Molecular & Biochemical Parasitology 188 (2013) 20–25

Contents lists available at SciVerse ScienceDirect

Molecular & Biochemical Parasitology

A quantitative high throughput assay for identifying gametocytocidal compounds

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ARTICLE INFO

Article history:
Received 1 November 2012
Received in revised form 4 February 2013
Accepted 8 February 2013
Available online 27 February 2013

Keywords:
Malaria
Gametocytes
AlamarBlue
High throughput screening
Malaria drug discovery

ABSTRACT

Current antimalarial drug treatment does not effectively kill mature *Plasmodium falciparum* gametocytes, the parasite stage responsible for malaria transmission from human to human *via* a mosquito. Consequently, following standard therapy malaria can still be transmitted for over a week after the clearance of asexual parasites. A new generation of malaria drugs with gametocytocidal properties, or a gametocytocidal drug that could be used in combinational therapy with currently available antimalarials, is needed to control the spread of the disease and facilitate eradication efforts. We have developed a 1536-well gametocyte viability assay for the high throughput screening of large compound collections to identify novel compounds with gametocytocidal activity. The signal-to-basal ratio and Z'-factor for this assay were 3.2-fold and 0.68, respectively. The IC_{50} value of epoxomicin, the positive control compound, was 1.42 ± 0.09 nM that is comparable to previously reported values. This miniaturized assay significantly reduces the number of gametocytes required for the AlamarBlue viability assay, and enables high throughput screening for lead discovery efforts. Additionally, the screen does not require a specialized parasite line, gametocytes from any strain, including field isolates, can be tested. A pilot screen utilizing the commercially available LOPAC library, consisting of 1280 known compounds, revealed two selective gametocytocidal compounds having 54- and 7.8-fold gametocytocidal selectivity in comparison to their cell cytotoxicity effect against the mammalian SH-SY5Y cell line.

Published by Elsevier B.V.

1. Introduction

Effective chemotherapy is a critical component of current antimalarial control efforts [1]. Consequently, recent reports of recrudescence and delayed clearance following the recommended course of artemisinin combination therapy (ACT) are a major concern and being carefully monitored [2,3]. The lack of alternatives to ACT has led to a number of small molecule and natural product library screens for the identification of new antimalarials [4–7]. However, none of the large scale screens (>100 compounds) have included the sexual stages of the parasite life cycle that are required for malaria transmission.

Sexual development begins in the red blood cell with the production of a single male or female gametocyte. Once taken up by a mosquito in a blood meal, gametocytes are triggered to emerge from the red blood cell (RBC), fertilize and begin sporogonic development [8]. In *Plasmodium falciparum*, the most virulent human malaria, gametocyte development takes 10–12 days and is resistant to most common antimalarials, except primaquine. [9]. This prolonged maturation allows the parasite to be transmitted for more than one week after the clearance of asexual parasites. The addition of primaquine to standard antimalarial therapy has been shown to block transmission of the parasite to the mosquitoes [10], but it has significant side effects in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency [11]. This toxicity has limited its widespread use and spurred the drive to identify alternatives with gametocytocidal activity.

Previous high throughput screens to identify anti-malaria drugs have utilized assays that assess DNA replication to monitor asexual growth and cannot be used to evaluate gametocytes, which do not proliferate. We have previously reported a viability assay that can be used to monitor gametocytocidal activity against any parasite line in a 96-well format. Recently several other groups have developed 96-well screens using parasite lines transformed with gametocyte-specific reporters [12,13]. These assays were a marked improvement over the conventional method used to examine gametocytes, the manual quantitation of Giemsa-stained blood

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0166-6851/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.molbiopara.2013.02.005

smears, which is very slow and labor intensive. However, all these assays continue to be limited to the analysis of 100s of compounds by the number of gametocytes that can be produced *in vitro*. In the work reported here, we describe the miniaturization of our gametocyte viability assay into a 1536-well format that requires 25-fold fewer gametocytes/well than the previously reported 96-well format [14]. This assay format is conducive to high throughput screening, and has been validated using the library of pharmacologically active compounds (LOPAC) made up of 1280 known compounds. Two active compounds with 54 and 7.8-fold gametocytocidal selectivity, in comparison to their cell cytotoxicity effect as observed against the human SH-SY5Y cell line, have been identified from this screen.

2. Materials and methods

2.1. Materials

RPMI-1640 supplemented with L-glutamine and 25 mM HEPES (Catalog No. CUS-0645) was purchased from K·D Medical Inc. (Columbia, MD). Gentamicin solution (Catalog No. 15710-064), sodium bicarbonate solution (Catalog No. 25080), AlamarBlue (Catalog No. DAL1100) and Opti-MEM reduced serum medium, no phenol red (Catalog No. 11058-021) were purchased from Life Technologies (Grand Island, NY). One liter of RPMI-1640 was supplemented with 11 μg/ml of gentamicin and 0.27% of sodium bicarbonate solutions to make incomplete media. O positive human blood and serum was obtained from Interstate Blood Bank, Inc. (Memphis, TN) and the 75-cm^2 flasks (Catalog No. 430720) used for parasite culture were purchased from Corning Inc. (Corning, NY). The 1536-well white sterile tissue culture treated polystyrene plates (Catalog No. 789270-C) were purchased from Greiner Bio-One (Monroe, NC).

2.2. Gametocyte cell culture

P. falciparum 3D7 strain parasites were set up for gametocyte production in incomplete RPMI-1640 media supplemented with 10% positive human serum as described previously [15]. Stage III–V gametocytes were selected and enriched with 50 mM N-acetyl glucosamine (NAG) and Percoll density gradient centrifugation, respectively. Briefly, asexual parasites were adjusted to 0.1% parasitemia and 6% hematocrit in 12.5 ml of complete media in a 75-cm^2 flask on day 1. On day 3, 12.5 ml of complete media was exchanged and then 25 ml of complete media were exchanged every day from day 4 to 11. To eliminate asexual parasites, 2.8 ml of a 0.5 M NAG suspension was added to culture from day 9 to 11. On day 12 gametocytes were enriched with 65% Percoll/PBS by density gradient centrifugation at 1860 × *g* for 10 min and maintained in 1.5 ml of complete media for compound library screening on day 13.

2.3. AlamarBlue assay optimization

All optimization and miniaturization experiments were performed in 1536-well plate format. Malaria gametocytes, in suspension with 90% RBCs, were plated at a seeding density of 10k, 20k, and 27.5k cells per well at a final volume of 5 μl per well using the Multidrop Combi (Thermo Fisher Scientific, Logan, UT). Cells were incubated for 72 h at 37 °C and 5% CO_2. AlamarBlue dye was used for cell viability measurements. Briefly, 5 μl of a 2-fold concentrated AlamarBlue solution (2 ml diluted in 8 ml of Opti-MEM media) was added per well, and plates were incubated for 4, 8, 10, and 24 h at 37 °C and 5% CO_2. The fluorescence intensity of assay plates was captured using a fluorescence protocol (Ex = 525 nm, Em = 598 nm) on the ViewLux plate reader (PerkinElmer, Shelton, CT). Table 1 outlines the finalized protocol used in the miniaturized gametocytocidal assay.

Table 1
Gametocyte assay protocol (1536-well plate).

Step	Parameter	Value	Description
1	Medium	2.5 μl/well	Incomplete growth medium
2	Compound	0.02 μl/well	Compound in DMSO solution
3	Gametocytes	2.5 μl/well	Suspension (8×10^6/ml) in incomplete growth medium with 20% human serum
4	Incubation	72 h	37 °C, 5% CO_2
5	Detection reagent	5 μl/well	AlamarBlue (1:5 dilution) in OPTI-MEM
6	Incubation	24 h	37 °C, 5% CO_2
7	Incubation	120 min	Room temperature
8	Plate reading	Ex = 525 nm Em = 598 nm	Fluorescence intensity

2.4. Compound screen

Screening experiments were performed in a similar fashion as the optimization experiments. Briefly, 2.5 μl per well of incomplete medium was dispensed into 1536-well plates using the Multidrop Combi. Compound libraries were transferred in a volume of 23 nl per well using the NX-TR Pintool (WAKO Scientific Solutions, San Diego, CA), and malaria gametocytes, in suspension with 90% RBCs and incomplete media supplemented with 20% human serum, were plated at a seeding density of 20k cells per well and a volume of 2.5 μl per well using the Multidrop Combi. Plates were incubated for 72 h at 37 °C and 5% CO_2. The AlamarBlue dye was used for cell viability measurements, where 5 μl of a 2-fold concentrated AlamarBlue solution (2 ml diluted in 8 ml of Opti-MEM media) was added per well, and plates were incubated for an additional 24 h at 37 °C in the presence of 5% CO_2. Plates were read using a fluorescence protocol (Ex = 525 nm, Em = 598 nm) on the ViewLux plate reader.

2.5. Compound library and instruments for liquid handling

The library of pharmacologically active compounds (LOPAC) containing 1280 compounds was purchased from Sigma–Aldrich. Compounds were dissolved in 100% DMSO as 10 mM stock solutions and were further diluted in 384 well plates to 7 concentrations at a 1:5 ratio followed by reformatting into 1536-well compound plates. A CyBi®-Well dispensing station with a 384-well head (Cybio Inc., Woburn, MA) was used to reformat compounds in 384-well plate to 1536-well plate. The 1–4 μl/well reagents were dispensed using the Multidrop Combi. Compounds in DMSO solution were transferred to 1536-well assay plates at 23 nl/well using the Pintool workstation.

2.6. Data analysis

The 100% signal was defined from wells devoid of compounds, and the basal signal was obtained from wells treated with 17 nM epoxomicin. The primary screen data were analyzed using customized software developed by the NIH Chemical Genomics Center (NCGC) [16]. IC_{50} values of compounds in the confirmation experiments were calculated using the Prism software (Graphpad Software, Inc. San Diego, CA). Signal-to-background ratio was calculated as a comparison of signal in the presence or absence of the epoxomicin control compound. All values were expressed as the mean ± SD (n = 3).