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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-μM activity (IC_{50} 0.6–0.9 μM) were identified as potential starting points for giardiasis drug discovery.

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-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT), resazurin (AlamarBlue®) ^{3}H-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% O_2 5% CO_2, in N_2 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; 2×10^4 to 5×10^3 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 mm^2 ± 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; 6×10^4 to 5×10^3 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% O_2 5% CO_2 in N_2 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 (3×10^4 to 3.75×10^3 parasites/well in 200 μL) and incubated in 3% O_2 5% CO_2, in N_2 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 mm^2 ± 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