Acanthamoeba castellanii: A new high-throughput method for drug screening in vitro

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ABSTRACT

Despite significant public health impact, there is no specific antiprotozoal therapy for prevention and treatment of Acanthamoeba castellanii infection. There is a need for new and efficient anti-Acanthamoeba drugs that are less toxic and can reduce treatment duration and frequency of administration. In this context a new, rapid and sensitive assay is required for high-throughput activity testing and screening of new therapeutic compounds. A colorimetric assay based on sulforhodamine B (SRB) staining has been developed for anti-Acanthamoeba drug susceptibility testing and adapted to a 96-well microtiter plate format. Under these conditions chlorhexidine was tested to validate the assay using two clinical strains of A. castellanii (Neff strain, T4 genotype [IC$_{50}$ 4.68±0.6 µM] and T3 genotype [IC$_{50}$ 5.69±0.9 µM]). These results were in good agreement with those obtained by the conventional Alamar Blue assay, OCR cytotoxicity assay and manual cell counting method. Our new assay offers an inexpensive and reliable method, which complements current assays by enhancing high-throughput anti-Acanthamoeba drug screening capabilities.

Keywords: Acanthamoeba; colorimetric assay; drug screening; sulforhodamine B

1. Introduction

Acanthamoeba, an opportunistic ubiquitous protist, exists in two morphological stages during its life cycle: a vegetative trophozoite and a resistant cystic stage when conditions become unfavorable [Lloyd et al., 2001; Coulon et al., 2010]. Acanthamoeba organisms, despite being free-life amoebas, can cause a spectrum of human diseases, including granulomatous amoebic encephalitis, a rare but fatal infection of the central nervous system (CNS), cutaneous lesions, and Acanthamoeba keratitis (AK), a chronic eye infection that affects contact lens wearers with an increasing frequency [Marciano-Cabral and Cabral 2003;...
Visvesvara et al., 2007]. Despite the social and clinical impact there is no vaccine, and current therapy for prevention and treatment of Acanthamoeba infection is limited or inadequate. The first-line of treatment of AK is topical therapy with biguanides, such as polyhexamethylene biguanide or in combination with diamidines, such as propamidine, aminoglycosides or imidazoles [Kumar and Lloyd 2002; Seal 2003]. However, current treatment of AK has non-specific effects, can be problematic and relapses are common [Elder et al., 1994; Tseng et al., 1998; Lorenzo-Morales et al. 2015]. These challenges make exigent the need for identification of new and more effective anti-Acanthamoeba drugs.

Research and development costs associated with successful market release of anti-Acanthamoeba therapeutics can be high, driving a continuous demand to develop new effective preclinical tools that can identify lead compounds and weed out weak candidates at an early stage in the drug discovery program. Any anti-Acanthamoeba drug susceptibility testing program aimed at discovering novel compounds requires the availability of reliable screening assays. In vitro drug testing platforms are valuable tools for screening of chemotherapeutic agents and provide preliminary data for in vivo studies. Trophozoite counting methods have been developed, but a major limitation of these assays is the lack of sensitivity [Buck and Rosenthal, 1996; Narasimhan and Madhavan, 2002; Ondarza et al., 2006]. Acanthamoeba-based screening assays are constantly undergoing improvements toward higher throughput and reduced reagent consumption. In this regard, drug susceptibility testing using colorimetric cell-based assays in microtiter plate format has been an essential methodology in drug discovery, allowing many samples to be analyzed rapidly and simultaneously using chemicals that stain the cells directly, or that are metabolized into colored products [Weyermann et al., 2005]. A few microtiter plate-based methods have been developed to screen novel compounds against Acanthamoeba trophozoites [McBride et al., 2005; Heredero-Bermejo et al., 2015]. However, there is still a need for the development of
more affordable assays with improved readouts in order to enable the testing of a larger number of compounds and identification of new drug candidates.

Sulforhodamine B (SRB) is a bright-pink aminoxanthene dye that binds to basic amino acids of cellular proteins under mild acidic conditions, and dissociates under basic conditions [Vichai and Kirtikara, 2006]. The binding of SRB is stoichiometric, and the amount of dye extracted from stained cells is directly proportional to the total protein mass and therefore correlates to cell number [Vichai and Kirtikara, 2006]. A spectrophotometer is used to quantify the intensity of the color, producing quantitative data (optical absorbance values) that correlates with the number of cells present. The SRB colorimetric assay was originally developed for in vitro anticancer-drug screening [Skehan et al., 1990; Shoemaker, 2006; Vichai and Kirtikara 2006] and was used for the evaluation of cell proliferation in a 96-well plate assay [Lin et al., 1999].

In the present study, we investigated whether a quantitative SRB colorimetric assay can be developed for testing molecules with anti-Acanthamoeba activity. Here, we describe the characteristics of this new SRB assay including defining the optimal culture conditions, seeding density and spectral measurement. To assess the sensitivity of the assay we determined the IC\textsubscript{50} of chlorhexidine, a widely used antimicrobial with a documented anti-Acanthamoeba activity. Our findings indicate that SRB protein staining is a sensitive in vitro high-throughput method that can be used to expedite the discovery of new anti-Acanthamoeba drug candidates.

2. Materials and Methods

2.1. Acanthamoeba strains and culture conditions
Two strains of *Acanthamoeba castellanii* were used as test organisms, a genotype T3 clinical AK strain was kindly provided by the University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna and Neff strain of genotype T4 that was originally obtained from American Type Culture Collection (ATCC; 30010). Strains belonging to genotypes T3 and T4 share close genetic similarity and have been frequently observed in AK infections. Each strain was maintained in axenic peptone-yeast-glucose (PYG) growth media containing 0.75% proteose peptone, 0.75% yeast extract and 1.5% glucose containing 40\(\mu\)g gentamicin ml\(^{-1}\) (Biochrom AG, Cultek, Granollers, Barcelona, Spain). Cultures were fed with fresh medium every week to maintain *A. castellanii* trophozoites in 75-cm\(^2\) tissue culture flasks.

### 2.2. Relationship between optical density and trophozoites' count

The choice of wavelength is an important consideration for correlating optical density with the number of *A. castellanii* trophozoites. Hence, we assessed the absorption spectra of serial dilutions of *A. castellanii* trophozoites and examined the relationship between seeding density (i.e. number of trophozoites) and absorbance within the wavelength range 450-630 nm. *A. castellanii* trophozoites were harvested by keeping culture flasks chilled on ice for 5 min to detach adherent trophozoites, which were then separated from PYG medium by centrifugation at 1000g for 5 min. From a stock solution of \(8 \times 10^5\) ml\(^{-1}\) *A. castellanii* trophozoites seven serial dilutions (50, 100, 250, 500, 1000, 2500 and 5000 trophozoites/well) were made and seeded in triplicates in a 96-well microtiter plate in order to generate a calibration curve. Control wells were filled with equal volume of culture medium, but without trophozoites. Adherence of trophozoites was checked using a Leica DM IL inverted microscope (Leica, Wetzlar, Germany). After 30 min trophozoites were fixed and stained with sulforhodamine B (SRB) as described below. Measurements were corrected by
blank wells containing PYG medium only. A logarithmic transformation was performed for both optical density (OD) values and the numbers of trophozoites to standardize the variance and balance the differences between the data points. These transformed data were used to fit a linear regression curve.

2.3. Acanthamoeba seeding density optimization

To determine the optimal density of *A. castellanii* to be used in the drug susceptibility testing, trophozoites ranging from 100 to 2000 trophozoites/well were prepared, in triplicates to a final volume of 100 µl/well in a 96-well tissue culture plate. After 30 min incubation, the trophozoites were fixed and stained with SRB assay using a method modified from that described previously [Vichai and Kirttikara, 2006]. Briefly, without removing the culture medium, 25 µl cold (4°C) Trichloroacetic acid (50% w/v) (TCA, Merck, Germany) was added to each well, already containing 100 µl PYG medium, and the plate was incubated at 4°C for 1 hr. The supernatant was then discarded, and the plate was washed gently three times with slow-running tap water to remove TCA, culture medium and dead (unattached) trophozoites and the plates were left to dry at ambient temperature. About 25 µl of sulforhodamine B solution (4 % w/v SRB dissolved in 1% acetic acid in water) (Sigma) was added to each well, and the culture was incubated for 15 min. Unbound SRB was removed by washing three times with 1% acetic acid. Then, the plates were air-dried completely. Cell protein-bound dye was solubilized by adding 150 µl Tris base [tris-(hydroxymethyl)-aminomethane] solution (10 mM, pH 10.5) to each well, followed by shaking the plate on a gyratore shaker for 5-10 min. Optical density (OD) of each well was measured using BioTek's PowerWave XS absorbance microplate reader in dual wavelength mode. The color absorbance was measured spectrophotometrically at 562 nm as the test wavelength. The reference wavelength was 630 nm. OD values were corrected for background OD from blank
wells containing PYG medium only. The OD value of each well was compared to obtain the optimal seeding concentration of *A. castellanii*.

2.4 *In vitro susceptibility testing*

The *in vitro* susceptibly of *A. castellanii* strains to chlorhexidine (CHX, Sigma) was tested based on the SRB method. The optimal seeding density number *A. castellanii* trophozoites, determined to be 500/well, was seeded in triplicates in 100 µl of PYG medium in 96-well culture plates. Trophozoites were allowed to adhere to the well surface for 30 min. Culture media was then removed and 100 µl of serial dilutions of chlorhexidine (0.15, 0.31, 0.62, 1.25, 1.5, 3, 6, 12 µM) in PYG medium was added to each well. Chlorhexidine was selected because it is commonly used in contact lens cleaning solutions due to its broad-band antimicrobial and topical disinfectant activities. After 96 hr of incubation the test plates were subjected to the SRB staining in order to assess the inhibitory activity of chlorhexidine. To validate the sensitivity of the SRB method a parallel experiment was performed under identical conditions to test the efficacy of chlorhexidine against *A. castellanii*, by direct-counting of trophozoites using a haemocytometer and an optical microscope at ×40 magnification. All experiments were performed in triplicates and were repeated independently at least three times.

2.5 *Statistical analysis*

The half maximal inhibitory concentration (IC$_{50}$) values were calculated by Probit regression. IC$_{50}$ values, from at least three separate experiments, were compared using Student’s *t*-test. Data are presented as the means ± standard error of the means (SEM). Differences were significant when the *P* value was <0.05. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).
3. Results and Discussion

In this study, we leveraged the simplicity and sensitivity of the sulforhodamine B (SRB) staining assay to develop a new method to facilitate high-throughput anti-*Acanthamoeba* drug screening. This method relies on the ability of SRB to bind to protein components of *A. castellanii* trophozoites adherent to culture plates. To adapt the SRB assay to *A. castellanii* the effect of absorbance range and the trophozoite’s seeding density on the performance of the assay was investigated.

We first evaluated the absorption spectra of serial dilutions of *Acanthamoeba* trophozoites and investigated the relationship between the concentration of trophozoites (i.e. trophozoite’s seeding density) and absorbance within the wavelength range 450-630 nm. As shown in Fig. 1a, the SRB method allows the detection of even small differences in number of *A. castellanii* trophozoites, confirming the sensitivity of the method. At the wavelength of maximal absorbance of SRB (562 nm) we were able to detect trophozoite densities at as low as 100 trophozoites per well. Regression analysis demonstrated a linear relationship between OD readings and the seeding density of *A. castellanii* trophozoites up to 2000 trophozoites per well (Fig. 1b). Next, we determined the optimal number of *Acanthamoeba* trophozoites per well to be used in the SRB assay. At cell seeding density of 500 cells per well, trophozoites reached confluence as indicated by the change in the slope of the curve (Fig.1c). Hence, 500 cells per well were considered the optimal seeding density for the drug testing experiment. This represents an improvement over the standard Alamar Blue assay, which requires a 100% reduction of the dye (in control wells), which is achieved with a very high density of trophozoites/well. Alamar Blue also requires a certain exposure time, depending on the concentration of trophozoites, for the dye to be reduced. In contrast, the SRB method does not require time-critical steps and sample preparation can be performed in less than 90 min.
Also, stained plates can be stored at the fixing and staining steps for at least one month for later processing. SRB staining is also less sensitive to environmental fluctuation, such as variations in pH or depletion of glucose, and is independent of intermediary metabolism - factors that may be influenced by the test compounds.

*Acanthamoeba castellanii* trophozoites vary in size from 25 to 40 µm [Marciano-Cabral and Cabral, 2003]. Therefore, it can be argued that *A. castellanii* trophozoites can vary in volume with growth phases or with different strains and that this subtle variation in the size of trophozoites could alter the protein content of trophozoites and thus influences the SRB measurements. However, even if *Acanthamoeba* strain- or subtype-dependent variability in the growth of trophozoites exists this was insufficient to affect the measurement of average protein concentration of a proliferating population of trophozoites. Also, the SRB assay has been developed using clonal strains and identical number of trophozoites in each well.

We further tested the anti-*A. castellanii* activity of serial dilutions of chlorhexidine using the SRB assay. Fig. 2 shows the dose-response effect of chlorhexidine on the growth of two clinical strains of *A. castellanii*. The IC₅₀ values of chlorhexidine using the SRB assay were 4.68±0.6 µM and 5.69±0.9 µM for Neff strain and T3 stain, respectively and by using manual count was 3.72±0.4 µM for Neff strain. Despite the fact that removal of PYG medium post-fixation and before the addition of the reagents might have caused loss of some trophozoites that are not firmly attached to the culture plate, chlorhexidine IC₅₀ values obtained with SRB staining in the present study were found comparable to the IC₅₀ reported using the Alamar Blue method [McBride et al., 2005; Martin-Navarro et al, 2008] and the OCR cytotoxicity assay [Heredero-Bermejo et al., 2015], further supporting the reliability of the SRB method. Apparent differences in the intensity of the colour between wells seeded by two *A. castellanii* strains are observed. Wells exposed to lower chlorhexidine concentrations developed more
intense colour and correlated with high OD readings, reflecting large number of trophozoites (Fig. 3).

A few methods have been developed to screen compounds against *A. castellanii* trophozoites. The traditional cell counting methods using haemocytometer and staining, such as trypan blue are simple and inexpensive but very time-consuming, labor intensive and sometimes inaccurate [Buck and Rosenthal, 1996; Narasimhan and Madhavan, 2002; Ondarza et al., 2006]. Measurement of mitochondrial metabolic rate using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay or MTS assay to indirectly reflect viable cell numbers has been applied [O'Toole et al., 2003]. MTT is a tetrazolium salt that is reduced to purple formazan crystals mainly by mitochondrial succinate dehydrogenase [Wang et al., 1996]. MTS assay utilizes a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] that is an alternative to MTT. The formazan formed from MTS is water-soluble and less toxic in contrast to the insoluble formazan product formed by MTT. In both assays, the colour intensity of the formazan dye correlates to the number of viable cells.

Although useful, viability assays can be prone to a number of factors that can adversely affect the results. For example, some chemicals or phytochemicals may interact with MTT directly even in the absence of cells [Lim et al., 2015]. Also, cell metabolic activity may be influenced by different culture conditions or chemical treatments, which can cause considerable variations in the results reported from these assays [Plumb et al., 1989]. The comparative efficacy of using PrestoBlue®, MTT and CellTiter96® in assessing the effect of chlorhexidine digluconate on *A. castellanii* and *A. polyphaga* indicated that results can be influenced by the strain used, the viability of trophozoites and the interference of the culture medium [Herdero-Bermejo et al., 2013]. However, these factors could also influence any test, not only PrestoBlue®, MTT or CellTiter96® assays. The colorimetric assay based on the
mitochondrial reduction of Alamar Blue® [McBride et al., 2005] requires expensive reagents and high trophozoite seeding density, limiting their ability to be used in high throughput screening of large library of chemicals. A newly developed microplate method based on the real-time measurement of oxygen concentration rate (OCR) of cultured trophozoites has proved to be sensitive and reliable for testing compounds with anti-Acanthamoeba activity [Heredero-Bermejo et al., 2015]. This OCR-based method enables real-time assessment of cytotoxicity while maintaining the high-throughput drug screening capability. Even though this method requires the addition of mineral oil to wells no addition of substrate is need, an improvement over other methods including the SRB assay.

In conclusion, our work represents the first application of the SRB staining method in anti-Acanthamoeba drug screening research. This high-throughput, colorimetric method can enable the screening of large libraries of chemical molecules against Acanthamoeba trophozoites in a microtiter plate format. The method promises an effective and inexpensive preclinical tool that can accelerate the screening of prospective therapeutics, while increasing the translational predictive value of in vitro drug testing studies and reducing the need for in vivo animal-based experiments. This SRB method also has potential to be used in the future for the rapid drug susceptibility testing of clinical A. castellanii isolates from infected patients. Since our interests lie in developing this new SRB assay we have only tested one drug in this study. A useful future direction would be to explore a library of compounds with different mechanism of actions in parallel with another established assay in order to test the utility of the SRB assay. Finally, because protein content, metabolism and OCR can all be influenced by cytotoxic drugs in different manners, multiplexing assays measuring these parameters simultaneously is the best way to get the true picture of response of A. castellanii trophozoites to toxic agents.
Acknowledgments

This research was co-financed by the EU Research Potential (FP7-REGPOT-2012-CT2012-31637-IMBRAIN), the European Regional Development Fund (FEDER) and the Spanish Instituto de Salud Carlos III (PI11/00840). We wish to thank Dr Kevin Webb from University of Nottingham for critical comments and constructive suggestions.

References


Fig. 1. Calibration curve of SRB assay on *Acanthamoeba castellanii* trophozoites was constructed of optical density (OD) values against the numbers of trophozoites for the validation of linearity. *A. castellanii* trophozoites were seeded at the indicated cell densities per well. The SRB method was performed 30 min after seeding as described in the materials and methods. (a) Profiles of the spectra of normalized OD between 450 and 630nm versus SRB-stained trophozoites in a series of seeding densities. (b) Calibration curve of trophozoite numbers against OD at 562 nm (OD$_{562}$), showing a linear relationship between trophozoite density and absorbance produced by the stained trophozoites with a $R^2$ correlation coefficient of 0.9988. (C) Relationship between trophozoite’s seeding density and absorbance. All results represent the mean of three independent experiments.
Fig. 2. Effect of chlorhexidine exposure on the growth rate of two clinical strains of Acanthamoeba castellanii. Dose-response curve of cultures of A. castellanii after 96 hr exposure to chlorhexidine at serial dilutions (0.15, 0.31, 0.62, 1.25, 1.5, 3, 6, 12 µM). The number of A. castellanii trophozoites was determined by the SRB staining method. Progressive inhibition of trophozoites’ growth was detected in Neff strain (triangles) and T3 strain (squares) with exposure to increasing concentrations of chlorhexidine. Each data point represents the mean ± SEM for nine samples (triplicate samples in three independent experiments).
Fig. 3. Representative micrographs of microtitre plates stained with SRB showing the inhibitory effect of chlorhexidine on trophozoites of two *Acanthamoeba castellani* strains (Neff and T3) at 72 hrs after exposure to chlorhexidine. The plate was designed as follows: (C) control untreated wells, (B) blank wells and the test wells showing serial dilutions (100 to 0.78 μM) of the tested chlorhexidine. Note the change in the color intensity (OD) of the wells, which increases with increasing the number of trophozoites per well.