IJP: Drugs and Drug Resistance 8 (2018) 295–303

Contents lists available at ScienceDirect

IJP: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Functional screening of selective mitochondrial inhibitors of *Plasmodium*

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ARTICLE INFO

Keywords:
Plasmodium falciparum
Plasmodium yoelii
Oxygen consumption
Mitochondrial inhibitors and cytochrome

ABSTRACT

Phenotypic screening has produced most of the new chemical entities currently in clinical development for malaria, plus many lead compounds active against *Plasmodium falciparum* asexual stages. However, lack of knowledge about the mode of action of these compounds delays and may even hamper their future development. Identifying the mode of action of the inhibitors greatly helps to prioritise compounds for further development as novel antimalarials. Here we describe a whole-cell method to detect inhibitors of the mitochondrial electron transport chain, using oxygen consumption as high throughput readout in 384-well plate format. The usefulness of the method has been confirmed with the Tres Cantos Antimalarial Compound Set (TCAMS). The assay identified 124 respiratory inhibitors in TCAMS, seven of which were novel anti-plasmodial chemical structures never before described as mitochondrial inhibitors.

1. Introduction

Malaria is still one of the deadliest infectious diseases in the world (WHO, 2015). Several *Plasmodium* species can infect humans, and of these, *P. falciparum* causes the most severe form of the disease. Over the past 50 years, antimalarial chemotherapy has depended on just four classes of drugs: antifolates, endoperoxides, quinolines (4 and 8 amino quinolines) and quinolones. The inevitable emergence and spread of resistance to all four classes has created an urgent need for new drugs at affordable prices. There are several possible approaches to the discovery of new therapeutic compounds. A strategy employed by academic (e.g. Guiguemde et al., 2010) and industry (e.g. Gamo et al., 2010; e.g. Plouffe et al., 2008) laboratories is screening for *P. falciparum* growth inhibitors *in vitro*, an approach that has produced a wider structural diversity to date. The current challenge is to prioritise tens of thousands of hit compounds for future development, in order to identify the drug candidates most likely to be successful. If the primary biochemical mechanisms responsible for the observed anti-plasmodial activity are established in an efficient manner, compounds can be prioritised, classes with redundant mechanisms likely to be affected by cross-resistance can be identified, and progression can focus on modes of action considered desirable for their clinical validation or parasitological effects.

One of the few validated targets in *P. falciparum* is mitochondrial function (Painter et al., 2007). Extensive analyses of the data from parasite genome sequence projects and biochemical and physiological studies have revealed the presence of an active electron transport chain that can generate the electrochemical potential necessary for coenzyme Q and iron-sulphur cluster biosynthesis, as well as for pyrimidine metabolism (Painter et al., 2007; Vaidya and Mather, 2009; van Dooren et al., 2006). The latter seems to be the critically essential role of the canonical mitochondrial electron transport chain under *in vitro* conditions (Painter et al., 2007) and during the intraerythrocytic cycle *in vivo*. It should be noted however that other life stages seem to have expanded requirements for a functional respiratory chain in *Plasmodium* species (Hino et al., 2012; Sturm et al., 2015). It has been known for quite some time that this essential function is also highly druggable and can be selectively inhibited with compounds such as atovaquone (Fry and Pudney, 1992; Hudson, 1993) and the more recently described 4-(1H) pyridones (Capper et al., 2015; Cowley et al., 2012; Yeates et al., 2008).

However, the development of biochemical methods to measure *Plasmodium* mitochondrial function and inhibition with enough throughput to test tens of thousands of compounds under comparable conditions in a reasonable time presents numerous complications, including: (i) the difficulty of purifying functional mitochondria, which

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https://doi.org/10.1016/j.ijpddr.2018.04.007
Received 17 October 2017; Received in revised form 20 April 2018; Accepted 26 April 2018
Available online 09 May 2018

underlies the low reproducibility between experiments from different laboratories; (ii) the low throughput of the techniques used, which traditionally require Clark-type electrodes or flow cytometry (Srivastava et al., 1997); and (iii) the intrinsically low *in vitro* respiratory rate of *P. falciparum* mitochondria, due to the parasite using the glycolytic pathway as its main source of metabolic energy during intraerythrocytic growth.

Here we describe a method capable of testing tens of thousands of compounds with whole-cell activity and identifying inhibitors of parasite mitochondrial metabolism by measuring oxygen consumption in red blood cells (RBCs) infected with *Plasmodium* species, using short incubation times and a high throughput format. The assay reliably differentiated compounds targeting the respiratory chain from those inhibiting *Plasmodium* growth through a different mechanism. The method has been validated using known mitochondrial inhibitors and standard antimalarial compounds and was used to identify novel inhibitors of the electron transport chain present in the TCAMS, set of phenotypic hits (Gamo et al., 2010).

2. Materials and methods

2.1. Biological material

Plasmodium yoelii 17XL was maintained *in vivo* in female CD1 mice. Four days after infection, blood from each mouse was collected in 4 ml of Hank's balanced solution (HBSS) containing 10 units of heparin/ml at approximately 50% parasitemia. Blood was washed three times with HBSS at 800 × g for 10 min. Cells were diluted 1:1 in HBSS and passed through a pre-washed small plasmodipur leukocyte-filter (Euro Diagnostica V. B.). Each filter was used to effectively remove leukocytes from a maximum of 15 ml of blood. The collected red blood cells (RBCs) were washed and resuspended again in HBSS. Infected erythrocytes were enriched by centrifugation over a solution of PBS with 70% (v/v) Percoll and 0.3 M glucose at 335 × g in a swinging bucket rotor for 45 min at room temperature. The interphase, containing trophozoites and schizonts almost exclusively, as determined by Giemsa-stained thin blood smears, was collected. Infected RBCs (iRBCs) were washed twice with RPMI 1640 medium containing 1% (v/v) foetal calf serum (FCS) and resuspended in the same medium at $7{\cdot}10^8$ iRBCs/ml for assays. During all these processes, unless otherwise noted, cells were maintained at 4 °C. All animal studies were ethically reviewed and carried out in accordance with European Directive 86/609/EEC and the GSK Policy on the Care, Welfare and Treatment of Animals. Guidelines and codes of conduct for animal care and research performed at the DDW Laboratory Animal Science facilities were approved and accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

3D7 and K1 strains of *P. falciparum* from MR4 (American Culture Type Collection) were grown in human type O + erythrocytes obtained from the Spanish Red Cross Blood Bank in complete medium: RPMI 1640 with 25 mM HEPES and $NaHCO_3$ (Sigma R5886), 20 mg/L hypoxanthine and 0.5% (v/v) Albumax II (Invitrogen) and grown in 5% CO_2, 5% O_2 and 90% N_2 at 37 °C. For each experiment, cultures of 250 ml at 4% haematocrit were grown in five 175 cm^2 flasks, with daily medium changes, and harvested at the highest possible trophozoite and schizont parasitemia (≥10%) by centrifugation at 800 × g at room temperature. iRBC were washed twice in RPMI containing 1% (v/v) FCS and adjusted in that media to $4{\cdot}10^8$ iRBCs/ml, 33% haematocrit. A total of $8.4{\cdot}10^7$ iRBC/well were used for screening and subsequent experiments, whereas different numbers *P. falciparum*-infected RBCs-well were used in the pilot experiments to develop the assay, as indicated in the *Oxygen consumption assay* section below and the legends to the figures. As mature forms (trophozoites and schizonts) are the most metabolically active and hence most likely to consume oxygen, cultures were harvested at time points when the proportion of mature forms was maximal (Supplementary Table 4).

Human epithelial HEK 293 cells were grown at 37 °C in SMEM (Sigma M8167) with 2 mM L-glutamine, 10% (v/v) FCS and 1% (w/v) Pluronic F127 (Sigma), using a continuous culture system. Cells were collected by centrifugation at 800 × g during 10 min at RT, washed twice in SMEM with 10% (v/v) FCS medium and then adjusted to $1.3{\cdot}10^7$ cells/ml.

2.2. Compounds

Atovaquone, endochin and TCAMS compounds (GSK), stigmatellin (Fluka), FCCP (Sigma) and proguanil (Molekula) were dissolved in DMSO. Sodium azide (Panreac), potassium cyanide (Sigma) and chloroquine (Sigma) in water and myxothiazol, antimycin and rotenone (Sigma) in ethanol. In the assay, the final concentration for DMSO or ethanol was 0.5% (v/v), a concentration that did not inhibit oxygen consumption and which was used as a 0% inhibition control (Control 1).

2.3. Oxygen consumption assay

To set up the assay, the first experiments with HEK 293, *P. yoelii* and *P. falciparum* cells were done in 96-well plates, to measure signal-to-background (S/B), timeframe needed to calculate Vmax and a starting point of the number of cells per well. As HEK 293 and *P. yoelii* were going to be used in a HTS campaign, assay was later set up in 384-well plate format. *P. falciparum* cells were only used in 96-well plates, as the amount of culture needed to observe oxygen consumption did not allow miniaturization the assay to 384-well plates.

Different amounts of cells were needed for each cell type to obtain the desired signal-to-noise ratio. $6.5{\cdot}10^5$ HEK 293 mammalian and $1.4{\cdot}10^7$ for *P. yoelii* iRBCs in 384-well plates and $8.4{\cdot}10^7$ iRBC for *P. falciparum* iRBCs in 96-well plates.

Plasmodium cells were incubated with the inhibitors at a wide range of concentrations for 20 min at 37 °C, and then transferred to the BD™ Oxygen Biosensor System (BD™ OBS) from BD Biosciences. 96-well (for *P. falciparum*) or 384-well (for *P. yoelii*). The pre-incubation step was introduced to reduce the lag-time observed when plates are incubated immediately following parasite addition. BD™ OBS plates were previously filled with RPMI 1640 medium, 1% (v/v) FCS, pre-warmed to 37 °C, to give a final volume of 200 µl (for 96-well plates) or 80 µl (for 384-well plate format), containing test compounds at the same concentration as in the pre-incubation step. HEK 293 cells were not pre-incubated to avoid losing adherent cells in this intermediate step, so they were directly seeded in the BD™ OBS plate that already had SMEM with 10% (v/v) FCS media and the inhibitors. The plates were placed in a Spectramax Gemini EM plate reader (Molecular Devices), and the fluorescence emitted at 630 nm was measured from the bottom, after excitation at 485 nm (cut-off of 495 nm), at 2 min intervals for HEK 293 and *P. yoelii*, or 5 min in the case of *P. falciparum*. Data were collected for 2 h for HEK 293 and *P. yoelii* and 12 h for *P. falciparum*. The intensity of fluorescence in each well correlates with the oxygen concentration present at each measurement. The V_{max}, expressed as the increase in fluorescence over time, was automatically calculated as the slope at the inflection point of the sigmoidal graph using 10 points in the case of HEK 293 and *P. yoelii* or 50 points for *P. falciparum*. The initial slope was constant between 8 and 50 min for HEK 293, between 30 and 90 min for *P. yoelii* and between 30 min and 8 h for *P. falciparum*. Percent inhibition by a compound concentration was calculated relative to the 0.5% DMSO (0% inhibition of oxygen consumption, Control 1) after subtracting the background fluorescence in wells treated with 1.25 µM atovaquone (100% inhibition oxygen consumption, Control 2) in the case of *Plasmodium* cells or stigmatellin at 10 µM for HEK 293 cells.