**In vitro** activity of pyrvinium pamoate against *Entamoeba histolytica* and *Giardia intestinalis* using radiolabelled thymidine incorporation and an SYBR Green I-based fluorescence assay

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**Objectives:** To assess the *in vitro* activity of the FDA-approved antihelminthic drug pyrvinium pamoate against *Entamoeba histolytica* and *Giardia intestinalis*.

**Methods:** A head-to-head comparison of a standard radiolabelled thymidine incorporation assay and the SYBR Green I-based fluorescence assay for determination of *in vitro* inhibition by pyrvinium and metronidazole was performed.

**Results:** The 50% inhibitory concentration (IC₅₀) for treatment of *E. histolytica* with pyrvinium was 4–5 μM for both assays compared with 1–2 μM for metronidazole. For pyrvinium treatment of *G. intestinalis*, an IC₅₀ of ~12 μM was determined by the radiolabelled thymidine assay alone, with maximum inhibition around 60%. In contrast, the IC₅₀ for metronidazole treatment using this assay was ~2 μM.

**Conclusions:** Pyrvinium is a potential gut lumen agent for treatment of intestinal amoebiasis, but possibly not for giardiasis. SYBR Green I is an alternative screening method for *E. histolytica*, but not *G. intestinalis*.

Keywords: drug assay, drug, protozoan

**Introduction**

A paucity of new therapeutics have been tested for treatment of giardiasis and amoebiasis in recent years, despite the discovery of metronidazole-resistant *Giardia* isolates and the potential for development of resistance in *Entamoeba histolytica*.¹ As drug resistance becomes a larger problem, an increased need for new therapeutics is apparent and will have to be met, facilitated by high-throughput drug screening. Pyrvinium pamoate is an antihelminthic drug that received FDA approval for the treatment of pinworm (*Enterobius vermicularis*) infections in 1955.² In a recent study, pyrvinium was shown to have potent activity against the enteric protozoan parasite *Cryptosporidium parvum* both *in vitro* and in a neonatal mouse model.³ Based on its promising activity against *Cryptosporidium*, we sought to determine with a head-to-head comparison of the [³H]thymidine incorporation assay⁴ and the SYBR Green I fluorescence assay⁵ whether pyrvinium was effective against *E. histolytica* and *Giardia intestinalis* in axenic cell culture.

**Materials and methods**

**Parasite isolates and drugs**

Axenic *E. histolytica* trophozoites, strain HM-1:IMSS (ATCC 30459), were maintained in TYI-S:33 and axenic *G. intestinalis* trophozoites, strain WB C6, were maintained in TYI-S:33 medium modified by the replacement of potassium phosphate with sodium bicarbonate supplemented with 0.5 g/L bile.⁶ Cultures were tested for bacterial contamination prior to *in vitro* experiments. Plates were wrapped in parafilm and sealed in GasPak³M EZ Gas Generating Pouches (BD, Franklin Lakes, NJ, USA) along with the provided gas-generating sachets in order to maintain anaerobic conditions throughout the incubation period. *E. histolytica* or *G. intestinalis* cultures in log phase were used for *in vitro* inhibition assays. Prior to isolation, dead or detached parasites were removed by aspiration. Parasite concentration by haemocytometer counts was adjusted to 10⁶ parasites/mL. The *Giardia* or *Entamoeba* suspension was added to each well for an initial seed of 10⁴ parasites per well. Control wells to determine background fluorescence or radioactivity received 200 μL of medium only.

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Pyrvinium pamoate [IUPAC name: 4-[(3-carboxy-2-hydroxy-naphthalen-1-yl)methyl]-3-hydroxynaphthalene-2-carboxylate; 2-[(E)-2-(2,5-dimethyl-1-phenylpyrrol-3-yl)ethenyl]-N,N,1-trimethylquinolin-1-ium-6-amine] shown in Figure 1(a), purchased as a dry powder from MP Biomedicals (Solon, OH, USA), was initially dissolved in DMSO due to poor solubility in aqueous solutions, 1 mg/mL and diluted 1:250 into culture medium. Metronidazole, purchased from Sigma (St Louis, MO, USA), was dissolved in water.

Inhibition determination by [³H]thymidine incorporation and SYBR Green I

Twenty-four hours after drug exposure, parasite wells were dosed with 0.2 μCi of methyl[³H]thymidine (40–60 Ci/mmol; GE Healthcare), then incubated for an additional 48 h at 37°C and frozen at −80°C. Plates were harvested onto filter paper using a Tomtec Harvester96 Mach III M cell harvester (Hamden, CT, USA). BetaPlate Scint scintillation fluid (PerkinElmer, Waltham, MA, USA) was added to dried filters and radioactivity was counted using a Wallac 1450 Microbeta Jet liquid scintillation and luminescence counter (PerkinElmer).

For SYBR Green I, medium was aspirated from wells and replaced with 100 μL of PBS. Plates were frozen at −80°C and thawed at 37°C. SYBR Green I was diluted 1:5000 in lysis buffer and 100 μL was added to each well. Plates were wrapped in foil to protect them from light and incubated for 30–45 min at room temperature with constant rocking. Fluorescence was measured using a HTS 7000 Bioassay Reader (PerkinElmer) with excitation at 485 nm, emission at 535 nm and gain set to 60. To avoid edge

Figure 1. (a) Structure of pyrvinium. (b) Linear relationship between numbers of parasites seeded overnight into microtitre plates and measured fluorescence. Values plotted are the means and standard deviations (SDs) of duplicate or triplicate wells after subtracting background fluorescence from wells receiving medium only. Lines represent regression curves for plotted data. Open circles represent data for *E. histolytica* and filled squares represent data for *G. intestinalis*. Growth inhibition curves for pyrvinium (c and d) and metronidazole (e and f) treatment of *E. histolytica* (c and e) and *G. intestinalis* (d and f). Inhibition percentages are from at least two independent experiments. Error (SD) was propagated from quadruplicate wells. Filled squares and continuous lines represent data generated using the [³H]thymidine incorporation assay and filled triangles and broken lines represent data generated using the SYBR Green I assay.
Activity of pyrvinium against Entamoeba and Giardia

Statistics

After subtracting background fluorescence, determined from wells that received medium only, mean percentage growth inhibition was calculated as $1 - \frac{([\text{mean cpm/RFU}])}{(\text{mean cpm/RFU in infected wells without drug})} \times 100$ and was plotted as a function of drug dose. Standard deviations of activity were determined from replicate wells and error was propagated for percentage inhibition estimates from replicate experiments. Sigmoidal curves were fitted to the data using SigmaPlot 9.0. IC50 and IC90 values were calculated from the equation of the fitted curve for $y=50\%$ or $90\%$. Confidence intervals (CIs) for IC50 and IC90 were determined from the 95% CI for the fitted curves.

Results

The relationship between numbers of parasites seeded into plates and relative fluorescence from SYBR Green I staining of parasite DNA shows a strong linear correlation although slopes were slightly different between the two parasite species. See Figure 1(b). The limit of detection was $\sim$5000 parasites for SYBR Green I staining with linear regression to SYBR Green I with linearity to $\Delta$S. The relationship was dependent on replacement of culture medium with PBS prior to the addition of the linear relationship was dependent on replacement of culture medium with PBS prior to the addition of SYBR Green I due to high background readings in wells containing only medium with bile (data not shown).

The comparative effects of pyrvinium and metronidazole treatment on parasite growth in vitro were investigated by exposure of axenic E. histolytica and G. intestinalis to the antimicrobial agents for 72 h. Growth inhibition curves calculated from $[^3H]$thymidine incorporation or SYBR Green I staining methods are shown in Figure 1(c–f). As demonstrated by the overlap of error bars around the data points, the inhibition curves generated using $[^3H]$thymidine and SYBR Green I were comparable for all experimental conditions except treatment of Giardia with pyrvinium, where SYBR Green I consistently produced lower inhibition measurements compared with $[^3H]$thymidine. IC50 and IC90 values calculated from the equations for the fitted curves are shown in Table 1. The IC50 for metronidazole inhibition of both parasite species was in the range of 1–2 $\mu$M while the IC50 for pyrvinium inhibition of E. histolytica was between 4 and 5 $\mu$M. Based on the CIs, there was no difference in the IC50 or IC90 values for pyrvinium or metronidazole treatment of E. histolytica calculated from the two different assays. By $[^3H]$thymidine incorporation, an IC50 for pyrvinium inhibition of G. intestinalis was calculated to be $\sim$12 $\mu$M but 50% inhibition levels were not attained using the SYBR Green I method. For metronidazole treatment of Giardia, although the IC50 and IC90 values for the two different assays were similar, there was no overlap in 95% CIs. Microscopic visualization of the results correlated with the assay determinations. In the case of E. histolytica, $>5 \mu$M metronidazole or 10 $\mu$M pyrvinium showed clumped and rounded protozoans consistent with death. In the case of G. intestinalis, $>2 \mu$M metronidazole showed few parasites with no motility. However, with 10–20 $\mu$M pyrvinium, microscopy of G. intestinalis showed 50–60% confluent parasites with motility.

Discussion

In the present study, we found that pyrvinium was able to inhibit growth of E. histolytica in axenic culture with an IC50 of 4–5 $\mu$M, which is approximately three times higher than the observed IC50 of metronidazole under the same conditions. However, pyrvinium is not completely soluble in aqueous solutions, which may reduce its activity compared with more soluble agents and also explains the larger standard deviations for pyrvinium compared with metronidazole. The normal human dosage for treatment of enterobiasis (E. vermicularis) with pyrvinium is 5 mg/kg, which results in an intestinal drug concentration of $\sim$100 $\mu$M, assuming a 70 kg person and a 3 L gut volume. We observed near 100% inhibition of E. histolytica by pyrvinium at drug concentrations $<$10 $\mu$M. Additionally, the observed IC50 of pyrvinium in this study was well below the reported IC50 of $\sim$100 $\mu$M paromomycin for E. histolytica. In the case of G. intestinalis, the in vitro inhibition curve with pyrvinium begins to level at around the 60% inhibition range, consistent with its known poor solubility. A high-throughput screen using propidium iodide for Giardia also did not show inhibition with pyrvinium from the National Institute of Neurological Diseases and Stroke (NINDS) custom collection at 10 $\mu$M drug. However, albendazole has shown activity against Giardia.

Table 1. Comparison of IC50 and IC90 values of pyrvinium and metronidazole for E. histolytica and G. intestinalis by inhibition assay

<table>
<thead>
<tr>
<th>Drug</th>
<th>Parasite</th>
<th>$[^3H]$Thymidine IC50 ($\mu$M)</th>
<th>SYBR Green I IC50 ($\mu$M)</th>
<th>$[^3H]$Thymidine IC90 ($\mu$M)</th>
<th>SYBR Green I IC90 ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrvinium</td>
<td>E. histolytica</td>
<td>4.2 (3.2, 5.1)</td>
<td>4.6 (4.1, 5.1)</td>
<td>6.6 (5.3, 7.9)</td>
<td>8.4 (7.0, 9.8)</td>
</tr>
<tr>
<td></td>
<td>G. intestinalis</td>
<td>11.8 (10.4, 14.6)</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>E. histolytica</td>
<td>1.1 (1.0, 1.2)</td>
<td>1.5 (1.1, 1.8)</td>
<td>2.0 (1.8, 2.2)</td>
<td>3.0 (2.3, 4.9)</td>
</tr>
<tr>
<td></td>
<td>G. intestinalis</td>
<td>2.2 (2.1, 2.3)</td>
<td>1.8 (1.72, 1.83)</td>
<td>3.6 (3.4, 3.8)</td>
<td>2.4 (2.39, 2.45)</td>
</tr>
</tbody>
</table>

IC50 and IC90 values were calculated from fitted curves using data points from at least two independent experiments run in quadruplicate. Values in parentheses represent the 95% CIs from the fitted curves. Dashes (—) indicate that an IC50 or IC90 inhibition level was not attained.
While the two assays gave comparable results for pyrvinium inhibition of *E. histolytica*, SYBR Green I produced consistently lower inhibition estimates than [3H]thymidine for *G. intestinalis* treated with pyrvinium. Although with metronidazole, bile alone did not interfere in the *Giardia* assay, fluorescence interference caused by the combination of pyrvinium and bile may account for the differences between the two inhibition curves. Incomplete washing of bile and pyrvinium will falsly elevate SYBR Green I fluorescence. For *Giardia*, the SYBR Green I assay is less optimal than the reported propidium iodide screen.

An important positive control for utilization of the SYBR Green I assay is inclusion of a high concentration of a known effective inhibitor to accurately determine the contribution of the parasite inoculum to fluorescence. High parasite inoculum and minimal growth can report similar fluorescence to low parasite inoculum and high growth between experiments. Subtraction of the baseline fluorescence resulting from maximum inhibition by effective drugs from the no drug control will control for this inoculum effect that may skew sigmoidal inhibition curves.

Based on the inhibition results from this study, we postulate that pyrvinium may be a potential therapeutic for treatment of intestinal amoebiasis but possibly not giardiasis. *In vivo* animal testing of pyrvinium in *Giardia* models is suggested. As pyrvinium is not appreciably absorbed from the gastrointestinal tract, it is not likely to be effective against amoebic liver abscesses and disseminated amoebiasis, but may be useful as a lumen-acting agent to eliminate intestinal amoebic colonization and cyt shedding. Though clinical and pre-clinical trials would be needed to determine the minimum effective and tolerable doses, the safety of pyrvinium up to daily doses for 2 weeks for treatment in humans has already been established, which will significantly shorten the approval process if pyrvinium is found to be effective against *E. histolytica* infections in humans.

### Acknowledgements

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### Transparency declarations

We have submitted a provisional patent application for use of pyrvinium for protozoan infections with Johns Hopkins University.

### References