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Research paper

First homology model of *Plasmodium falciparum* glucose-6-phosphate dehydrogenase: Discovery of selective substrate analog-based inhibitors as novel antimalarial agents

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ABSTRACT

In *Plasmodium falciparum* the bifunctional enzyme glucose-6-phosphate dehydrogenase–6-phosphogluconolactonase (*Pf*G6PD–6PGL) is involved in the catalysis of the first reaction of the pentose phosphate pathway. Since this enzyme has a key role in parasite development, its unique structure represents a potential target for the discovery of antimalarial drugs. Here we describe the first 3D structural model of the G6PD domain of *Pf*G6PD–6PGL. Compared to the human enzyme (hG6PD), the 3D model has enabled the identification of a key difference in the substrate-binding site, which involves the replacement of Arg365 in hG6PD by Asp750 in *Pf*G6PD. In a prospective validation of the model, this critical change has been exploited to rationally design a novel family of substrate analog-based inhibitors that can display the necessary selectivity towards *Pf*G6PD. A series of glucose derivatives featuring an α-methoxy group at the anomeric position and different side chains at position 6 bearing distinct basic functionalities has been synthesized, and their *Pf*G6PD and hG6PD inhibitory activities and their toxicity against parasite and mammalian cells have been assessed. Several compounds displayed micromolar affinity (K_i up to 23 μM), favorable selectivity (up to > 26-fold), and low cytotoxicity. Phenotypic assays with *P. falciparum* cultures revealed high micromolar IC_{50} values, likely as a result of poor internalization of the compounds in the parasite cell. Overall, these results endorse confidence to the 3D model of *Pf*G6PD, paving the way for the use of target-based drug design approaches in antimalarial drug discovery studies around this promising target.

1. Introduction

Malaria caused by Plasmodium spp. remains one of the major causes of death worldwide, with over two-hundred million new infections each year and hundreds of thousands of deceases in 2015 [1]. Most deaths occur in sub-Saharan Africa (90%) and in children under 5 years old (70%) by infection with *Plasmodium falciparum*

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(*Pf*), the deadliest of the five malaria parasite species that affect humans [1]. Despite the huge current health and economic impact of malaria, the past two decades have witnessed a tremendous advance in its management. Indeed, malaria has been or is in the process of being eradicated in 30 countries and its incidence, childhood prevalence, and mortality have significantly decreased, mainly as a consequence of improved vector control measures, chemoprophylaxis, diagnosis, and chemotherapy. However, *P. falciparum* has developed resistance to standard antimalarial drugs, including artemisinin, i.e. the core component of the current first-line treatment (artemisinin combination therapies), which poses a serious risk to the recent advances in management and eradication of malaria [2,3]. To overcome the increasing emergence of resistance, new drugs that operate by novel mechanisms of action and feature novel chemotypes, avoiding cross-resistance to current antimalarial drugs, are urgently needed [4–11].

G6PD is a housekeeping enzyme that catalyzes the first and rate-limiting step of the pentose phosphate pathway (PPP), where glucose-6-phosphate (G6P) is converted into 6-phosphogluconolactone, thereby leading to the production of nicotinamide adenine dinucleotide phosphate (NADPH). Next, 6-phosphogluconolactonase transforms the product into 6-phosphogluconate, which in turn is converted into ribulose-5-phosphate by the following enzyme in the PPP, 6-phosphogluconate dehydrogenase, producing another molecule of NADPH. This process contributes to maintain the cell redox homeostasis, which is of particular importance in RBC, since they do not contain mitochondria and therefore any other source of NADPH [12–15]. In *P. falciparum* the bifunctional enzyme glucose-6-phosphate dehydrogenase–6-phosphogluconolactonase (*Pf*G6PD–6PGL) catalyzes the first step in the parasitic PPP route, which affords reducing equivalents for biosynthetic reactions, anabolic pathways, and protection against reactive oxygen species [16–18]. Although other enzymes can contribute to NADPH production in Plasmodium spp. [19,20], *Pf*G6PD–6PGL is essential for parasite survival during infection, as supported by profuse experimental studies, including reverse genetics, enzymatic inhibitory studies, chemical and RNAi targeting, and metabolic profiling [21–23]. Moreover, *Pf*G6PD–6PGL (107 kDa) evolutionary and structurally differs notably from its human counterpart (hG6PD, 59 kDa), since it combines G6PD and 6PGL activities into a single protein [23–28]. *Pf*G6PD–6PGL also differs from the human enzyme in substrate affinity and kinetic mechanism [23,24,29]. However, the lack of detailed 3D atomic information has precluded the development of target-based rational design of inhibitors, even though high throughput screening studies have led to the identification of a family of selective inhibitors, which are active at the submicromolar range and competitive with respect to G6P (Fig. 1) [30–32].

Malaria parasites have exerted selective pressure on the cellular phenotype of human erythrocytes, driving to the strongest known evolutionary adaptation behind sickle-cell trait, thalassemia, G6PD deficiency, and other erythrocyte pathologies that coexist in areas where malaria is present [33,34]. Concurrently, G6PD deficiency in the human host provides some degree of tolerance against malaria, partially protecting from severe clinical manifestations [35]. G6PD deficiency is an X-linked recessive hereditary disorder in RBC caused by missense mutations at the housekeeping G6PD gene. More than 160 mutations have been described for this deficiency, giving rise to clinical phenotype from mild to severe dysfunction of the red cells [12,13]. Polymorphic distribution of G6PD-deficient alleles in different malaria endemic areas -with over 400 million people carrying polymorphic variants-supports the original malaria protection hypothesis [36–40], which is suggested to be the consequence of natural selection processes [12–14].

The association of the RBC redox homeostasis maintenance, the polymorphic G6PD selection in human populations to protect against malaria, and the biological significance of *Pf*G6PD–6PGL in the parasite cycle and in response to oxidative stress, make *Pf*G6PD–6PGL a promising target for the development of novel antimalarial drugs. Hence, we describe here the first 3D structural model of the G6PD domain of *Pf*G6PD–6PGL, which has unveiled a critical difference in the substrate binding site compared to the hG6PD enzyme. To validate this structural model, a prospective study has been carried out, involving the synthesis of a series of substrate analog-based inhibitors, which have been rationally designed on the basis of the homology model, and their biological evaluation, including enzymatic inhibition assays against *Pf*G6PD and hG6PD enzymes, phenotypic assays in cultured *P. falciparum*, and the assessment of their cytotoxic activities against a mammalian cell line.

2. Results and discussion

2.1. 3D structural model of PfG6PD

In order to build a 3D model of the Plasmodium enzyme, the sequence homology of *Pf*G6PD was compared with the sequences of the G6PD enzyme from human, *Mycobacterium avium*, *Trypanosoma cruzi*, and *Leuconostoc mesenteroides*, taking advantage of the availability of X-ray structures of the enzymes of these organisms (Supplementary Material Table S1), using the multiple alignment COBAL tool [41] implemented in BLAST [42,43].

The multiple alignment of the G6PD sequences of these four organisms revealed sequence similarities in the range of 33–50% and 50–67% considering both identities and conservative changes between residues, respectively (Supplementary Material Fig. S1). Nevertheless, there is a high structural resemblance in the 3D fold of the protein skeleton of these proteins, as it can be stated from the structural superposition of the X-ray structures (Fig. 2A). This structural analysis also revealed the large resemblance between the residues that are directly implicated in the binding of the substrate G6P (Fig. 2B). As expected from the negative charge of G6P, the binding of the phosphate group is assisted by interactions with positively charged residues, such as Lys205 and Arg365, and hydrogen-bond interactions with His201 [44,45]. On the other

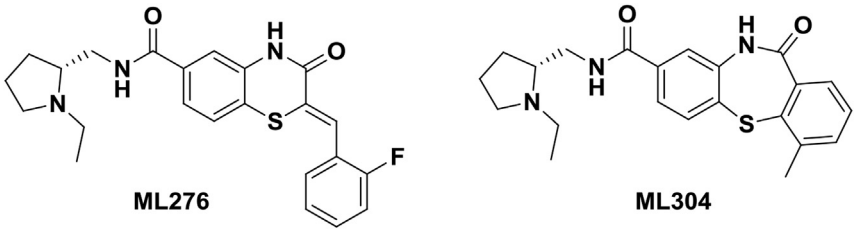

Fig. 1. Chemical structures of the selective *Pf*G6PD inhibitors ML276 and ML304.