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Plasmodium falciparum: Stage specific effects of a selective inhibitor of lactate dehydrogenase

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Abstract

Plasmodium falciparum lactate dehydrogenase (*Pf*LDH) is essential for ATP generation. Based on structural differences within the active site between *P. falciparum* and human LDH, we have identified a series of heterocyclic azole-based inhibitors that selectively bind within the *Pf*LDH but not the human LDH (hLDH) active site and showed anti-malarial activity in vitro and in vivo. Here we expand on an azole, OXD1, from this series and found that the anti-*P. falciparum* activity was retained against a panel of strains independently of their anti-malarial drug sensitivity profile. Trophozoites had relatively higher *Pf*LDH enzyme activity and *Pf*LDH-RNA expression levels than rings and were the most susceptible stages to OXD1 exposure. This is probably linked to their increased energy requirements and consistent with glycolysis being an essential metabolic pathway for parasite survival within the erythrocyte. Further structural elaboration of these azoles could lead to the identification of compounds that target *P. falciparum* through such a novel mechanism and with more potent anti-malarial activity.

Index descriptors and abbreviations: PfLDH, *Plasmodium falciparum* lactate dehydrogenase; hLDH, human lactate dehydrogenase; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide; PBS, phosphate buffer saline; APAD, 3-acetyl pyridine adenine dinucleotide; RBC, red blood cells; URBC, uninfected red blood cells; IRBC, infected red blood cells; PCR, polymerase chain reaction; IC_{50}, fifty percent inhibitory concentration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Keywords: Protozoa; *Plasmodium falciparum*; Lactate dehydrogenase; Inhibition; Intraerythrocytic cycle; Stage specificity

1. Introduction

Malaria remains a major threat to public health worldwide. It is estimated that 1–2 million children die each year mostly in Sub-Saharan Africa from the severe complications of *Plasmodiun falciparum* malaria (Guerin et al., 2002) Attempts to reduce the rates of morbidity and mortality have been hampered by the increasingly limited efficacy of current anti-malarial drugs to which *P. falciparum* has developed resistance (Olliaro and Taylor, 2003). Thus, renewed efforts are required to develop novel and affordable anti-malarials to overcome the detrimental effects of drug resistance, particularly in developing countries (Ridley, 2003).

Glycolysis represents the main source of ATP generation during the asexual intraerythrocytic cycle (Lang-Unnasch and Murphy, 1998). Glucose uptake is between 30 and 100 times higher than that of the uninfected

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erythrocyte (Jensen et al., 1983), almost all of which is converted to lactate (Homewood and Neame, 1983). The increased metabolic activity of the parasite during this period is reflected in the high levels and activity of glycolytic enzymes compared to those of the erythrocyte (Roth, 1990). As there appears to be no functioning citric acid cycle in the erythrocytic stages of the parasite life cycle, the NADH required for glycolysis to proceed is regenerated from NAD^+ via the conversion of pyruvate (the product of glycolysis) to lactate. This reaction is catalyzed by lactate dehydrogenase (LDH), the final enzyme of the glycolytic pathway in *Plasmodium*. Crystallographic analysis of *Pf*LDH (Dunn et al., 1996) revealed an enlarged active site cavity in comparison to mammalian forms of the enzyme, suggesting that highly selective inhibitors could be designed for the parasite enzyme. In contrast to compounds such as gossypol derivatives that inhibit *Pf*LDH by binding to the co-factor site (Gomez et al., 1997), we have identified a series of heterocyclic azole-based compounds that selectively inhibit *Pf*LDH at sub-micromolar concentrations, typically at concentrations about 100-fold lower than those required to inhibit human LDH (Cameron et al., 2004). Crystallographic analysis of enzyme–azole complexes showed that this class of compounds bind to the substrate binding site and are competitive with lactate. These compounds showed low micromolar activity against drug sensitive 3D7 and drug resistant K1 strains of *P. falciparum* in vitro, low cytotoxicity in mammalian cells and were effective in suppressing parasitemia during infection with the rodent malaria parasite *P. berghei* (Cameron et al., 2004). In this paper, we describe the *P. falciparum* stage-specific effects of the most active and *Pf*LDH-specific compound of this series, the oxadiazole 4-hydroxy-1,2,5-oxadiazole-3-carboxylic acid (OXD1), the correlation between inhibition of *Pf*LDH enzyme activity by OXD1 and *Pf*LDH RNA expression levels, and in addition, attempt to establish if the parasites retain their growth potential after OXD1 exposure during the intraerythrocytic development of the asexual stages. These data support the principle that specific inhibitors that bind into the active site of *Pf*LDH can form viable and effective anti-malarial compounds.

2. Materials and methods

2.1. Plasmodium falciparum in vitro culture and synchronization

All parasite clones, isolates and strains were acquired from MR4 (Malaria Research and Reference Reagent Resource Center, Manassas, Virginia, USA). Strains/isolates used in this study were: the drug sensitive 3D7 clone of the NF54 isolate (unknown origin); the drug sensitive strains FCR-8 (The Gambia) and FCC2 (China); the chloroquine, pyrimethamine and cycloguanil resistant K1 strain (Thailand); the chloroquine and pyrimethamine resistant FCR3 strain (The Gambia); the chloroquine resistant FCB strain (Colombia) and the chloroquine, pyrimethamine, cycloguanil, and mefloquine resistant isolate Tm90C2A (Thailand). In vitro culture of *P. falciparum* was carried out following standard methods (Trager and Jensen, 1976) with modifications as described (Cameron et al., 2004). Synchronization of parasite growth was achieved by Percoll and sorbitol treatment as described previously (Fleck et al., 2003).

2.2. In vitro parasite growth inhibition assays

The method used to assess parasite growth was based on the [^{3}H]hypoxanthine incorporation assay (Desjardins et al., 1979) with modifications including the use of Albumax instead of 10% human plasma to supplement the culture medium. Due to the absence of hypoxanthine in Albumax-based medium, addition of unlabelled hypoxanthine was found to be essential for optimal parasite growth and [^{3}H]hypoxanthine incorporation in asynchronous cultures during the 48 h growth-assay period. Addition of unlabelled hypoxanthine at a concentration 15 μM at the beginning of the assay, ensured that [^{3}H]hypoxanthine (0.048 μM, specific activity 17.40 Ci/mmol) uptake was linear up to 1% parasitemia, thus enabling detection of small reductions in the parasitemia (within the range of 0.1–1%). Stock drug solutions were dissolved in 100% dimethyl sulfoxide (Sigma, Dorset, UK) and 50 μl of a 2-fold dilution series (192, 96, 48, 24, 12, 6, and 3 μM) of the drugs prepared in assay medium (RPMI 1640 supplemented with 0.5% Albumax II (Invitrogen), 0.2% w/v glucose, 0.03% L-glutamine, and 15 μM hypoxanthine) added to each well of 96-well plates in triplicate. Fifty microlitres of asynchronous (65–75% ring stage) *P. falciparum* culture (0.5% parasitemia) or uninfected erythrocytes were added to each well reaching a final volume of 100 μl per well, a final hematocrit of 2.5% and final dimethyl sulfoxide concentrations $\leqslant$0.01%. Plates were incubated at 37 °C in 5% CO_2, and 95% air mixture for 24 h, at which point 20 μl (0.1 μCi/well) of [^{3}H]hypoxanthine (Perkin-Elmer, Hounslow, UK) was added to each well. The addition of 0.1 μCi per well instead of 0.5 μCi as described by Desjardins et al. was found to provide a satisfactory range of CPM between the maximum incorporation in untreated cultures and that of uninfected red cells. Plates were mixed for 1 min using a plate shaker and returned to the incubator. After an additional 24 h incubation period, the experiment was terminated by placing the plates in a −80 °C freezer. Plates were thawed and harvested onto glass fibre filter mats using a 96-well cell harvester (Harvester 96, Tomtec, Oxon, UK) and left to dry. After the addition of MeltiLex solid scintillant (Perkin–Elmer,