.

3. Results

3.1. High-throughput screening

Two duplicate screens of 480 compounds from the BIOMOL ICCB Known Bioactives 2 compound library and 1040 compounds from the NINDS Custom Collection 2 (Supplement) were performed for a total of four replicate screens comprising 6080 individual assays on 24 384-well plates. The visual inspection of color coded heat maps of trophozoite counts revealed a significant edge effect; trophozoite counts in edge wells were generally lower, perhaps a result of an edge-center oxygen gradient. Computational correction of this artifact, performed as described above, enabled the identification of active compounds regardless of their position on the plate. In addition to color-coding screen quality was also visually assessed by plotting trophozoite counts from two replicate screens on scatter plots (Fig. 1A). Cumulative trophozoite counts were also calculated for each well and their frequency distribution was plotted (Fig. 1B). These plots illustrate the ability of the assay to easily identify inhibitors. In contrast, the ability to identify of enhancers of trophozoite was less obvious due to the limited dynamic range of the assay and to the fact that cultures reached near-saturation.

The number of agonist of trophozoite multiplication identified by HTS depends on the threshold (Table 1). Using the most stringent cut-off of 6 standard deviations 48 hits were found among 1520 compounds screened. No false positive were found among the 784 empty wells present on the six plates, although with a less stringent cut-off of 3 standard deviations, 3 false inhibitors were found among 784 control wells without compound. As the threshold was lowered to 3 standard deviations, the number of positives increased to 65. The results from the HTS and the identity of 48 inhibitors are summarized in Table 2.

Although the activities of compounds identified as inhibitors are diverse, most are known toxins or inhibitors. For instance, actinomycin D, a well-known inhibitor of RNA polymerase, and aphidicholin, a potent DNA polymerase inhibitor both strongly inhibited trophozoite growth. Some of the hits, such as taxol, nocodazole and colchicine have been previously shown to inhibit trophozoite growth, the former two in a reversible fashion [11]. Sig-

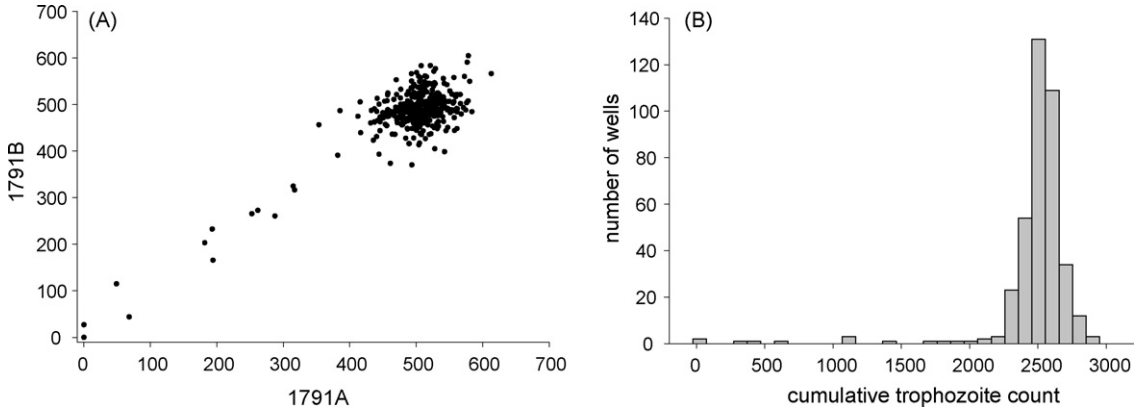

Fig. 1. Trophozoite counts visualized on a scatter plot of replicate screens and as a frequency distribution histogram. (A) Scatter plot showing trophozoite counts from two replicate screens. Assay readouts for screens A and B are plotted on *x*-and *y*-axis, respectively. (B) Frequency distribution of cumulative trophozoite counts from four replicate screens of plate 1791. Counts were corrected for edge effect.

nificantly, most hits were not previously known to affect *G. lamblia* and their mode of action remains to be elucidated.

In its current configuration, the power of the HTS assay to identify enhancers of trophozoite mitosis is limited. Using the least stringent cut-off of 3 standard deviations no agonists of trophozoite multiplication was identified (Fig. 2). However, when edge-adjusted and ranked counts were visually inspected for compounds associated with high trophozoite counts, one compound, h11 on Plate 1792 (indirubin), was noticed for ranking among the top 10% in all four screenings. Although h11 does not meet the primary screen cut-off, we conducted confirmatory analysis for indirubin as described below.

3.2. Secondary screens

Two inhibitors of trophozoite growth indentified by HTS were randomly selected among available compounds for secondary screening. For secondary screens cultures were grown in 2 ml screw-cap glass vials in the presence of compound. The inhibitory activity of DPI and gliotoxin were confirmed (Fig. 3). No trophozoite survived in cultures containing these compounds at a concentration of 5 μg/ml. After 24 h, the treated cultures were essentially devoid of recognizable trophozoites, whereas the control culture grew normally. We further investigated if the inhibitory effect of DPI and gliotoxin were reversible following a 24-h exposure to 5 μg/ml.

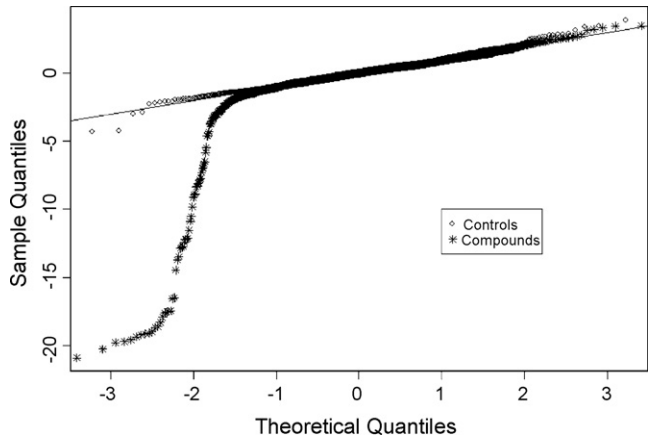

Fig. 2. Normal Quantile-Quantile plot of trophozoite counts from wells containing no compounds (circles) and wells with compounds (*). Compounds deviating from the normal distribution, indicated with a continuous line, are putative hits.

In addition, nocodazole, which also was strongly inhibitory in HTS, was also included because its effect on trophozoites, and on the cytoskeleton in particular, has been investigated previously [11–13]. Contrary to prior observations with lower concentration and shorter exposure time [13], the effect of nocodazole could not be reversed by replacing the medium. Similarly DPI at 5 μg/ml was also irreversibly toxic. In contrast, a culture treated with the same concentration of gliotoxin resumed normal growth 48 h after gliotoxin was removed. Secondary screens can thus be used to identify lethal compounds, and identify concentrations which arrest growth without killing the trophozoites.

Because indirubin was identified as a possible agonist of trophozoite proliferation, and the biological interest in identifying agonists of trophozoite multiplication, we tested this compound in four independent secondary screens at concentrations ranging from 5 to 20 μg/ml (Fig. 4). Due to the poor water solubility of indirubin, the equivalent concentration of the DMSO solvent was added to control cultures. In all experiments, the highest indirubin concentration had a slight enhancing effect on trophozoite multiplication. We applied Poisson regression to model the cell counts as a smooth function of time. The estimated growth rates in the groups exposed to 10 or 20 μg/ml indirubin ranged from 34% (p-value < .001) to 8% (p-value < .01) higher than the DMSO controls in experiments A–D. We also observed a difference between experiments with respect to the concentration of indirubin needed to promote proliferation and assume that this effect originates from the fact that in experiments B–D the indirubin solution was sterilized by heating to 68 °C, whereas filtration was used in the first experiment. The former treatment may have partially inactivated indirubin, requiring more compound to achieve a growth promoting effect. In experiment A, the same culture tubes were periodically chilled and aliquots removed for counting, whereas in experiments B–D multiple tubes were seeded with parasites, and counted only once. This may explain the fact that in the first experiment, the cultures had not reached peak density after 73 h. Although the indirubin effect is modest, we conclude that this compound promotes *G. lamblia* proliferation in culture and that, in the presence of this compound, cultures peak at a higher trophozoite density.

4. Discussion

Our motivation for screening bioactive compounds with a *G. lamblia* proliferation assay is twofold: to explore the feasibility of applying chemical genetics to study trophozoite multiplication,

Table 2
Inhibitors based on a cut-off of 6 standard deviations

Plate (compounds)	Location	Compound name or code	Supplier and number[a]	Standardized trophozoite count
1791	d4	Ebselen	B EI-207	−7.0
(320)	n4	Calyculin A	B EI-192	−17.6
	b8	β-Lapachone	B GR-308	−19.6
	f8	Actinomycin D	B GR-300	−10.9
	n8	Cantharidin	B PR-105	−7.0
	b12	*Unnamed compound*	B PI-102	−9.8
	b14	Helenalin	B EI-301	−20.3
	h16	17AAG	B EI-308	−16.6
	p16	Cycloheximide	B GR-310	−11.6
	h18	Anisomycin	B ST-102	−13.7
	l18	SB-415286	B EI-311	−6.6
	h20	Aphidicolin[b]	B CC-101	−19.6
1792	b3	Nocodazole[b]	B T101	−19.2
(160)	g3	GO6976	B EI-269	−19.1
	j7	Staurosporin	B EI-156	−19.8
	m7	LY83583	B CN-200	−17.5
	c9	Diphenylamineiodonium	B CN-240	−18.3
	i9	ICRF-193	B GR-332	−16.5
	l9	tolbutamide	B AC-117	−10.5
	m9	5-Iodotubercidin	B EI-293	−8.3
	n9	Vinblastine sulfate	B T-116	−17.5
	o9	Mitomycin C	B GR-311	−7.8
	c11	2,5-Di-*tert*-butylhydro quinone	B CA-420	−8.9
	k11	Latrunculin B	B T-110	−7.7
	j13	Taxol[b]	B T-104	−19.7
	e15	Glendanamycin	B EI-280	−8.3
	e17	Genistein[b]	B EI-147	−12.1
	m19	Gliotoxin	B PI-129	−18.0
	i21	K252a	B EI-152	−12.4
1920	b4	Nitrofurazone[b]	N 1500434	−12.2
(320)	i12	Pyrithion zinc	N 1500260	−8.1
	n16	Quinacrine HCl[b]	N 1500522	−12.2
	o22	Furazolidone[b]	N 1500309	−12.9
1921	d3	Fenbendazole[b]	N 1501016	−18.7
(320)	f3	Mebendazole[b]	N 1501110	−20.9
	o3	Phenylmercuric acetate	N 1500644	−18.6
	l4	Cycloheximide	N 1502112	−14.5
	e7	Thimerosal[b]	N 1500572	−17.5
1922	k6	Anisomycin	N 1503906	−9.1
(320)	b8	Oxfendazole[b]	N 1505296	−13.5
	k8	Taxol[b]	N 1503908	−19.0
	o9	Nocodazole	N 1503266	−19.2
	a12	Thiram	N 1503322	−8.9
	n18	Formononentin	N 102007	−19.4
	p18	Diadzein	N 200789	−12.8
	a22	Oxybendazole	N 1503373	−8.1
1923	e9	Griseofulvin	N 200046	−6.5
(80)	a13	Celastrol	N 201664	−12.7

[a] Library names: B, BIOMOL ICCB Known Bioactives2—High Conc.; N, NINDS Custom Collection 2.
[b] Asterisk indicates known activity against *G. lamblia*.

and to identify new *G. lamblia* drug targets. We anticipate that chemical genetics will uncover pathways which regulate mitosis, as well as numerous metabolic pathways which directly or indirectly maintain trophozoite viability. As expected from the complexity of the trophozoite cytoskeleton [2,11,14,15], several compounds which interfere with microtubules, including nocodazole, vinblastine, latrunculin B, taxol, and mebendazole, were identified among the inhibitors. With respect to nocodazole, in HTS trophozoites were exposed to 5.5 μg/ml drug, comparable to the 3 μg/ml concentration used by Mariante et al. [12], but almost 200-fold higher than used for synchronizing trophozoites in culture [13].

Although the purpose of HTS is the identification of active compounds, an inspection of the libraries (Supplement) may uncover previously unknown differences between *G. lamblia* and cells from other species. The identification of anti-fungals or anti-bacterials which showed no activity against *G. lamblia* would not only indicate biologically interesting differences in metabolism, but also be of practical significance. Such compounds could facilitate the axenification of new isolates by controlling contanimating microorganisms without affecting trophozoite proliferation, or could be used to rescue contaminated cultures.

We envisage that the creative design of assays for various phases of the *G. lamblia* life cycle will enable the application chemical genetics to dissecting the entire life cycle. This parasite is amenable to this approach as all phases of its life cycle can be reproduced in vitro. For instance, assays suitable for HTS can be developed for excystation, encystation, and trophozoite attachment. Together, the information derived from these assays may lead to the development of a comprehensive map of the metabolic network controlling the parasite's life cycle. Microarray analysis comparing parasite gene expression in the presence and absence of perturbing compounds will be relevant to interpreting these results. Trophozoite division

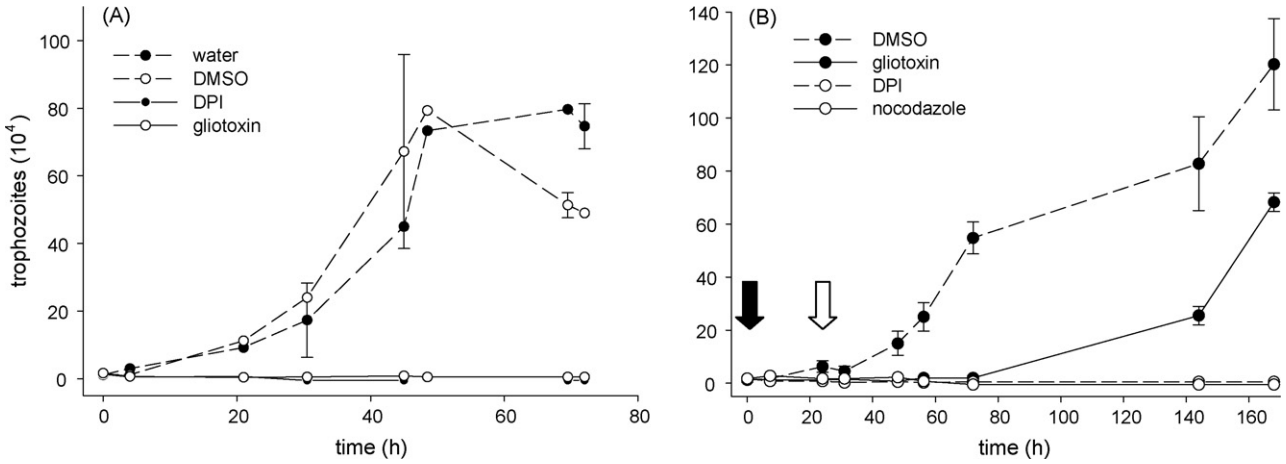

Fig. 3. Secondary screens of inhibitory compounds. (A) Growth curves of trophozoite cultures exposed to DPI and gliotoxin. To improve the clarity of the graph, only selected error bars (S.D.) are shown. (B) Reversibility of gliotoxin inhibition. Secondary screens were performed as described except that the medium was replaced with compound-free medium 24 h post-inoculation. The filled and empty arrows mark the time at which compounds were added and removed.

was selected for the initial proof of feasibility, not only because of the relative simplicity of the assay, but also because trophozoite proliferation is directly related to the pathogenesis of giardiasis.

HTS may also enable a comprehensive functional study of the trophozoite adhesive disk by identifying molecules which inhibit or increase attachment. An assay capable of separately quantifying adhering and free trophozoites based on gravity [16] is currently being developed. Chemical genetics could also be used to investigate other unique aspects of the *G. lamblia* metabolism, such as its sensitivity to oxygen [17], or mechanisms conferring resistance to metronidazole. Compounds which differentially affect *G. lamblia* assemblages could be found in a comparative HTS, and serve

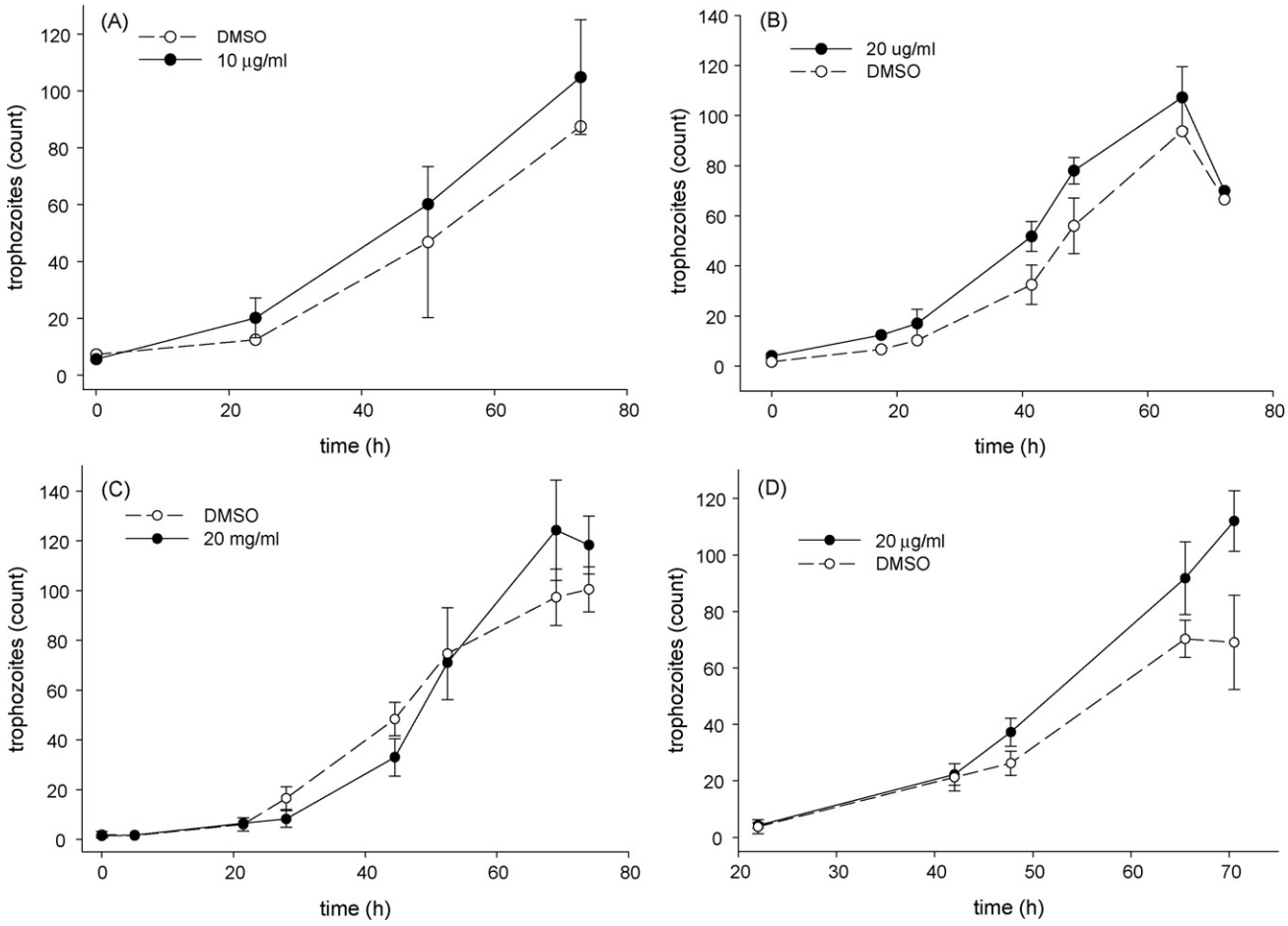

Fig. 4. Indirubin promotes trophozoite proliferation in secondary screens. Trophozoite cultures were grown in the presence of indirubin or DMSO as indicated. A–D represent four replicate and independent experiments. In experiment A trophozoites were enumerated by repeatedly chilling and sampling a single culture; in experiments B-D replicate culture tubes were chilled at subsequent time points and the trophozoites counted.

to identify phenotypic differences between assemblages and their relevance to disease.

Since the high-throughput assay in its current format does not discriminate between live and dead trophozoites, the toxicity of inhibitory compounds to *G. lamblia* trophozoites is unknown. As demonstrated by the secondary screens, the effect of certain compounds can be reversed, a fact which is well known, and is sometimes used to synchronize trophozoite cultures [13,18,19]. The mechanism of trophozoite inhibition by gliotoxin has to our knowledge not been investigated, nor its suitability for synchronizing trophozoite cultures. This compound is an epipolythiodioxopiperazine immunosuppressive mycotoxin produced by certain fungi belonging to the genera *Aspergillus*, and *Gliocladium*. Its effect in many biological systems has been studied, but, as with many compounds identified in our screen, its activity against *G. lamblia* has apparently not been reported. DPI, the second compound which could not be reversed, is known to irreversibly inhibit nitric oxide synthase with a 50% inhibitory concentration in cultured macrophages of only 30 nM [20]. NOS activity in *G. lamblia* has been reported [21] and its sequence identified in the *G. lamblia* genome (giardiadb.org; gene GL50803_91252). Together these observations imply that NOS is essential for trophozoite survival and multiplication.

Compounds which can arrest trophozoite multiplication without killing the parasite are of particular interest for identifying pathways which modulate trophozoite mitosis. A refined HTS assay, in which the compound is present only for a portion of the growth cycle, would enable a systematic differentiation of reversible and toxic inhibitors. An analysis of the morphology of growth arrested trophozoites, together with knowledge about the mode of action of such compounds in other systems, will lead to testable models of metabolic networks regulating trophozoite mitosis and multiplication in general.

Agonists of trophozoite proliferation are of particular interest as they may provide new insights into the regulation of mitosis. In its present configuration the assay is poorly suited to identify enhancers of trophozoite proliferation that might be present in compound libraries. The assay is limited by it's dynamic range, primarily the maximum concentration of trophozoites that can accurately be measured. The assay could be adapted for detecting agonists by either reducing the initial trophozoites concentration, or reducing the incubation time, or limiting growth rates by growing trophozoites in sub-optimal conditions such as culture in diluted medium. We have observed that growth rates and peak trophozoite density are reduced approximately in proportion to the concentration of the growth medium, suggesting that screens in diluted medium may uncover additional agonists. However, agonists of trophozoite growth are likely to be rare, as this phenotype is probably regulated by multiple interacting metabolic pathways. Activators are therefore less likely to be encountered than inhibitors, as inhibition results from the perturbation of any one of a multitude of essential pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2008.08.005.

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