Validation of novel fluorescence assays for the routine screening of drug susceptibilities of *Trichomonas vaginalis*

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**Objectives:** To evaluate existing protocols, based on Alamar blue (resazurin), for the routine determination of drug susceptibility in trichomonads, develop new ones and validate these by screening small antiprotozoal libraries.

**Methods:** The resazurin-based assay was evaluated by determining fluorescence development in *Trichomonas* media with various cell densities after various intervals and in the presence of metronidazole. Similar investigations were performed with the alternative fluorophores propidium iodide (PI) and resorufin. The optimized protocols were used to screen for new antitrichomonal compounds.

**Results:** Anaerobic cultures of *Trichomonas vaginalis* rapidly reduced blue resazurin to red, fluorescent resorufin. However, the ascorbic acid in the culture medium produced similar effects, even in the absence of cells, causing high background fluorescence and variability. Moreover, *T. vaginalis* rapidly metabolized resorufin to the non-fluorescent and colourless metabolite dihydroresorufin, making the fluorescent signal transient. In contrast, resorufin proved to be an excellent viability probe for *Trichomonas* due to its chemical stability in media and rapid metabolism by the parasite. We also show that staining with PI after cell permeabilization similarly constitutes a reliable measurement of trophozoite numbers. Using the PI and resorufin assays we determined reproducible EC⁵₀ values and identified potent antitrichomonal compounds from a limited screen of phosphodiesterase inhibitors and phosphonium salts.

**Conclusions:** The resorufin- and PI-based assays are suitable for routine and high-throughput drug screening, whereas resazurin-based assays are not. These assays constitute a major advance in the current protocols as demonstrated by a successful screen for new antitrichomonal lead compounds.

**Keywords:** Alamar blue, resorufin, resazurin, trichomoniasis, drug screening, propidium iodide

**Introduction**

Trichomoniases, caused by the anaerobic flagellated protozoan *Trichomonas vaginalis*, is the most common curable sexually transmitted disease in the world, with an estimated 174 million new cases annually.¹ However, the disease is often asymptomatic, especially in men, and is habitually underestimated in terms of global disease burden. However, trichomoniases is important in terms of disease-associated complications such as infertility,² preterm delivery, low birth weight or premature rupture of membranes.³,⁴ Moreover, trichomoniases increases HIV transmission rates⁵,⁶ and the risk of cervical neoplasia.⁷,⁸

Metronidazole is the first line of treatment against *T. vaginalis*.⁹,¹⁰ Other members of the nitro-imidazole compound family can be used, all functioning through similar mechanisms.¹⁰ However, reports of resistance to metronidazole are frequent¹¹,¹² and metronidazole resistance appears to be associated with cross-resistance to other 5-nitro compounds, including tinidazole and nitazoxanide.¹⁰,¹³ Thus there is an urgent need for new trichomonicidal compounds.

In order to improve the current chemotherapy of *T. vaginalis* infection, natural products as well as synthetic compounds should be screened for antitrichomonal activity and low toxicity. However, no drug evaluation procedures that lend itself to...
high-throughput screening have been described for trichomonads, the standard procedure being microscopic evaluation of trophozoite cultures to determine MICs.14,15 This approach has obvious disadvantages, making it unsuitable for serious drug development programmes.

Recently it has been proposed that resazurin (Alamar blue®), which has been used extensively for drug susceptibility tests for other unicellular eukaryotes including Leishmania spp.16 and Trypanosoma spp.,17,18 Candida spp.,19 as well as various mammalian cell types,20–22 could be adopted to assess drug susceptibility in T. vaginalis.23,24 This assay is inexpensive and can easily be scaled up for screening purposes, as has been demonstrated for other cell types.25 The assay is performed in a multwell format and produces a readout, by either fluorescence or absorbance, which is linear with cell number over a considerable range,26 and from which reproducible EC50 values (50% effective concentrations) can easily and reproducibly be determined.

However, attempts to validate this method in our laboratory indicated that this assay poses several inherent challenges for T. vaginalis, leading to irreproducible or misleading results. Possible factors contributing to these discrepancies were therefore investigated and were found to be associated with the rapid reduction of the resazurin dye to resorufin by components of the Diamond’s culture medium used, even in the absence of cells. Resorufin, in turn, was stable in this medium, but was rapidly converted into non-fluorescent dihydroresorufin by live T. vaginalis trophozoites. An alternative method using the fluorophore propidium iodide (PI) was investigated and adapted from a protocol recently developed for other protozoa for drug susceptibility tests and for real-time cell survival assays.26 Cell membranes are impermeable to the positively charged PI, which therefore only enters dead or disintegrating cells, upon which it forms a complex with nucleic acids, generating a fluorescent signal that is directly proportional to the number of cells.26 PI can thus be used to assay drug susceptibility after a fixed incubation time followed by cell permeabilization to allow the formation of a PI/nucleic acid complex, or for real-time monitoring of cell survival during drug exposure.26 We propose a reliable, standardized PI-based protocol for the routine screening of drug susceptibility of T. vaginalis. In addition, we validate a resorufin-based drug susceptibility protocol. The assays were used to screen two small compound libraries, identifying several compounds with in vitro antitrichomonal activity similar or superior to that of metronidazole.

Materials and methods

Chemicals

Resazurin sodium salt, resorufin sodium salt, PI, metronidazole and digitonin were all purchased from Sigma. Resazurin and resorufin were prepared as 500 μM solutions in PBS, and filter-sterilized with a 0.22 μm filter. The mixture was kept away from light and stored in a freezer at −20 °C until required. Stock solutions (20 mM) of PI, of metronidazole and of digitonin were prepared in DMSO.

Culturing and media

The metronidazole-susceptible G3 strain of T. vaginalis was kindly supplied by Jeremy Mottram (University of Glasgow, UK) and was cultured as described previously.27 The strain is a clonal line derived from an original isolate from 1973.28 The organisms were cultured in vitro in standard media for T. vaginalis: Hollander’s modification of TYM,29 a common modification30 of the original Diamond’s medium.31 This medium is often referred to as modified Diamond’s medium (MDM) and was supplemented with 10% (v/v) heat-inactivated horse serum (HIHS) (Invitrogen). Cultures were routinely incubated at 37 °C in sterile 25 mL bottles filled completely and capped airtight to create an anaerobic environment. The exact composition of 1 L of our medium was 20 g of trypticase peptone (BD, Sparks, USA), 10 g of yeast extract (Formedium Ltd, UK), 5 g of maltose monohydrate (Sigma), 1 g of l-ascorbic acid (Sigma), 1 g of KCl, 1 g ofKHCO3, 1 g of KH2PO4, 0.5 g of K2HPO4 and 0.1 g of FeSO4.7H2O (pH adjusted to 6.3 with HCl). The culture was passaged into fresh MDM every 24 h to ensure the cells were at an appropriate cell concentration of ~2 × 106 cells/mL, determined through microscopic cell counts using a haemocytometer. When culturing in multwell plates, the plates were sealed with Nescofilm and inserted into BD GasPak EZ pouches (BD Diagnostics, UK) to create anaerobic conditions, as they produce CO2 while absorbing O2.

Determination of EC50 values by fluorescence

Sterile 96-well opaque culture plates (Greiner BIO-ONE Ltd, Germany) were used except where otherwise indicated and two rows were filled with a doubling dilution of test compound, usually metronidazole, in MDM/HIHS, with the final well receiving MDM/HIHS without any drug. This gave a concentration range, usually of 200 μM–0.024 nM, across the plate in a volume of 100 μL. T. vaginalis trophozoites (5 × 104 cells/well) were then added in 100 μL of MDM/HIHS, giving a final volume of 200 μL. The outside columns and rows of wells were filled with 200 μL of sterile water each to increase humidity and prevent evaporation in the test wells during the incubation. The plate was sealed with Nescofilm and incubated in anaerobic conditions using BD EZ Pouches at 37 °C for 24 h, unless stated otherwise. At the end of the incubation period, 20 μL of the filter-sterilized assay dye solution (either 500 μM resazurin or resorufin in PBS, or 90 μM PI/200 μM digitonin in PBS) were added. Plates were kept in the incubator until full colour development of the dye. Fluorescence was read at the end of each incubation period using a FLUOstar Optima (BMG LABTECH GmbH, Offenburg, Germany). In some cases multiple readings were taken, at 1 min, 1 h and 2 h after addition of the dye. All fluorescence in resazurin and resorufin assays was determined at an excitation wavelength of 544 nm and an emission wavelength of 599 nm; PI fluorescence was read using 544 nm and 620 nm filters for excitation and emission, respectively. EC50 values were determined by non-linear regression using an equation for a sigmoid curve (GraphPad Prism 5.02).

Monitoring of resorufin fluorescence and absorbance in real time

An aliquot of 200 μL of cell suspension in MDM/HIHS was added to the first well of a transparent 96-well plate. An aliquot of 100 μL of the same medium was added to all the subsequent wells, and the cells were diluted sequentially to give doubling cell dilutions. The last well was left cell-free (medium only) as a control. An aliquot of 20 μL of 125 μg/mL resazurin in PBS was added to each well, and alternate fluorescence (544/599 nm) and absorbance (590 nm) measurements were taken for 180 2 min cycles; this experiment was performed using clear-bottomed plates. In a separate set of experiments, different media and media components, without cells, were incubated with either resazurin or resorufin and fluorescence was monitored in real time over 3 h to assess the effect of various media components on the fluorescence of these dyes.
Results

**Determination of EC$_{50}$ values with resazurin and resorufin**

It has previously been reported that the Alamar blue (resazurin) assay can be used to determine the effects of drugs on populations of *T. vaginalis*, much as it has been routinely used for many other cell types.\(^{21,26}\) This assay is based on the reduction of resazurin, which is blue and non-fluorescent, by live cells to resorufin, which is red and brightly fluorescent. Thus this reaction can be monitored by either absorbance or fluorescence at the appropriate wavelengths. However, the assay is based on two assumptions: first, that indeed only live cells catalyse the resazurin to resorufin, and second, that resorufin is stable under the assay conditions. In some cell types, it has been observed that resorufin could be further reduced to dihydroresorufin (see Figure S1, available as Supplementary data at JAC Online).\(^{21,26}\) Given the strongly reducing growth conditions and unusual biochemistry of *T. vaginalis*, there is a clear need to address these assumptions.

We performed a standard dose–response resazurin assay with *T. vaginalis* trophozoites exposed to doubling dilutions of metronidazole (20 μM–0.08 nM) for 24 h in fresh MDM/HiHS under anaerobic conditions. Upon addition of resazurin, colour change was almost immediate, and a fluorescence reading taken just 1 min after addition produced a sigmoidal curve with a steep negative slope, with an EC$_{50}$ value of 0.46 μM (Figure 1a, 1), consistent with the characteristic change from blue resazurin to red resorufin (Figure 1b, 1). The interpretation of ‘live’ and ‘dead’ cells was confirmed by manual microscopic counting of cells (Figure 1a, 3), which gave the same MIC as the sigmoidal resazurin curve. However, we observed that the colour continued to rapidly change, even in wells where all the cells had succumbed to the high metronidazole concentration, and even where no cells had been added (Figure 1b, 2). The blue colour disappeared rapidly in all wells, while the red wells lost colour until they were faintly yellow. Apparently, the wells with live cells continued to metabolize resorufin to dihydroresorufin and those without live cells continued to reduce resazurin to at least resorufin. This led to an inversion of the EC$_{50}$ curve, which now assumed an equally steep positive slope, when fluorescence was measured again after 1 h (Figure 1a, 2), with the dead cells now identified by the highest fluorescence level, as the live cells had converted all the fluorescent resorufin into non-fluorescent dihydroresorufin.

This interpretation was confirmed by adding resorufin instead of resazurin as the indicator dye, in a parallel experiment. Resorufin was stable for at least 1 h without live cells, leading to stable, high levels of fluorescence; however, live cells almost instantaneously converted resorufin into non-fluorescent dihydroresorufin, leading to identical EC$_{50}$ curves with steep positive slopes after 1 min or 1 h of incubation (Figure 1a, 4–6). The photographs of the plates at 1 min and 1 h (Figure 1b, 4 and 5) confirm this interpretation, with the red colour stable in wells receiving the highest drug concentrations and a pale yellow colour in wells with any number of live cells as assessed by microscopic counts (Figure 1a, 6).

It thus appears that both resazurin and resorufin could potentially be used to generate reliable EC$_{50}$ values, but that resorufin generates the more stable fluorescence output. Nonetheless, we found that, if determined immediately after colour change, incubation with resazurin produces a fluorescence signal proportional to cell number (Figure 2a). However, the procedure suffers from the obvious drawback that the fluorescence output is a function of the incubation time and changes from a ‘fluorescence equals live cells’ scenario to a ‘fluorescence equals dead cells’ state. A resorufin-based assay would not suffer from such a drawback, and we found that resorufin fluorescence was also proportional to cell numbers (but with low fluorescence indicating high cell numbers) and at least as sensitive as resazurin for the detection of live *T. vaginalis* trophozoites (Figure 2b).

**Effect of media composition on fluorescence**

We directly compared within a single experiment the utility of the fluorescent dyes resazurin and resorufin to generate *T. vaginalis* EC$_{50}$ values. As we had observed that MDM/HiHS changes colour from light brown to a much darker brown within days of preparation when stored at 4°C, which may reflect some change in the medium composition, we tested this both in freshly prepared media and media stored for 3 weeks (sterile, unopened, protected from light) at 4°C.

Figure 3 shows that both dyes produced highly similar EC$_{50}$ values for metronidazole (0.13–0.30 μM). As shown in Figure 1, the sigmoid curve obtained using resazurin in fresh media changed from a negative to a positive slope upon further incubation for 30 min at room temperature, whereas the fluorescence levels of resorufin remained stable over the same time. A close observation of the resazurin metabolism shows that the dye is almost instantly reduced to resorufin by the trophozoites, showing a high fluorescence in the presence of live cells after 1 min. It is unclear whether the resazurin needs to be taken up by the parasites for this reaction to occur. At 5 min this reaction is complete, resulting in a further increase in fluorescence with live cells, but fluorescence in the wells containing dead cells only, due to prior exposure to metronidazole, more than doubled in this short time, as the culture medium also reduces the resazurin to resorufin, albeit more slowly in the absence of *T. vaginalis*. At the 30 min point, the media has reduced all resazurin, but the live trophozoites have started to further metabolize the dye to dihydroresorufin, leading to a reduction in fluorescence in those wells—inverting the dose–response curve.

Interestingly, this did not happen using the same cells incubated in ‘stored’ medium, although fluorescence in the well containing live cells decreased as it did in fresh medium (conversion of resorufin into dihydroresorufin by the parasites) and fluorescence in wells containing dead cells had started to increase (conversion of resazurin into resorufin by the medium). We conclude that one or more ingredients of MDM/HiHS are capable of rapidly reducing resazurin, and this component is itself unstable upon storage at 4°C in solution.

This was further investigated following the development of fluorescence after addition of resazurin to MDM/HiHS medium, and to cultures containing a range of cell densities up to 5 × 10$^5$ cells/well in a 96-well plate, concomitantly monitoring absorbance at 590 nm (red). Fluorescence increased immediately and dramatically at all cell densities (including cell-free MDM), but reached the highest level in the culture with the highest cell density (Figure S2A, available as Supplementary data at JAC
Online). Absorbance initially declined as well due to the dissipation of the strong blue colouring of resazurin, and in a second phase due to reduced specific absorbance at 590 nm, caused by metabolism of resorufin (Figure S2B, available as Supplementary data at JAC Online).

We investigated whether the observed rapid reduction of resazurin was specific to MDM/HIHS, as several related growth media for T. vaginalis have been described since the original publication of the first medium, by Diamond, in 1957.31 We prepared all varieties of the medium from relevant literature and found

![Graphs and tables showing drug susceptibility assays with resazurin and resorufin.](https://i.imgur.com/3G5.png)

**Figure 1.** Drug susceptibility assays with resazurin and resorufin. (a) T. vaginalis cultures of $5 \times 10^4$ cells/mL were incubated under anaerobic conditions at 37°C for 24 h with various concentrations of metronidazole (doubling dilution from 20 μM final concentration in well 1 to 0.08 nM in well 19; well 20 received no metronidazole). At the end of the incubation, 20 μL of either resazurin or resorufin solution were added to each well (45 μM final concentration) and the plates were read after 1 min (frames 1 and 4) and again at 1 h (frames 2 and 5). After the 1 h read, cells were counted using a haemocytometer (frames 3 and 6). The graphs shown are representative of numerous highly similar repeats. (b) Corresponding photographs of the plates used in frames 1 and 2 and frames 3 and 4 from (a), showing colour changes with resazurin and resorufin, respectively, after 1 min and 1 h. In frame 1 the numbering of the wells is indicated as well as the sequence of dilution. Black bars indicate the apparent MIC based on visual observation of the colour. These closely matched the cell counts displayed in frames 3 and 6 from (a).
that all of these (but not PBS), including the original Diamond's medium and TYI-S-33, rapidly reduce resazurin to resorufin (Figure S3, available as Supplementary data at JAC Online) in the presence and absence of various T. vaginalis cell densities. These media were all prepared to pH 6.0, and all were complemented with 10% heat-inactivated bovine serum for the experiment (Table S1, available as Supplementary data at JAC Online). The reduction of resazurin was not due to bacterial or fungal media contamination, as treatment with antibiotics or ultrafiltration (pore size 0.22 μm) did not change the rate of

Figure 2. Linear correlation between fluorescence and cell count. Cells were grown in standard MDM/HIHS for 24 h under anaerobic conditions at 37°C and were subsequently doubly diluted in a 96-well plate (200 μL final volume). (a) To each well was added 20 μL of 500 μM resazurin in PBS and fluorescence was determined immediately after colour change occurred. (b) To each well 20 μL of 500 μM resorufin sodium salt were added (final concentration in well 45 μM), in which the cells were incubated for 2 h at 37°C, after which fluorescence was determined.

Figure 3. Storage of media profoundly affects resazurin fluorescence. T. vaginalis trophozoites were grown in either freshly made MDM/HIHS or the same medium stored at 4°C for 3 weeks. Cells were incubated for 24 h in 200 μL MDM/HIHS with doubling dilutions of metronidazole exactly as described in Figure 2; to each well either resazurin or resorufin was subsequently added to 45 μM final concentration. Fluorescence was read after 1 min, 5 min, 30 min and 1 h (not all shown). The experiment is representative of three similar experiments with near-identical outcomes.
and absence (Figure 4b) of trophozoites in the medium.

in the presence of resazurin, both in the presence (Figure 4a) and absence (Figure 4b) of trophozoites in the medium. However, in all cases the rate at which fluorescence developed was drastically reduced when the medium had been stored for 2 weeks at 4 °C (Figure 4c). These results show that the reducing agent is common to all the four different media, i.e. could be trypsinase, yeast extract and L-ascorbic acid (see Table S1).

The medium component responsible for reducing resazurin was identified by preparing a series of MDM media lacking single components and monitoring fluorescence after addition of resazurin. The same experiment was performed concomitantly with each medium component separately and various combinations of components (all at the same concentrations as in MDM and dissolved in PBS at pH 6.0). Some of the results are shown in Figure 5. PBS alone, HIHS, Tris base solution or the trypanosome medium HMI-9 all failed to affect resazurin fluorescence, but ascorbic acid (alone or in combination with FeSO₄ or yeast extract) had a dramatic effect on fluorescence. Complete MDM also rapidly reduced the resazurin, but to a lower apparent fluorescence due to the strong colour of fresh MDM/HIHS, which quenches part of the signal. Omission of iron sulphate from MDM did not change the dynamics of resazurin reduction, but after omission of ascorbic acid fluorescence increased only very slowly, identical to the rate of yeast extract in PBS—showing that a component of the extract is capable of reducing the dye. We conclude that ascorbic acid is the component of MDM responsible for the rapid reduction of resazurin. As some other Trichomonas media have substituted much of the ascorbate for cysteine, we also tested cysteine in PBS at 1 g/L, the concentration used in Diamond’s original recipe (Table S1). Cysteine, which has a redox potential of −0.34 V at pH 7 is a much stronger reducing agent than ascorbic acid (+0.06 V at pH 7), and appeared to reduce resazurin completely to dihydroresorufin, leading to a transient fluorescence when monitored over several hours (Figure 5).

Optimization of a PI-based drug test for T. vaginalis

We have previously shown that the fluorophore PI can be used as an alternative to the Alamar blue/resazurin dye. PI generates a fluorescent signal upon binding to nucleic acids in cells permeable to it—i.e. those either dead or permeabilized with a chemical reagent. PI fluorescence has been shown to be proportional to DNA concentration and cell number for digitonin-permeabilized Trypanosoma brucei, and here we demonstrate the same for T. vaginalis (Figure 6a). Fluorescence was also directly proportional to the concentration of PI (Figure 6b), showing that nucleic acid binding sites were not limiting at the cell density used in the experiment (2 × 10⁵ cells/mL).

The level of fluorescence of T. vaginalis trophozoites upon incubation with PI was dependent on preincubation with digitonin (or another permeabilizing agent), and we next investigated the optimal digitonin concentration and incubation time with T. vaginalis trophozoites (Figure 7). Concentrations of 12.5–50 μM digitonin caused rapid lysis, and associated PI fluorescence, reaching maximum emission at 6–10 min after digitonin addition. At the next dilution (6.25 μM), fluorescence was slightly lower and an incubation time of 20 min was required to reach peak fluorescence; lower concentrations did not cause cell lysis over the duration of the experiment (4 h). Fluorescence gradually declined over this period; this may be due to bleaching of the dye, or more likely to the degradation of nucleic acids in the permeabilized cells. This reinforces the need for a PI assay to be read at a standardized time after incubation with standardized concentrations of PI and digitonin. In Figure 8(a) we

Figure 4. Development of resazurin fluorescence over 6 h in various T. vaginalis media. (a) Fresh media with 5 × 10⁵ cells/mL. (b) Fresh media without cells. (c) Two-week old media (stored at 4 °C). 1, MDM; 2, TYM; 3, Diamond’s; 4, TYI-S-33; 5, PBS. Cells were grown and fluorescence was recorded as indicated in Figure 2.

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show an EC50 determination with metronidazole using either freshly prepared or stored (3 weeks, 4°C) MDM/HIHS medium, using 8.2 mM PI and 18.2 mM digitonin, and an incubation time of 1 h at 37°C. The EC50 values were validated with manual microscopic cell counts (Figure 8b). Using this protocol, EC50 values were entirely reproducible and fluorescence levels were sufficient to produce very good signal-to-noise ratios.

**Screening of small compound libraries using the resazurin and PI assays**

To establish the utility of the new protocols we next screened two small compound libraries for activity against *T. vaginalis* trophozoites in vitro. For the resazurin assay we used a 45 μM resazurin final concentration and for the PI assay we used 8.2 μM PI plus 18.2 μM digitonin. The two assays were performed on four independent occasions, in parallel, i.e. using the same batch of cells for both assays, using a drug exposure time of 24 h exactly and anaerobic culture conditions in fresh MDM/HIHS medium at 37°C. The EC50 values were entirely reproducible and fluorescence levels were sufficient to produce very good signal-to-noise ratios.

Table 1 summarizes the results of the screening. CDIQM compounds 1–11 are phosphonium salts (structures in Table S2, available as Supplementary data at JAC Online). This class of compounds was recently shown to have potent activity against *Leishmania*33 and *Trypanosoma* species (C. Dardonville and H. P. de Koning, unpublished results). Several of these compounds showed low micromolar activities, with the PI assay consistently giving slightly lower EC50 values than the resorufin assay, including for metronidazole (Table 1). Reproducibility was excellent, leading to low error values. The highest activity in this series was for CDIQM-6, which displayed EC50 values of 1.5 ± 0.2 and 0.69 ± 0.11 μM (n=4) by resorufin and PI assays, respectively. The data displayed in Table 1 were obtained by reading the plates 1 h after addition of the fluorescent dye. Tables S3 and S4 (available as Supplementary data at JAC Online) also show the EC50 values obtained after reading the
drugs. Without this, ab initio drug development for decades, in part because the perceived lack of urgency has not stimulated sufficient efforts to develop in vitro or in vivo systems for high-throughput screening of drug candidates. Without this, ab initio drug development is virtually impossible, and this situation explains why only direct analogues of metronidazole have been brought to market in the last half century. However, the above status quo is increasingly being challenged as unsatisfactory, in part due to an increasing incidence of metronidazole-refractory infections that are almost impossible to treat due the lack of alternative drugs.6,12,35 And while trichomoniasis itself is indeed almost always a mild infection, it will be of long duration if not treated,36,37 and there are clear associations with severe conditions due to chronic vaginal bleeding, inflammation and cervical pathology, contributing to increased transmission rates of HIV-15,6,10 and virally induced cervical cancer,7,8 in addition to adverse effects on fertility and pregnancy.3,4

While the need for new trichomicidal drugs that are not cross-resistant with 5-nitroimidazoles is now widely accepted, the lack of a standardized protocol for drug screening has continued to forestall large-scale efforts such as screens of compound libraries. In 2005 Campos Aldrete et al.23 proposed the widely used viability indicator dye Alammar blue (resazurin) for the evaluation of drug action against Trichomonas and showed that the parasites were able to reduce the non-fluorescent blue dye to red resorufin, generating a fluorescent signal that correlated well with cell numbers. They also noted that the red colour quickly disappeared, resulting in a yellow medium colour instead,21 but this was not further investigated. However, it is known that further reduction of resorufin to dihydroresorufin can result from over-incubation of resazurin with some cell

Discussion

Despite the very high infection rates and limited pharmacopoeia against trichomoniasis, there has been little effort towards new drug development for decades, in part because the perceived lack of urgency has not stimulated sufficient efforts to develop in vitro or in vivo systems for high-throughput screening of drug candidates. Without this, ab initio drug development is virtually impossible, and this situation explains why only direct analogues of metronidazole have been brought to market in the last half century. However, the above status quo is increasingly being challenged as unsatisfactory, in part due to an increasing incidence of metronidazole-refractory infections that

plates immediately after addition of the dye (1–5 min) and after 30 min. The values were highly similar, showing that the outcome of the assays is not dependent on time like the resazurin assay, but the signal-to-noise ratio was significantly improved after incubation for 30 or 60 min at 30°C.

We also tested a small custom-made library kindly provided by Mercachem (Nijmegen, The Netherlands) of 16 compounds that incorporated features both of inhibitors of cAMP phosphodiesterases (PDEs) and nitro-heterocycles. We have recently shown potent antipiroplasm activity by Cpd-A, an inhibitor of Trypanosoma brucei PDE-B.34 Although Cpd-A displayed only modest activity against T. vaginalis trophozoites (EC50 20 μM; n = 3), several of the nitro analogues displayed highly promising activity. Two examples given in Table 1 displayed similar activity to metronidazole (30-001 and 30-006; structures in figure S5, available as Supplementary data at JAC Online), whereas two other compounds (30-002 and 30-003) were an order of magnitude more potent than the standard treatment, with EC50 values of 37 and 44 nM, respectively.

![Figure 7](https://academic.oup.com/jac/article-abstract/67/4/933/859605)

**Figure 7.** Digitonin-induced lysis of T. vaginalis trophozoites monitored using PI. T. vaginalis trophozoites were grown for 24 h at 37°C under anaerobic conditions and added to a 96-well plate at 5 × 10^6 cells/mL in MDM with 10% HIHS in the presence of a doubling dilution of digitonin. To each well PI was added to a final concentration of 9 μM and fluorescence was recorded at wavelengths of 544 nm (excitation) and 620 nm (emission). Digitonin concentrations of ≥6.25 μM rapidly lysed the parasites, whereas concentrations of ≤3.12 μM had no effect on the integrity of the cells over the duration of the experiment.

![Figure 8](https://academic.oup.com/jac/article-abstract/67/4/933/859605)

**Figure 8.** Validation of a PI-based drug assay for the determination of IC50 values. T. vaginalis trophozoites in MDM/HIHS (5 × 10^6 cells/mL) were incubated in 24-well plates at 37°C in the presence of a doubling dilution of metronidazole for 24 h, after which samples were taken from each well for microscopic cell counts. PI and digitonin were added to each well to final concentrations of 8.2 μM and 18.2 μM, respectively, and incubated for another hour at 37°C. Fluorescence (λexc = 544, λem = 620) was read and plotted against the metronidazole concentration (a). Virtually identical EC50 values were obtained when the cells were grown in parallel in freshly prepared medium or in 3-week-old medium stored at 4°C (1.60 μM and 1.14 μM, respectively). (b) Corresponding cell counts from the wells before addition of digitonin (filled circles, fresh media).
Resazurin reduction was not due to any component of the sera we used, or to bacterial or mycoplasma contamination. A systematic investigation of media components identified L-ascorbic acid, a mild reducing agent, as the chemical that almost instantaneously reduces resazurin to resorufin. L-Ascorbic acid is present at high concentrations in all current Trichomonads media (0.2–1.0 g/L), but not in PBS, HOMEM (Leishmania), HMI-9 (Trypanosoma) and mammalian culture media such as RPMI-1640. The high levels of L-ascorbic acid in Diamond’s media are essential to sustain the high reducing potential required for anaerobic Trichomonads growth. The discovery that L-ascorbic acid is responsible for the reduction of resazurin is consistent with the observation that media stored for a few weeks at 4°C reduces resazurin at a slower rate, presumably due to the slow oxidation of L-ascorbate to L-dehydroascorbate.

In contrast to resazurin, which is easily reduced and has an oxidation potential that is about 100 mV higher than that of resorufin, both resazurin and PI were completely stable in all Trichomonads media, and all components thereof, for at least 3 h. Thus changes in fluorescence using these dyes were indeed a function of cell numbers only, without the confounding factors. Resorufin reduction was exclusively by live cells, establishing this dye for the first time as a viability indicator in its own right. This can be attributed to the unique biochemistry of trichomonads, which possess a hydrogenosome containing several reductases for the production of hydrogen, in lieu of mitochondria. Reductases within the hydrogenosome, including thioredoxin reductase, pyruvate-ferrodoxin oxidoreductase and NAD-dependent malic enzyme, are also responsible for the reduction of the produg metronidazole to its active free radical form.

An assay based on incubations with resorufin, as demonstrated in Figure 1, would see high levels of fluorescence associated with dead cells (un-metabolized resorufin) and none with live cells, which quickly convert the dye into dihydroresorufin. In contrast to the resazurin assay, the outcome was not influenced by the age or exact composition of the media, and produced highly reproducible EC₅₀ values that correlated very well with MIC values obtained by microscopic cell counts. Moreover, resorufin is, to the best of our knowledge, not appreciably reduced by microorganisms that might potentially contaminate a clinical Trichomonads culture, making resorufin a trichomonad-specific viability indicator much superior to resazurin. Indeed, rapid reduction of resorufin has only been reported as an NADH-dependent reaction in mitochondria of stimulated motor nerve terminals.

The assay proposed here is cheap and easy to standardize and scale up, as the only addition to the cell cultures is a fixed volume of resorufin stock solution, followed by a short development time.

The PI-based assay was also validated as a potential drug susceptibility assay for trichomonads. It requires the addition (simultaneous or separate) of a permeabilizing agent and of the PI dye itself. We achieved reliable and reproducible results using 8.2 μM PI and 18.2 μM digitonin, added after a 24 h anaerobic incubation of T. vaginalis trophozoites with test compounds, followed by a 1 h incubation with the dye at 37°C, but the use of other permeabilizing agents or different PI concentrations would certainly be possible. Once lysed, the level of fluorescence is not indefinitely stable however, probably due to the degradation of nucleic acids in the permeable cells. Fundamentally, the PI

<table>
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<tr>
<th>Compound</th>
<th>EC₅₀ value (μM) ± SEM</th>
<th>n</th>
<th>EC₅₀ value (μM) ± SEM</th>
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<tbody>
<tr>
<td>CDIQM-1⁰</td>
<td>&gt;100</td>
<td>4</td>
<td>&gt;100</td>
<td>4</td>
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<tr>
<td>CDIQM-2⁰</td>
<td>≥52 ± 18</td>
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<td>≥34 ± 2</td>
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<tr>
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<tr>
<td>CDIQM-4⁰</td>
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<td>≥19 ± 4</td>
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<tr>
<td>CDIQM-6⁰</td>
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<td>≥0.69 ± 0.11</td>
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<tr>
<td>CDIQM-7⁰</td>
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<tr>
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<tr>
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<tr>
<td>CDIQM-10⁰</td>
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<td>≥4.0 ± 0.7</td>
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<tr>
<td>CDIQM-11⁰</td>
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<td>≥63 ± 18</td>
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<tr>
<td>30-001⁹</td>
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<tr>
<td>30-002⁹</td>
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<td>30-006⁹</td>
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<tr>
<td>Metronidazole</td>
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<td>4</td>
<td>0.35 ± 0.01</td>
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</table>

ND, not determined.
⁰For structure, see Figure S5.
⁹See Andrew Stephan Tomcufcik, Patrick Thomas Izzo and Paul Frank Fabio, 3-nitroimidazo[1,2-b]pyridazine, Patent application 2208830, 1972. Structure in Figure S5.
⁵Compounds information confidential pending patent application.

Table 1. Effect of selected compounds on T. vaginalis trophozoites in vitro as determined by resorufin and PI assays measured 60 min after addition of the dye.
assays measure cell numbers based on PI-binding of their DNA and RNA, whereas the resorufin assay indicates the presence of any live *T. vaginalis* trophozoites through the disappearance of fluorescence and red colour. The PI assay is not as specific for trichomonads as resorufin (unless the permeabilizing agent is specific), as the dye binds similarly to all nucleic acids.

In conclusion, we present here two novel assays for the high-throughput testing of chemical compounds against *T. vaginalis*, both superior to the only test proposed to date, based on resazurin, for which we have identified serious problems in scale-up and reliability. Both of these assays, but particularly the resorufin assay, can be used cheaply and reliably to routinely test metronidazole susceptibility in clinical cultures, as the resorufin assay is highly sensitive to even very low cell numbers and trichomonad specific. It is hoped that this development will contribute to renewed interest in both epidemiological studies of metronidazole resistance and, crucially, new drug development against this serious sexually transmitted infection. We show here that the identification of new compounds with strong antitrichomonal activity is straightforward using either protocol, leading to highly reproducible EC\(_{50}\) values. As a first step, other compound libraries with known antiprotosal activity should now be screened for activity against trichomonads.

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**Supplementary data**

Figures S1–S5 and Tables S1–S4 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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