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Human dihydrofolate reductase influences the sensitivity of the malaria parasite *Plasmodium falciparum* to ketotifen – A cautionary tale in screening transgenic parasites

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ABSTRACT

Ketotifen has recently been reported to inhibit the growth of both asexual and sexual malaria parasites. A parasite transporter, PfgABCG2, has been implicated in its mechanism of action. Human dihydrofolate reductase (hDHFR) is the most commonly used selectable marker to create transgenic *Plasmodium falciparum* cell lines. Growth assays using transgenic *P. falciparum* parasites with different selectable markers revealed that the presence of hDHFR rather than the absence of PfgABCG2 is responsible for a shift in the parasite's sensitivity to ketotifen. Employing a range of *in vitro* assays and liquid chromatography-mass spectrometry we show that ketotifen influences hDHFR activity, but it is not metabolised by the enzyme. Our data also highlights potential pitfalls when functionally characterising transgenic parasites.

1. Introduction

Ketotifen, a tricyclic antihistamine, suppresses the proliferation of rodent malaria parasites *in vivo* (Milner et al., 2012) and human malaria parasites (*P. falciparum*) *in vitro* (Eastman et al., 2013). Both ketotifen and its metabolite norketotifen kill schizonts and liver-stage *P. berghei* parasites (Milner et al., 2012). Ketotifen and other antihistamines have also been shown to reverse chloroquine resistance in *P. falciparum* (Basco et al., 1991) and in *P. yoelii* (Singh and Puri, 2000). The potential of ketotifen as an antimalarial is therefore of significant interest.

Dihydrofolate reductase (DHFR) converts dihydrofolate (DHF) into tetrahydrofolate (THF) in the folate pathway. This pathway is essential for DNA synthesis and amino acid metabolism in the parasite (Hyde, 2005) and DHFR inhibitors such as pyrimethamine have been widely used for the treatment of malaria. Another antifolate, WR99210, inhibits *P. falciparum* growth by inhibiting *P. falciparum* DHFR (Kinyanjui et al., 1999) and is used as a selectable marker for the transfection of *P. falciparum*. Human dihydrofolate reductase (hDHFR) is insensitive to WR99210 (De Koning-Ward et al., 2000; Fidock and Wellems, 1997) and parasites transfected with a plasmid containing the gene encoding hDHFR are resistant to WR99210 and survive WR99210-selection pressure.

Eastman et al. reported that disruption of the gene encoding the ABC-transporter PfgABCG2 reduces the sensitivity of asexual blood-stage 3D7 parasites to a range of tricyclic compounds, including ketotifen (Eastman et al., 2013). From this, the authors concluded that PfgABCG2 plays a role in the parasite response to these compounds. In this study, we investigated the sensitivity to ketotifen of an independently-generated 3D7 parasite line lacking PfgABCG2 (Tran et al., 2014), comparing it with that of a number of other parasite lines.

2. Material and methods

2.1. Parasites

Transfections were performed on chloroquine sensitive 3D7

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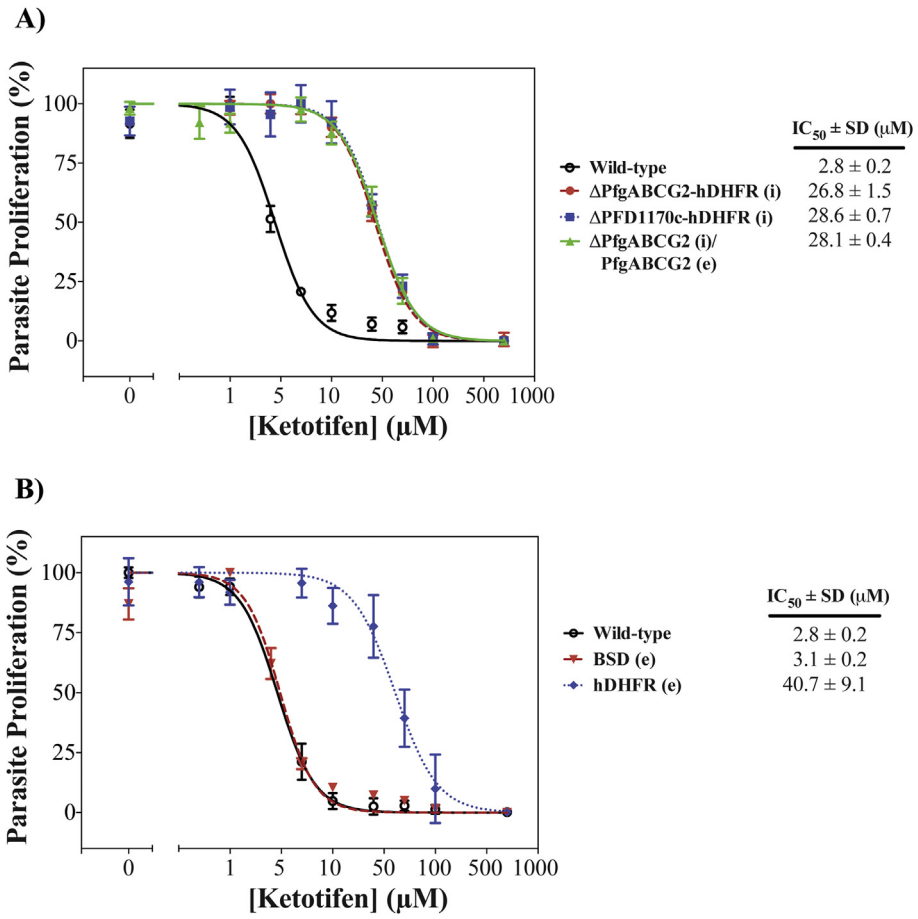

Fig. 1. Expression of the selectable marker hDHFR antagonises the inhibition of *P. falciparum* parasite proliferation by ketotifen. The proliferation of asexual blood-stage parasites in the presence of a range of ketotifen concentrations was assessed over 72 h. Selectable marker cassettes integrated into the genome are indicated by (i), selectable marker cassettes located on episomal plasmids are indicated by (e). A) Sensitivity of parasites to ketotifen is independent of the presence of PfgABCG2. Wild-type: 3D7 parental line; ΔPfgABCG2-hDHFR (i): PfgABCG2 knock-out parasites expressing the hDHFR selectable marker. ΔPFD1170c-hDHFR (i): PFD1170c knock-out parasites expressing the hDHFR selectable marker. ΔPfgABCG2-hDHFR (i)/PfgABCG2-BSD (e): PfgABCG2 knock-out parasites expressing the hDHFR selectable marker and PfgABCG2 expressed from an episomal plasmid, under the control of the PfgABCG2 promoter, using blasticidin-S deaminase (BSD) as the selectable marker. B) Sensitivity to ketotifen is modulated by the presence of hDHFR. BSD (e): parasites expressing actin II (tagged by red fluorescent protein) and the blasticidin-S deaminase (BSD) selectable marker from an episomal plasmid. hDHFR (e): parasites expressing the hDHFR selectable marker from an episomal plasmid. Mean and standard deviation (SD) values of biological triplicates are shown.

wild-type parasites as previously described with some modifications (Fidock and Wellems, 1997; Rug and Maier, 2013). Six different lines were used (Table S1): (I) wild-type parasites; (II) parasites containing an episomal human dihydrofolate reductase (*hDHFR*) selection cassette (hDHFR (e)) (Tran et al., 2014) ((e) referring to an episomal locus, (i) to integration into the genome); (III) PfgABCG2 knock-out parasites generated by genomic integration of the *hDHFR* selection cassette into the gene encoding PfgABCG2 (ΔPfgABCG2-hDHFR (i)) (Tran et al., 2014); (IV) ΔPfgABCG2 parasites complemented with an episomal copy of gABCG2 (ΔPfgABCG2-hDHFR (i)/PfgABCG2-BSD (e)) (Tran et al., 2014); (V) PFD1170c knock-out parasites (ΔPFD1170c-hDHFR (i)) (Nguyen et al., manuscript in preparation), generated by genomic integration of the *hDHFR* selection cassette into the gene encoding PFD1170c (an exported protein unrelated to PfgABCG2; see Supplementary Fig. S1 for the integration strategy); and (VI) PF14_0124-RFP-BSD (e) parasites, containing an episomal plasmid pRREP-4/PF14_0124 (see Supplementary Fig. S2 for a schematic representation of the episomal plasmid) expressing both *Aspergillus terreus* blasticidin-S deaminase (BSD) and *P. falciparum* actin II (encoded by *PF14_0124*) fused to red fluorescent protein (BSD (e)). BSD confers resistance to blasticidin-S (Yamaguchi et al., 1965; Mamoun et al., 1999) and the gene encoding BSD thereby serves as a selectable marker.

The parasites were cultured using standard methods (Trager and Jensen, 1976) with slight modifications (Maier and Rug, 2013). Parasites and erythrocytes were grown in RPMI 1640-Hepes medium with Glutamax (ThermoFisher Scientific #72400120) supplemented with 10 mM glucose (Sigma), 480 μM hypoxanthine (Sigma), 20 μg/ml gentamicin (ThermoFisher Scientific), 0.25% (w/v) Albumax II (ThermoFisher Scientific), and 5% heat inactivated human serum. The use of human erythrocytes was approved by the ANU Human Ethics committee 2011/266. Ring-stage parasites were synchronized by sorbitol treatment (Lambros and Vanderberg, 1979).

2.2. In vitro proliferation assay

Synchronous ring-stage cultures (100 μL, 0.2% parasitemia, 2% haematocrit) were incubated with ketotifen fumarate (Sigma) at a range of concentrations for 72 h at 37 °C, after which parasitised erythrocytes were stained with 1 μM SYTO16 (Invitrogen) at 37 °C for 30 min, then counted using a flow cytometer (BD LSR II, BD Biosciences) on the FITC channel (488/525 nm). Each parasite cell line was assayed in triplicate and 50,000 events (total RBCs) were counted for each sample and processed using FlowJo v887