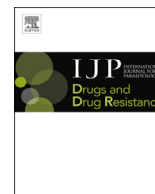




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A novel *in vitro* image-based assay identifies new drug leads for giardiasis



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ABSTRACT

Giardia duodenalis is an intestinal parasite that causes giardiasis, a widespread human gastrointestinal disease. Treatment of giardiasis relies on a small arsenal of compounds that can suffer from limitations including side-effects, variable treatment efficacy and parasite drug resistance. Thus new anti-*Giardia* drug leads are required. The search for new compounds with anti-*Giardia* activity currently depends on assays that can be labour-intensive, expensive and restricted to measuring activity at a single time-point. Here we describe a new *in vitro* assay to assess anti-*Giardia* activity. This image-based assay utilizes the Perkin-Elmer Operetta[®] and permits automated assessment of parasite growth at multiple time points without cell-staining. Using this new approach, we assessed the “Malaria Box” compound set for anti-*Giardia* activity. Three compounds with sub- μ M activity (IC₅₀ 0.6–0.9 μ M) were identified as potential starting points for giardiasis drug discovery.

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1. Introduction

Giardiasis causes significant worldwide morbidity with an estimated 184 million symptomatic cases annually (Pires et al., 2015) and an associated 171,100 disability-adjusted life years (DALYs) (Kirk et al., 2015). While giardiasis is more prevalent in the developing world it is also a burden in developed countries, with hospital based treatments in the United States of America costing \$34.4 million (USD) annually (Collier et al., 2012). Giardiasis is commonly associated with clinical symptoms including nausea, vomiting and acute diarrhoea (Nash et al., 1987; Farthing, 1996). However it can manifest as a chronic disease and cause malabsorption, weight loss and failure to thrive in children (Al-Mekhlafi et al., 2005, 2013; Bartelt et al., 2013). There is also mounting evidence that *Giardia* infection may be linked to irritable bowel syndrome, food allergies and obesity (Di Prisco et al., 1998; Hanevik et al., 2009; Guerrant et al., 2013).

As there is no currently available vaccine for humans, the control of giardiasis is dependent on chemotherapy. Current chemotherapeutic options are limited to a small number of compounds which

are associated with treatment failures and clinical resistance (reviewed in Ansell et al., 2015). The 5-nitroimidazole class of compounds, typically metronidazole, are the most commonly used treatment agents (Watkins and Eckmann, 2014). However, these compounds have reported clinical failure rates of up to 40% (Oren et al., 1991; Farthing, 1996; reviewed in Watkins and Eckmann, 2014; Nabarro et al., 2015) and can also cause significant side-effects including neurological disorders and sudden death (Escobedo and Cimerman, 2007). Alternative agents including the benzimidazoles, such as albendazole, can also be used. However, the efficacy of these drugs varies widely (e.g. Hall and Nahar, 1993; Escobedo et al., 2003). In addition, the benzimidazole drugs appear particularly susceptible to the development of drug resistance, with data suggesting that parasite resistance can be easily selected *in vitro* (Gardner and Hill, 2001). New anti-*Giardia* agents with improved efficacy and toxicity are needed to improve this position.

A number of low to high throughput *in vitro* assays have been developed to identify new compounds active against *Giardia*. However, most rely on metabolic indicators or manual cell counting. Activity assays that rely on manual cell counting via microscopy have the advantage of permitting the assessment of growth at multiple time-points and provide useful morphological information, but are time consuming and may be subjective. While the more automated assays that make use of growth indicators

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including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT), resazurin (AlamarBlue[®]), ³H-thymidine, ATP content or the assessment of glucuronidase activity in transgenic parasites (Müller et al., 2009) are more rapid, they inherently increase assay cost, provide limited activity/morphology information and permit only single time-point of assessment. Activity assays reliant on transgene expression are also limited to assessing activity against genetically manipulated parasites.

Efforts to improve current growth assay methods have included combining microscopy with automated image analysis software to decrease time limitations associated with manual enumeration methods (Bonilla-Santiago et al., 2008; Faghiri et al., 2011; Gut et al., 2011). For example, in an approach reported by Gut et al. (2011) parasites are stained with 4',6-diamidino-2-phenylindole (DAPI) to automatically distinguish and enumerate living trophozoites without bias. While this significantly reduces assay evaluation time, parasites must still be fixed and stained which necessitates extra handling and eliminates the possibility of multiple time-point evaluations.

In this study, we developed an automated live-cell digital phase-contrast microscopy assay to assess the activity of compounds against *Giardia* trophozoites *in vitro*. The Perkin-Elmer Operetta[®], with its associated Harmony[®] and PhenoLOGIC[™] software, was used to exploit the power of automated digital phase-contrast microscopy and image analysis as a mechanism to identify and enumerate parasites based on their morphology without the need for a cell marker. A particular advantage of this approach is the ability to assess parasite growth at multiple time-points. This assay was used to assess the anti-*Giardia* activity of compounds from the "Malaria Box". The "Malaria Box", a set of compounds with known activity against mammalian cells (Kaiser et al., 2015) multiple parasite species including *P. falciparum* (Spangenberg et al., 2013), *Toxoplasma gondii*, *Entamoeba histolytica* (Boyom et al., 2014), *Cryptosporidium parvum* (Bessoff et al., 2014), *Leishmania major* (Khraiwesh et al., 2016) and *Trypanosoma* spp. (Kaiser et al., 2015) has never previously been assessed for anti-*Giardia* activity.

2. Materials and methods

2.1. Parasites and culture

G. duodenalis (strain BRIS/91/HEPU/1279; metronidazole sensitive; assemblage B (Upcroft et al., 1995; Nolan et al., 2011)) was grown axenically (3% O₂ 5% CO₂, in N₂ at 37 °C) in Kiesters-modified TYI-S-33 media in 8 mL borosilicate vials (Pyrex glass, No. 9825; VWR) as previously described (Keister, 1983; Meloni and Thompson, 1987). Media was prepared on a weekly basis and stored at 4 °C. When required for use, aliquots were supplemented with 10% foetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin.

2.2. Compounds

Albendazole, metronidazole and furazolidone were obtained from Sigma-Aldrich, USA and prepared in 100% DMSO to stock concentrations of 10–50 mM. Stocks were stored at –20 °C until required. Malaria Box compounds were obtained from the Medicines for Malaria Venture (MMV; www.mmv.org) as 10 mM stocks prepared in 100% DMSO.

2.3. Establishing assay conditions

2.3.1. Comparing automated parasite enumeration with manual counting

Giardia parasites were grown in stock 8 mL borosilicate tubes (Section 2.1) to ≤ 80% confluence. Parasites were detached from culture vials by incubating on ice for 30 min. After detachment, parasites were collected, counted using a haemocytometer and seeded in 96-well micro titre plates (Corning Costar 3596; total volume 200 µL; 2 × 10⁴ to 5 × 10³ cells/well). Outside wells of plates contained phosphate-buffered saline to reduce evaporation (PBS; 200 µL). Plates were incubated at 37 °C in sealed, activated Anaerocult[®] C mini bags as per manufacturer instructions as previously described (Upcroft and Upcroft, 2001). Growth of parasites seeded in triplicate wells on two separate occasions was assessed at 24 and 48 h by digital phase-contrast microscopy, enumerated using Harmony[®] and PhenoLOGIC[™] software (Section 2.6) and by the manual counting of bright-field images. Data from all experiments were combined (mean parasite count/1.7 mm² ± SD) and manual versus automated counts were compared using a student's t-test (Graphpad Prism 7[®]).

2.3.2. Assessing parasite growth in assay conditions

Giardia parasites were grown and prepared as described above. After detachment, parasites were collected, counted using a haemocytometer and seeded in 96-well micro titre plates (Corning Costar 3596; total volume 200 µL; 6 × 10⁴ to 5 × 10³ cells/well). However, as a reliable source of Anaerocult[®] C mini bags (Merck, Millipore) could not be obtained, microaerophilic conditions were established by incubating plates at 37 °C in air-tight chambers filled with 3% O₂ 5% CO₂ in N₂ as previously described (Gut et al., 2011). Growth of parasites was assessed at 24 and 48 h by digital phase-contrast microscopy and enumerated using Harmony[®] and PhenoLOGIC[™] software (Section 2.6). Data are presented as mean trophozoite count ± SD of 4 separate experiments, each carried out in triplicate wells. The average doubling time between 24 and 48 h for each seeding concentration was calculated using the equation, $t^d = (24) \times \log(2) / \log(c^2/c^1)$ where t^d = doubling time, c^1 was the average 24 h count and c^2 was the average 48 h count.

2.3.3. Assessing the impact of imaging on parasite growth

As assay plates were outside of anaerobic conditions during imaging (Section 2.6; ~20 min) and then returned to culture post-imaging for further incubation and assessment, the impact of imaging on parasite growth was assessed. In these assays two identical 96-well micro titre plates were prepared. One plate was imaged at 24 and 48 h and the other only at 48 h. In brief trophozoites were seeded into 96-well plates (3 × 10⁴ to 3.75 × 10³ parasites/well in 200 µL) and incubated in 3% O₂ 5% CO₂, in N₂ at 37 °C until imaging (Section 2.6). The 24 and 48 h imaged plate was returned to culture conditions after imaging at 24 h and re-imaged again at 48 h whereas the 48 h only plate remained in microaerophilic conditions until imaging at 48 h. Each cell seeding concentration was plated in six technical replicates on a single plate and each assay was repeated on three separate occasions. Data are presented as mean parasite count/1.7 mm² ± SD and cell counts were compared using a student's t-test (Graphpad Prism 7[®]).

2.4. Assessing the activity of control anti-*Giardia* compounds

The activity of albendazole, metronidazole and furazolidone against *Giardia* trophozoites was assessed. Each compound was serially diluted in triplicate wells (100 µL; 8 point dilution series for albendazole and furazolidone and 15 point dilution series for

metronidazole), and all wells except media only controls, were seeded with 1.5×10^4 *Giardia* trophozoites (100 μ L; 200 μ L final volume). Plates were incubated in 3% O₂ 5% CO₂, in N₂ at 37 °C until imaging and growth analysis (Section 2.6) at 24 and 48 h. Each assay included no drug with vehicle (0.2% DMSO) and no vehicle controls and in each case three independent assays were carried out. The concentration of DMSO in drug dilutions was kept constant at 0.2% and as previously shown (Johns et al., 1995) had no impact on parasite growth. Mean percentage growth inhibition compared to vehicle (0.2% DMSO) and background controls was determined for each assay. IC₅₀ values were calculated using log-linear interpolation (Huber and Koella, 1993).

2.5. Assessing the Malaria Box compounds for activity against *G. duodenalis*

All Malaria Box compounds were screened for activity against *G. duodenalis* BRIS/91/HEPU/1279 at a final concentration of 10 μ M in singlicate, in two independent experiments. Each plate included, background media, vehicle (0.2% DMSO), no vehicle and albendazole (10 μ M) controls. Assays were performed under the same

conditions as those used to assess the activity of control anti-*Giardia* compounds (Section 2.4; 1.5×10^4 parasites/well in final volume 200 μ L; imaged at 24 and 48 h). Compounds demonstrating greater than 50% inhibition at this concentration were assessed for activity at 5 μ M in duplicate (n = 2). Z-factors were calculated for each plate of each screening assay as previously described (Zhang et al., 1999). Compounds showing $\geq 50\%$ inhibition at 5 μ M were further investigated to determine IC₅₀ values as described for control compounds (Section 2.4; each titration was performed in duplicate on three occasions; 8 point dilution series; compound concentration range 10,000–78 nM).

2.6. Digital phase-contrast microscopy, image acquisition and analysis

Individual wells on assay plates were imaged using the PerkinElmer Operetta[®]. Plates were removed from incubation and each well was imaged using brightfield and phase-contrast microscopy (total area imaged 1.7 mm²; <20 min/plate) before being returned to incubation if required. Brightfield images were taken 1 μ m from the base of each well, with exposure set to 100 ms. Digital phase-

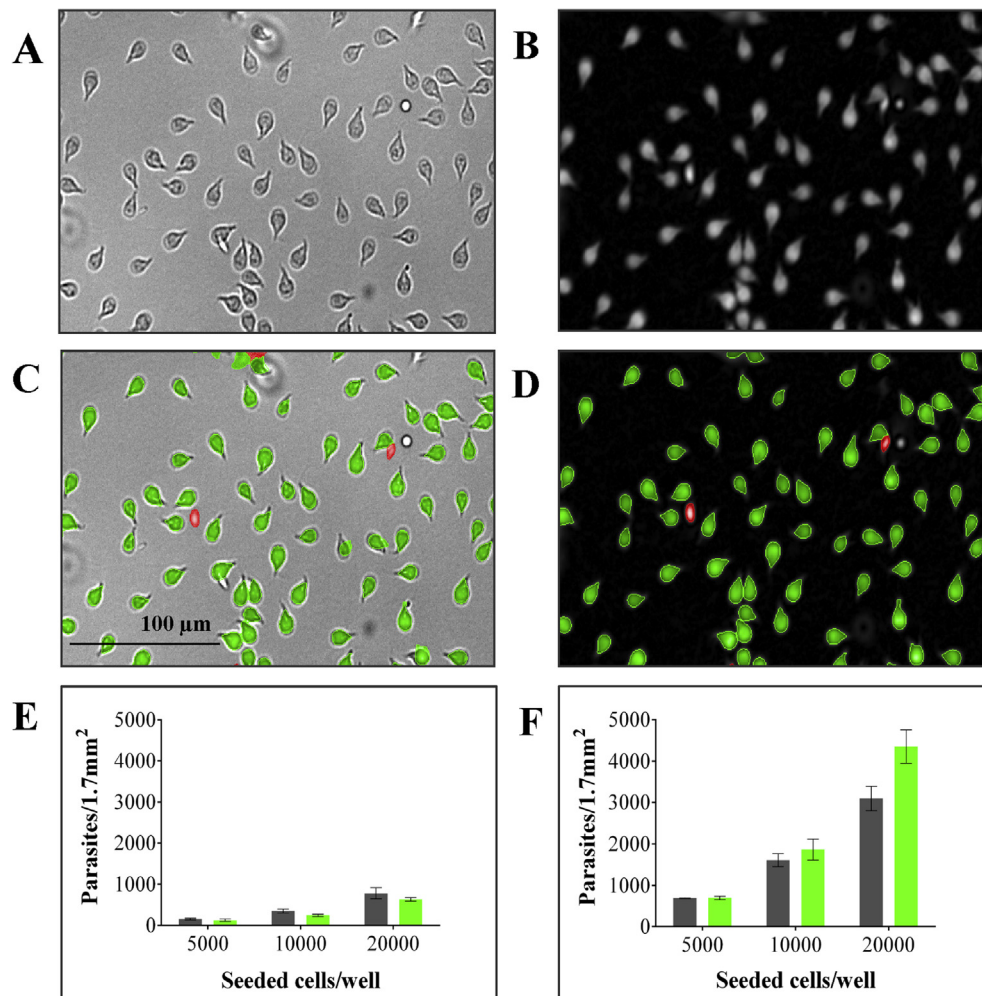


Fig. 1. Automatic enumeration of *Giardia* trophozoites by digital phase-contrast microscopy paired with Harmony[®] and Phenologic[™] automated counting. *Giardia* trophozoites seeded in 96-well micro-titre plates were imaged using brightfield (A) and digital phase-contrast microscopy (B). Images were automatically assessed by Harmony[®] and Phenologic[™] to identify and count trophozoites (green) amongst other signals (red) (C; brightfield and D; digital phase-contrast images). The effectiveness of the automated counting strategy was determined by comparing automated counts (E and F; green bars) to manually determining parasite numbers (E and F; grey bars). Parasite cultures were initiated at seeding concentrations of 2×10^4 – 5×10^3 cells/well and cell numbers were determined at 24 (E) and 48 h (F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

contrast images were taken with 40 ms exposure between $-5 \mu\text{m}$ and $5 \mu\text{m}$, with a speckle scale of $10 \mu\text{m}$. Images were automatically analysed and parasites enumerated with Harmony[®] and Phenologic[™] software manually trained to identify and count trophozoites. Manual training was performed within Harmony and Phenologic[™] using the “Select Population” building block and the “Linear Classifier” method. After training, an algorithm to identify trophozoites based on properties including their size and morphology was generated and used to assess all subsequent images.

3. Results

3.1. Establishing assay conditions

Growth assessment experiments showed that enumeration of *Giardia* parasites by digital phase-contrast microscopy and automated image analysis is as least as effective as examining parasite growth by manual cell counting (Fig. 1). Trophozoites were reliably identified by trained software (Fig. 1 C and D) and there was no statistically significant difference in parasite numbers when assessed manually or automatically using the Harmony[®] and Phenologic[™] software (Fig. 1 E and F; $p > 0.05$ in all cases).

Data describing trophozoite growth in assay culture conditions suggested that 1.5×10^4 cells/well was the highest initial cell density able to maintain adequate BRIS/91/HEPU/1279 growth for 48 h, the duration of planned screening assays (Fig. 2A). When seeded at higher or lower cell concentrations mean parasite doubling times increased (Fig. 2A insert). Imaging was also found to have no significant impact on parasite growth as growth of parasites on plates imaged at 24 and 48 h was not significantly different to those grown on plates imaged only at 48 h (Fig. 2B; $p = 0.68$).

3.2. Assessing the activity of control anti-*Giardia* compounds

The *in vitro* anti-*Giardia* activity of albendazole, metronidazole and furazolidone was assessed using digital phase-contrast microscopy and automated enumeration (Table 1). None of the IC₅₀ values determined using the assay described here fell further than a single standard deviation from the published range at either 24 h or 48 h (Table 1).

3.3. Assessing the anti-*Giardia* activity of Malaria Box compounds

Preliminary screens of the Malaria Box compound set identified 122 compounds with >50% inhibition at 24 or 48 h when assessed at $10 \mu\text{M}$ (Fig. 3A and B; Table S1). The Z factor of all assays plates in the $10 \mu\text{M}$ screen was >0.5 (average \pm SD; 0.74 ± 0.11). Further analysis of the 122 compounds identified 22 with >50% growth inhibition at $5 \mu\text{M}$ (Fig. 3C and D; Table S1). The Z factor of all assays plates in $5 \mu\text{M}$ assays was also >0.5 (average \pm SD; 0.73 ± 0.10). Further dose response analysis of the 22 compound with >50% inhibition at $5 \mu\text{M}$ identified three compounds (MMV007384, MMV019690 and MMV006203) with sub- μM IC₅₀ values (Table 2, Table S1 & Fig. S1) at either time-point.

4. Discussion

We have developed an *in vitro* medium throughput assay that permits *Giardia* drug susceptibility testing in real-time without any need to stain parasites. This assay is unique in that it harnesses the power of digital phase-contrast microscopy and dedicated analysis software to identify and count parasites thereby permitting speedy, multi-time point analysis of live parasite numbers. A comparison of automatically generated parasite counts with manual counts

demonstrates that the system can quickly and reliably assess trophozoite numbers (Fig. 1). As this assay permits the activity of compounds to be assessed at multiple time-points without any impact on parasite growth (Fig. 2) it also provides an opportunity to optimize data generation and limit cost. Additional information regarding the time course of compound activity and morphological effects, which can be derived from acquired images, may aid in compound triage and mechanism of action studies. Further reductions in cost and additional data acquisition may also be possible given that the assay is likely to be amenable to miniaturization and longer assessment periods (up to 72 h (Upcroft and Upcroft, 2001; Kulakova et al., 2014)).

While a potential limitation of the current assay may be in its assessment of parasite number rather than a metabolic parameter linked to viability, compounds with static activity can be of use therapeutically (Pankey and Sabath, 2004). In addition, metabolic assays can be associated with the same liability in the case of dormancy or when the compounds assessed interfere with the metabolic process used to quantitate inhibition (Collier and Pritsos, 2003; Ulukaya et al., 2004). Indeed, as a result of continued growth and the enhanced metabolic activity of controls over time, both assay types are likely to identify compounds with static activity as inhibitors. More specialized methods designed to assess mode of action are therefore more adequately placed to examine the nature of compound activity, post-identification. An additional limitation of the current assay that should be considered is its assessment of

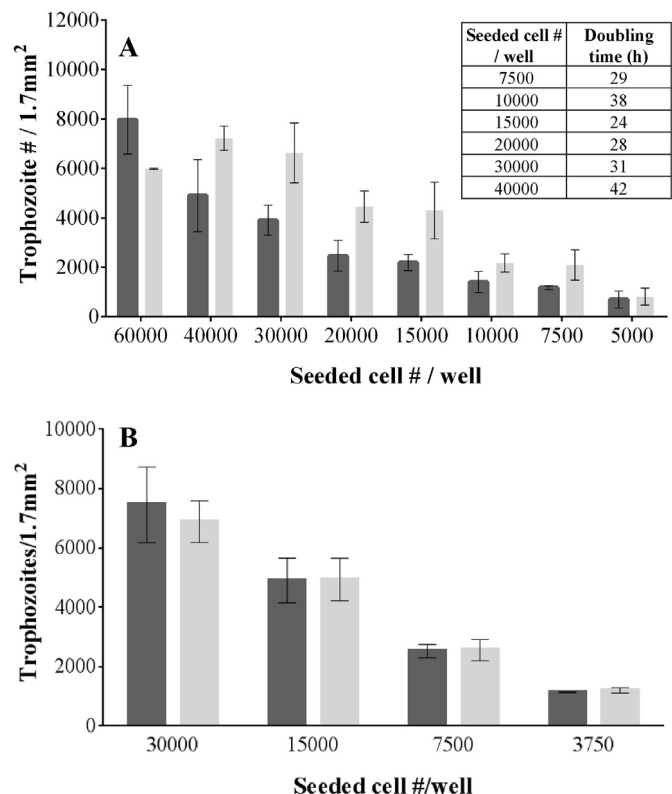


Fig. 2. Parasite growth assessment. The growth of *Giardia* trophozoites in assay conditions was assessed by digital phase-contrast microscopy (A). Parasite cultures were established in 96-well micro-titre plates at different seeding concentrations and growth was assessed at 24 h (dark grey) and 48 h (light grey). Data are presented as mean parasite count \pm SD of three independent experiments, each carried out in triplicate wells. In separate assays, the impact of parasite imaging on growth was assessed by comparing growth in plates imaged at both 24 and 48 h (B; dark grey) to those imaged only at 48 h (B; light grey). Data are presented as mean parasite count \pm SD of three independent experiments. There was no significant difference in the growth of parasites grown on plates imaged once at 48 h or at both time points ($p = 0.68$).

Table 1
In vitro anti-*Giardia* activity of control compounds.

Compound	IC ₅₀ 24 h		IC ₅₀ 48 h	
	Operetta (Mean ± SE)	Published range	Operetta (Mean ± SE)	Published range
Metronidazole	74 ± 3 μM	2 ^a –75 ^b μM	3 ± 1 μM	1 ^c –9 ^d μM
Albendazole	93 ± 15 nM	27 ^a –9600 ^b nM	89 ± 9 nM	38 ^e –377 ^f nM
Furazolidone	0.40 ± 0.06 μM	0.43 ^g –1.4 ^h μM	0.20 ± 0.04 μM	0.4 ⁱ –1.1 ^c μM

^a Cruz et al., 2003.

^b Arguello-Garcia et al., 2004.

^c Houkang et al., 2011.

^d Edlind et al., 1990.

^e Cedillo-Rivera et al., 2002.

^f Cedillo-Rivera and Munoz, 1992.

^g Boreham et al., 1984.

^h Townson et al., 1992.

ⁱ Tejman-Yarden et al., 2011.

parasite number based on adherence. While this is an inherent limitation of other assays including those that require the removal of culture media and well-washing prior to activity assessment, this would mean that the assay is likely to identify compounds that effect attachment in addition to compounds that effect replication. Although the consequences of this anti-attachment activity in the *in vivo* setting may be limited, more specialized assays would be required to discriminate between compounds that effect attachment versus those that inhibit replication. Nevertheless, the ability

of the current image-based assay to effectively examine the activity of compounds against *Giardia* parasites was demonstrated by assessing the activity of control anti-*Giardia* compounds albendazole, metronidazole and furazolidone, with IC₅₀ values generated by the automated imaging and enumeration system being within the range of previously published studies (Table 1). The suitability of the assay as a mechanism to identify compounds with activity against *Giardia* parasites was also demonstrated by assessing the Malaria Box compound set for potential anti-*Giardia* activity. The

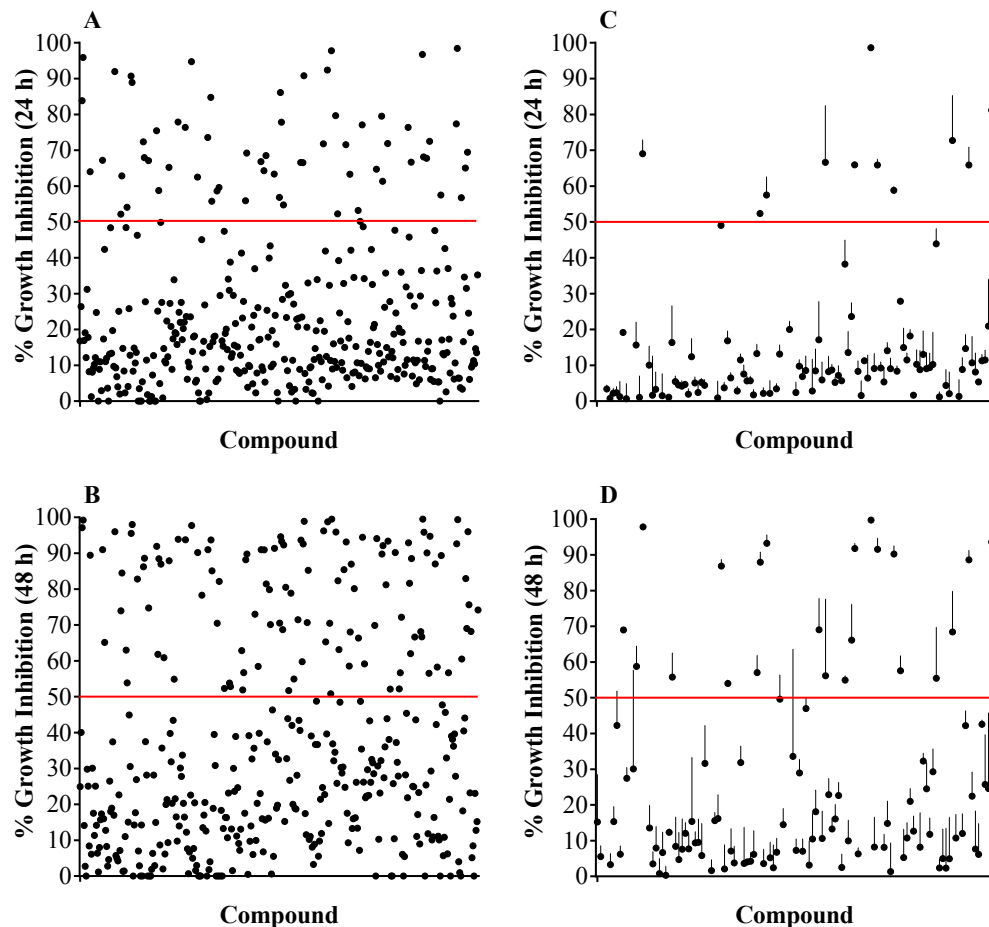
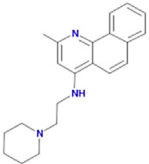
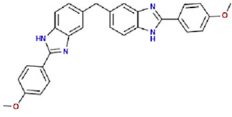
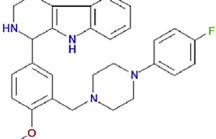


Fig. 3. Anti-*Giardia* activity of “Malaria Box” compounds. The anti-giardial activity of compounds in the ‘Malaria Box’ was determined using a primary 10 μM screen for 24 (A) and 48 h (B) and a secondary 5 μM screen for 24 (C) and 48 h (D) of compounds active (>50% inhibition) at 10 μM. All data are presented as mean % inhibition with standard error of the mean provided for 5 μM data (C & D).

Table 2
In vitro activity of Malaria Box compounds with sub μM IC_{50} against *Giardia* BRIS/91/HEPU/1279 parasites.

Compound Structure	Compound Name	IC_{50} (μM ; mean \pm SE)		SI ^a
		24 h	48 h	
	MMV006203	3.1 \pm 1.1	0.7 \pm 0.2	25.7
	MMV007384	0.8 \pm 0.2	0.6 \pm 0.2	8.7
	MMV019690	2.8 \pm 0.2	0.9 \pm 0.1	4.8

^a SI determined by comparing 48 h *Giardia* IC_{50} to existing MRC-5 fibroblast IC_{50} data (Kaiser et al., 2015).

mean Z factor for all plates in these assays (0.74 in 10 μM and 0.73 in 5 μM assays) suggest that the assay is of excellent quality (Zhang et al., 1999) and a promising new tool for *Giardia* parasite drug discovery. Of interest a previously described image-based *Giardia* assay which is dependent on parasite staining and hence an endpoint assay, reported a Z Factor of 0.54 (Gut et al., 2011). The identification of anti-*Giardia* compounds within the Malaria Box set that have structural similarities to known anti-*Giardia* compounds provides additional evidence that the current assay is suitable for compound activity assessment. MMV007384, the most potent of the anti-*Giardia* hits identified (Fig. S1, Table 2; 24 h IC_{50} 0.8 μM and 48 h IC_{50} 0.6 μM) is a benzimidazole. In addition MMV667492 (Table S1; 24 h IC_{50} 3.7 μM and 48 h IC_{50} 2.6 μM) is a naphthoquinone similar to menadione that has been shown to have promising efficacy against *G. duodenalis* trophozoites and cysts *in vitro* (Paget et al., 2004).

Two Malaria Box compounds, in addition to MMV007384 were identified to have sub μM IC_{50} values against *Giardia* parasites in the current study. These compounds were MMV019690 (Table 2; 24 h IC_{50} 2.8 μM and 48 h IC_{50} 0.9 μM) and MMV006203 (Table 2; 24 h IC_{50} 3.1 μM and 48 h IC_{50} 0.7 μM). While the selectivity index for MMV019690 (4.8; Table 2), generated using IC_{50} data against MRC-5 fibroblasts (Kaiser et al., 2015) suggest this compound may be associated with toxicity, the selectivity index of MMV006203, (25.7; Table 2), was more favourable, falling within recently published lead criteria range (Katsuno et al., 2015). Importantly, the identification of cell debris in images acquired during the assessment of MMV006203 and MMV019690 (Fig. S1) suggest that these compounds are cidal and warrant further investigation.

The current study has validated digital-phase contrast microscopy and automated parasite enumeration as a method to investigate *Giardia* drug susceptibility and identified new chemical scaffolds with anti-*Giardia* activity that may warrant further investigation. Unlike previously published image-based assessment *Giardia* assays, the method described in this study negates the need for cell staining and permits multiple-time point activity assessment which can improve screening costs and only add value to current drug discovery efforts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ijpddr.2017.01.005>.

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