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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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**Highlights**

The first structure of *P. falciparum* glucose-6-phosphate dehydrogenase is reported.

A key difference in the binding pocket of Plasmodium and human enzymes has been found.

A series of substrate analogs was designed as inhibitors of the Plasmodium enzyme.

They target selectively the Plasmodium enzyme with moderate potency and low toxicity.

The 3D structural model paves the way for the design of novel antimalarial drugs.

**Keywords:** malaria, glucose-6-phosphate dehydrogenase, *Plasmodium falciparum*, homology modeling, selective inhibitors, drug design.
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*<sub>i</sub> up to 23 µM), favorable selectivity (up to >26-fold), and low cytotoxicity. Phenotypic assays with *P. falciparum* cultures revealed high micromolar IC<sub>50</sub> 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 (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 (PfG6PD–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], PfG6PD–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, PfG6PD–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]. PfG6PD–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].

![Fig. 1. Chemical structures of the selective PfG6PD inhibitors ML276 and ML304.](image-url)
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 PfG6PD–6PGL in the parasite cycle and in response to oxidative stress, make PfG6PD–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 PfG6PD–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 PfG6PD 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 PfG6PD 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 Figure 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 hand, the hydroxyl groups of the glucose moiety participate in hydrogen bonds with several residues in the binding pocket, such as Asp258, Glu239 and Lys360, which in turn are also bound through salt bridges.
**Fig. 2.** Representation of selected X-ray structures of G6PD enzymes that contain G6P in the substrate-binding site from *H. sapiens* (PDB ID 2BHL and 5UKW), *T. cruzi* (PDB ID 5AQ1 and 4EM5), and *L. mesenteroides* (PDB ID 1E77 and 1E7Y). A) Superposition of the protein backbone (grey cartoon). G6P is shown as sticks (carbon atoms coloured in green). B) Superposition of key residues involved in the binding of G6P in these proteins. Numbering of residues in the human enzyme (see Table 1 for correspondence with residues in the other proteins).

The functional relevance of these residues is supported by the high degree of conservation between the enzymes of the distinct organisms (Table 1 and Supplementary Material Fig. S1), as the only alteration is the conserved replacement of Arg365 in *H. sapiens* (Arg408 in *T. cruzi*) by Lys344 in *L. mesenteroides*, thus enabling the electrostatic stabilization of the substrate-bound state via the formation of a salt bridge with the G6P phosphate group. Another difference concerns residue Gln395 (Gln437 in *T. cruzi*), which is replaced by Asp375 in *L. mesenteroides*, but this residue, which does not form a direct interaction with G6P, appears to be implicated in assisting
the proper arrangement of Lys205 in the human enzyme (Lys183 in \textit{L. mesenteroides}). Overall, the conservation of the residues involved in substrate binding accounts for the similar $K_M$ values determined for human (52 $\mu$M \cite{27}; 72 and 69 $\mu$M in RBC and recombinant G6PD, respectively \cite{28}) and \textit{L. mesenteroides} enzymes (69 and 114 $\mu$M with NAD$^+$ and NADP$^+$, respectively \cite{46,47}).

**Table 1**


<table>
<thead>
<tr>
<th>\textit{H. sapiens}</th>
<th>\textit{T. cruzi}</th>
<th>\textit{M. avium}</th>
<th>\textit{L. mesenteroides}</th>
<th>\textit{P. falciparum}</th>
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<tbody>
<tr>
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<td>Glu285</td>
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<td>Lys403</td>
<td>Lys321</td>
<td>Lys339</td>
<td>Lys465</td>
</tr>
<tr>
<td><strong>Arg365</strong></td>
<td><strong>Arg408</strong></td>
<td>Lys326</td>
<td>Lys344</td>
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<tr>
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<td>Gln437</td>
<td>Asp359</td>
<td>Asp375</td>
<td>Gln779</td>
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</table>

In light of these considerations, the sequence of \textit{Pf}G6PD–6PGL was compared with the sequence of the four organisms mentioned above. The multiple alignment shows that the \textit{Pf}G6PD domain, which roughly corresponds to the range of residues 335-910, has similarities in the range of 28-44\% and 46-62\% for identities and positive changes,
respectively (Supplementary Material Fig. S2). Because these values are only slightly lower than the results obtained for the proteins of *H. sapiens*, *T. cruzi*, *M. avium*, and *L. mesenteroides* (see above), it is reasonable to expect that the overall fold of the G6PD domain should resemble the common 3D structure of the four known proteins. Furthermore, there is a close conservation of the residues implicated in G6P binding, but for Arg365 (human G6PD), which unexpectedly is aligned to Asp750 in *Pf*G6PD (Table 1 and Supplementary Material Fig. S2). Noteworthy, this specific change is also observed in the enzyme of other Plasmodium species (Supplementary Material Table S2 and Fig. S3).

Because the Arg365 → Asp750 mutation represents a drastic alteration in both the size and charged nature of the side chain between human and Plasmodium enzymes, a homology model of *Pf*G6PD was built using SWISSMODEL [48] in order to assess the potential structural effect on the binding of G6P. The 3D structural model was built using the X-ray structure of the human G6PD complexed to G6P (PDB ID 2BHL [27]) as template (Supplementary Material Table 1). The results depicted in Fig. S4 (Supplementary Material) led to a model with a global quality estimate of 0.64 (QMEAN Z-score of -3.36; a detailed list of the individual parameters that contribute to the QMEAN score is provided in Fig. S4) [49, 50]. These values can be explained by the existence of three insertions in the *Pf*G6PD sequence. The longest insertion involves 61 residues (Glu448-Pro508), whereas the other two correspond to shorter stretches (Leu400-Ser408 and Lys692-Glu700). However, these insertions are located in areas distinct from the substrate binding site, which suggests that they do not alter the spatial arrangement of the residues involved in G6P binding (Fig. 3). To further check this finding, an additional model was built after deletion of the inserted stretches in the *Pf*G6PD domain. The 3D model had improved global quality estimate (0.74) and
QMEAN Z-score (-2.09) (further information available in Supplementary Material Fig. S5). Note that these values compare well with the QMEAN Z-score obtained for the human enzyme (QMEAN Z-score of -2.10; Fig. S6 in Supplementary Material). Noteworthy, the structure of the substrate binding site remained unaltered, with the only exception of the replacement of Arg365 in hG6PD by Asp750 in PfG6PD (Fig. 3).

Fig. 3. Representation of the 3D structural model of PfG6PD. A) Superposition of the X-ray structure of hG6PD with G6P in the substrate binding site (PDB ID 2BHL; white cartoon), and the homology model built for PfG6PD (residues 335-910; light blue cartoon), and after elimination of insertion loops (dark blue cartoon). The inserted regions in PfG6PD are shown in orange. B) Superposition of key residues involved in the binding of G6P in these proteins. Numbering of residues in the human and Plasmodium enzymes are given in bold and italics, respectively.

After homology modeling, molecular dynamics (MD) simulations were used to assess the structural and energetic stability of G6P bound to both human and *P. falciparum*
enzymes. This was deemed necessary to ascertain the potential impact of the \text{Arg365} \rightarrow \text{Asp750} mutation on the binding of G6P, given the availability of experimental data for the two enzymes [25, 27, 28, 51]. The simulation run for the hG6PD-G6P complex was stable along the whole trajectory, as noted in RMSD values of 2.3 Å for the protein backbone, 1.5 Å for the residues that define the binding pocket, and 1.3 Å for G6P (Supplementary Material Fig. S7). The structural stability of the complex is also reflected in the resemblance between the arrangement of G6P in the X-ray structure and the last snapshot of the MD simulation (Fig. 4A).

As expected from the structural relaxation of the homology model, a larger RMSD value was obtained for the protein backbone in the first 30 ns of the trajectory run for \textit{P}fG6PD, but it remained stable for the rest of the simulation (Supplementary Material Fig. S7). It is important to point out that the residues in the binding pocket showed small fluctuations (RMSD of 1.7 Å), preserving the integrity of the binding site. With regard to the ligand, the glucose ring of G6P rearranges in the binding site after the first 20 ns, but then remains stable (RMSD of 2.9 Å), as noted in the superposition of the snapshots taken at 60, 80 and 100 ns (Fig. 4B). Noteworthy, despite the replacement of \text{Arg365} by \text{Asp750}, a dense network of interactions maintains G6P tightly bound in the binding pocket (Fig. 4B).

Finally, the binding affinity of G6P for the two enzymes was determined by using the Solvation Interaction Energy (SIE) method [52], which is a MM/PBSA approach with weighting factors for the free energy components that were parameterized to reproduce the experimental binding affinities for a diverse set of protein-ligand complexes. The results point out that G6P binds both human and \textit{P. falciparum} enzymes with similar binding affinities (i.e., -7.4 and -7.7 kcal/mol; Supplementary Material Table S3). This finding is in agreement with the slightly larger $K_M$ determined experimentally for G6P.
upon binding to PfG6PD, which has been estimated to be 11-27 μM [25, 51], compared to the human enzyme (\(K_M\) of 52-72 μM [27, 28]), which gives confidence to the structural model of the PfG6PD-G6P complex.

Fig. 4. Representation of sampled structures of the complexes between human and P. falciparum G6PD enzymes with G6P. A) Superposition of the last MD snapshot with the X-ray structure (PDB ID 2BHL) for the hG6PD-G6P complex. B) Representation of MD snapshots taken at 60, 80 and 100 ns from the MD trajectory run for the PfG6PD-G6P complex. Average values of selected distances between G6P and residues in the binding pocket are shown in bold (Å; standard deviation given in parenthesis).

2.2. Chemistry

Keeping in mind the local difference in the substrate binding site model afforded by the Arg365 → Asp750 mutation between hG6PD and PfG6PD, it may be inferred that replacement of the phosphate group of the substrate G6P by a short side chain terminated with a basic functionality, protonatable at physiological pH, should be a
useful strategy to inhibit selectively the \( P.f \) G6PD enzyme. Such a chemical modification should enable the formation of an ionic interaction with Asp750 in \( P.f \) G6PD, whereas the enlarged size and the positive charge of the protonatable group should be detrimental for the interaction with Arg365 in the human enzyme, thereby making it possible the selective inhibition of the parasite enzyme. It may be expected that the success of this strategy, especially regarding the selective inhibition of \( P.f \) G6PD versus the human enzyme, will be dictated by the nature of the protonatable basic moiety, which should interact with Asp750, and by the linker that connects the glucose unit and the protonatable group, which should permit the proper arrangement of these groups in the substrate binding site.

Accordingly, taking advantage of the 3D model of \( P.f \) G6PD, we envisaged the synthesis of several substrate (G6P) analogs in which the phosphate group was replaced by different hydrogen bond donor and/or acceptor groups, namely thioether or sulfone (Scheme 1) or sulfonamide (Scheme 2), with short side chains terminating with protonatable amino or guanidino groups, to exploit the putative selective interaction with the negatively charged \( P.f \) G6PD Asp750 residue. For comparison purposes, some nitrile precursors, i.e. nitriles 4 and 6 (Scheme 1), were also subjected to biological evaluation.

During the enzymatic reaction catalyzed by G6PD, the anomeric position of the substrate G6P is oxidized by proton abstraction from the hydroxyl group to form a carbonyl group (in 6-phosphogluconolactone), with the departure of the anomeric hydrogen atom as a hydride that is transferred to the nicotinamide ring of the cofactor NADP\(^+\), which is thereby reduced to NADPH. To avoid the possibility of being transformed by G6PD, generating NADPH, and to avoid glucopyranoside ring opening and, hence, the potential epimerization at the anomeric position, the anomeric hydroxyl
group of the substrate G6P was replaced by an $\alpha$-methoxy group in the target inhibitors. Although this may be somewhat detrimental for the binding affinity of the designed inhibitors compared to the unsubstituted glucose moiety, this chemical modification was deemed convenient in order to increase the stability of the target inhibitors.

The synthesis of thioether derivatives 2–5 and sulfones 6–8 was undertaken starting from the known chloroderivative 1 [53], which was readily prepared by reaction of commercially available methyl $\alpha$-D-glucopyranoside with methanesulfonyl chloride in DMF at 65 ºC [54]. Amine 2, a compound recently described as an intermediate for the preparation of glycoclusters [55], was prepared in quantitative yield by nucleophilic substitution reaction of chloroderivative 1 with cysteamine (Scheme 1). The novel guanidine 3 was synthesized from amine 2 upon reaction with 1H-pyrazole-1-carboxamidine hydrochloride and Et$_3$N in refluxing acetonitrile, and isolated in the form of its hydrochloride salt.
Scheme 1. Synthesis of thioether and sulfone derivatives 2–8. Reagents and conditions: (i) cysteamine hydrochloride, NaH, DMF, 0 ºC, 10 min; then, 1, room temperature, 48 h, quantitative yield; (ii) 1H-pyrazole-1-carboxamidine hydrochloride, Et$_2$N, acetonitrile, reflux, 6 h to overnight; then, HCl/MeOH, 39% yield (3·HCl), 62% yield (8·HCl); (iii) 3-mercaptopropanenitrile, NaH, DMF, 0 ºC, 10 min; then, 1, room temperature, 72 h, quantitative yield; (iv) H$_2$ (16 atm), Raney-Ni, MeOH, room temperature, overnight, 41% yield (5), 28% yield (7); (v) m-chloroperbenzoic acid, NaHCO$_3$, CH$_2$Cl$_2$, 0 ºC; then, room temperature, 3 h, quantitative yield.

Reaction of chloroderivative 1 with the thiolate formed by deprotonation of 3-mercaptopropanenitrile with NaH in DMF afforded the novel cyanothioether 4 in quantitative yield, from which the novel thioether 5 and sulfones 6–8 were subsequently synthesized following standard procedures (Scheme 1). Thus, the aminothioether 5 was synthesized in moderate yield upon hydrogenation at 16 atm of cyanothioether 4 using Raney-Ni as the catalyst. M-Chloroperbenzoic acid oxidation of 4 gave in quantitative yield the corresponding cyanosulfone, 6, from which the aminosulfone 7 was synthesized by Raney-Ni-catalyzed hydrogenation. Treatment of 7 with 1H-pyrazole-1-carboxamidine hydrochloride and Et$_3$N in refluxing acetonitrile afforded the guanidinosulfone 8 in 68% yield.

For the synthesis of the novel amino- and guanidinosulfonamides 11 and 12, the known methyl 6-amino-6-deoxy-α-D-glucopyranoside, 9 [54], was used as starting material (Scheme 2). In turn, amine 9 was readily synthesized by reaction of chloroderivative 1 with potassium phthalimide in DMF at 100 ºC for 7 days, followed by hydrazinolysis. Reaction of amine 9 with 2-phthalimidoethanesulfonyl chloride [56], previously prepared by reaction of taurine with phthalic anhydride, followed by reaction with PCl$_5$,
gave in excellent yield the phthalimidosulfonamide 10. Hydrazinolysis of 10 in refluxing MeOH afforded aminosulfonamide 11, in low yield, after two tedious silica gel column chromatography purifications. Finally, aminosulfonamide 11 was converted into the corresponding guanidinosulfonamide, 12, under the usual guanidinylation conditions (Scheme 2).

Scheme 2. Synthesis of sulfonamido derivatives 11 and 12. Reagents and conditions: (i) 2-phthalimidoethanesulfonyl chloride, Et₃N, DMF, 0 ºC; then, room temperature, 6 days, 96% yield; (ii) hydrazine monohydrate, MeOH, reflux, overnight, 29% yield; (iii) 1H-pyrazole-1-carboxamidine hydrochloride, Et₃N, acetonitrile, reflux, 6 h; then, HCl/MeOH, 29% yield.

2.3. Biological profiling

The novel compounds designed to differentially bind at the G6P site of the catalytic pocket of the PfG6PD–6PGL enzyme were tested for in vitro inhibition of the parasite enzyme and the homologous human enzyme. Four out of the 9 tested compounds displayed a considerably higher affinity towards the parasite enzyme, i.e. lower $K_{i}^{G6P}$ values in the parasite enzyme than in the human enzyme. Thus, compounds 2, 3, 6, and 11 showed $K_{i}^{G6P}$ values between 23 and 76 μM in the parasite dual enzyme, but > 200 μM to > 2000 μM in the human enzyme (Table 2). Conversely, the human enzyme was
significantly inhibited by compounds 7, 8, and 12 at $K_i^\text{G6P}$ values below 40 μM, very similar or even lower to the $K_i^\text{G6P}$ values obtained for the malaria parasite enzyme.

As expected, those compounds that feature a terminal protonatable amino or guanidino group display higher affinities towards $Pf$G6PD than those with a nonbasic cyano group, and among them, amino derivatives are more selective than guanidino derivatives for $Pf$G6PD over hG6PD. Regarding the inner functionality of the side chain at position 6, the affinity towards $Pf$G6PD follows the order sulfonamide > sulfone > thioether, with the sulfonyl oxygen atoms of sulfonamides and sulfones likely mimicking the hydrogen acceptor capability of the phosphate oxygen atoms of the substrate G6P.

### Table 2

Affinity values toward $Pf$G6PD and hG6PD and antiplasmodial activity of the novel compounds$^a$

<table>
<thead>
<tr>
<th>compound</th>
<th>$K_i^{\text{G6P}}$ (μM)</th>
<th>$K_i^{\text{G6P}}$ (μM)</th>
<th>SI$^b$</th>
<th>IC$_{50}$ (mM)</th>
<th>$P. falciparum$</th>
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<td>6</td>
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<td>7</td>
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</tbody>
</table>

$^a K_i$ values are those of the forward enzymatic reaction using G6P as non-saturating substrate (between 10 and 300 μM) and saturating NADP concentrations (200 μM)
using the purified parasite ($K_i^{G6PD}$ [PfG6PD–6PGL]) or human ($K_i^{G6PD}$ [hG6PD]) enzyme. IC$_{50}$ values were obtained in triplicate in *P. falciparum* cultures in human erythrocytes. IC$_{50}$ values were obtained in triplicate in *P. falciparum* cultures in human erythrocytes.

b SI: selectivity index = $K_i^{G6PD}$ [hG6PD] / $K_i^{G6PD}$ [PfG6PD–6PGL].

Kinetic studies performed for compounds 2 and 11 using either PfG6PD or hG6PD enzymes, a fixed concentration of NADP (200 μM) and variable concentrations of G6P and vice versa, have shown that these compounds are competitive with the substrate G6P, but not with NADP$^+$. Indeed, regarding the parasite enzyme, the $K_i^{G6PD}$ values are clearly lower than the $K_i^{NADP}$ values (76 μM vs 364 μM for compound 2; 23 μM vs 269 μM for compound 11). The same trend is found for hG6PD, with compound 11 displaying $K_i^{G6PD}$ and $K_i^{NADP}$ values of 440 μM and 1.26 mM, respectively, whereas in compound 2 both $K_i$ values were above 2.0 mM.

Overall, the results point out that compounds 2 and 11, both bearing a terminal amino group, connected through 4-mer and 5-mer tethers to the glucose ring, respectively, fit the empty room generated by the Arg365 → Asp750 mutation in the binding pocket, leading to an effective salt bridge interaction with Asp750 in PfG6PD. At this point, it is worth noting that the $K_i$ obtained for these compounds (76.1 and 22.8 μM for 2 and 11, respectively; Table 2) compares well with the $K_M$ of G6P in PfG6PD, which varies between 11 and 27 μM [25, 51]. This can be realized from a similar binding of the glucose moiety in the substrate binding cavity, and by the replacement of the salt bridge between the phosphate group of G6P and the guanidinium moiety of Arg365 by the interaction formed between the protonated amino group of 2 and 11 with the carboxylate group of Asp750.

The *in vivo* phenotypic assay of the novel compounds on *P. falciparum* cultures showed curves clearly indicating a dose response effect, although with IC$_{50}$ values in the high micromolar range from 330 μM to 1.75 mM (Table 2). To trace the targeting of the
inhibitors on the *P. falciparum* intraerythrocytic cycle, the phenotype of the parasites growing in the presence of some selected inhibitors (3, 4, 6, and 12) was analysed at 2 mM (Fig. 5). Interestingly, these G6PD–6PGL inhibitors showed a full effect in killing the parasites since upon 48 h incubation most parasite forms showed unviable pyknotic forms, mostly in mature stages (trophozoites). This result suggests a different stage of action of these compounds relative to that of chloroquine, which kills the parasite at the stage of rings, the immature form, i.e. earlier than the new inhibitors. This is in agreement with the increased oxidative stress along the developmental erythrocytic cycle of the parasite [56], where inhibition of *Pf*G6PD–6PGL impedes to cope with the ROS generation upon heme release, leading to its death at mature stages.

**Fig. 5.** Phenotypic analysis of *P. falciparum* intraerythrocytic cycle upon exposure to compounds 3, 4, 6, and 12 in comparison to chloroquine (CQ) and a control (C). *In vitro* culture of *P. falciparum* during a complete erythrocytic cycle (48 h) was performed starting from young rings and incubated with the G6PD–6PGL inhibitors at 2 mM concentration and CQ at 10 μM in parallel to a control culture in the absence of inhibitors.
Cytotoxicity of the novel G6PD–6PGL inhibitors towards mammalian cells was also assayed using HEPG2 human hepatoma cells and concentrations of the inhibitors between 0.2 and 2.0 mM (Fig. 6). The novel compounds did not lead to significant loss of viability of HEPG2 human hepatoma cells, which showed percentages of survival above 80% for the range of inhibitor concentrations between 0.2 and 1.0 mM. Moreover, most of the inhibitors showing selectivity towards PfG6PD, with $K_i^{G6P}$ values for the human enzyme above 200 μM (2, 3, 4, 6, and 11) still maintained these viability values at the highest tested concentration of 2 mM. In contrast, those inhibitors showing a higher selectivity towards hG6PD, with $K_i^{G6P}$ values for the human enzyme below 200 μM (5, 7, 8, and 12) decreased cell viability below 80% at 1.5 and 2 mM concentrations.

![Fig. 6. Cytotoxicity assays of the novel G6PD–6PGL inhibitors. HEPG2 human hepatoma cells were incubated with the inhibitors at five different concentrations (0.2, 0.5, 1.0, 1.5, and 2.0 mM). Viability of the HEPG2 human hepatoma cells after 24 h is](image-url)
plotted in comparison with 100% viability of control cultures in the absence of inhibitors.

2.4. Binding mode of ML276

To further calibrate the reliability of the 3D structural model of PfG6PD, we attempted to identify the potential binding mode of compound ML276. To the best of our knowledge, ML276 and ML304 (Fig. 1) are the only compounds that have been found to inhibit PfG6PD through a competitive mechanism with the substrate, with IC$_{50}$ values of 0.9 and 0.19 µM, respectively. Furthermore, they are selective inhibitors of PfG6PD (IC$_{50}$ > 80 µM for the human enzyme). However, further development of novel drug candidates has been limited by the lack of information about the binding mode in PfG6PD.

Keeping in mind the selective and competitive inhibition of ML276 and ML304, it may be expected that they must bind to the enzyme in a similar way, reflecting their structural resemblance. Furthermore, we hypothesize that the PfG6PD/hG6PD selectivity arises from the interaction between the protonated tertiary amine of these compounds and Asp750. Assuming this hypothesis, we performed a computational study that combined guided docking and refinement by MD simulations in order to find a stable binding mode for ML276 in the substrate-binding pocket of PfG6PD (see Experimental section). The proposed binding mode (Fig. 7) retained the structural integrity along an unbiased 0.5 µs MD simulation, maintaining the interaction with the carboxylate group of Asp750, which formed stable hydrogen-bond contacts with the protonated amine and the amide NH groups of ML276. On the other hand, the fluorobenzene ring was firmly bound in a hydrophobic pocket shaped by Val585 and
Leu778. It is worth noting that this binding mode would impede the binding of the substrate due to the severe steric clash between the fused ring of ML276 and the phosphate group of G6P, which would justify the competitive inhibition by this compound.

**Fig. 7.** Representation of the putative binding mode of ML276 to the substrate binding cavity of *Pf*G6PD. A) Superposition of the *Pf*G6PD/ML276 complex and the X-ray structure G6P-bound hG6PD (PDB ID 2BHL). The protein backbone is shown as light blue cartoon, and selected residues are shown as blue sticks. ML276 and G6P are represented using sticks (with carbon atoms shown in green and white, respectively). Selected distances are shown as dashed line (average value in Å, and standard deviation...
in parenthesis are given in italics). B) Superposition of the ML276 orientation sampled at 0.1, 0.2, 0.3, 0.4, and 0.5 μs along the MD simulation. C) Time (μs) evolution of the RMSD (Å) of the residues in the binding pocket (black), and the ligand (green). D) Time (μs) evolution of the distances (Å) from the carboxylate oxygen of Asp750 to the protonated amine (red) and amide NH group (blue) of ML276.

3. Conclusions

Selective inhibition of the PfG6PD enzyme has recently emerged as a promising novel therapeutic approach, which is expected to derive new drugs that may circumvent one of the main drawbacks of the current antimalarial drugs, namely the development of resistance. In the absence of experimental 3D structural information of PfG6PD-6PGL, we have provided proof of concept for the selective substrate competitive inhibition of PfG6PD as a promising therapeutic approach. To this end, we have built a homology model with the aim of boosting the rational design of novel inhibitors. Unexpectedly, the homology model of PfG6PD revealed the presence of Asp750 instead of the arginine residue (Arg365), which is present in the equivalent position in the human enzyme, thus paving the way for the design of substrate analogs that selectively inhibit PfG6PD. In agreement with this hypothesis, introduction at position 6 of the glucose moiety of a short chain terminating with a basic amino or guanidino group has led to inhibitors with moderate two-digit micromolar affinity and up to > 26-fold selectivity for PfG6PD over hG6PD. These compounds exhibit a competitive inhibition mechanism with regard to the substrate G6P but not to NADP⁺, and are nontoxic to mammalian cells up to a concentration as high as 2 mM. In phenotypic assays with cultured P. falciparum parasites, the compounds exhibit submillimolar to low millimolar IC₅₀ values, reflecting a poor permeation across cell membranes as a
consequence of their high polarity. Interestingly, these compounds seem to act at a different stage of the parasite development than chloroquine, killing the parasites at the mature stages instead of at the earlier ring stage. Overall, these findings provide a basis for the application of target-based drug design techniques that drive the design of novel selective PfG6PD inhibitors, with improved physicochemical properties for ameliorating the pharmacokinetic profile, and valuable for combination therapies with sequential action at different stages of the parasite life cycle.

4. Experimental section

4.1. Molecular modeling

Multiple sequence alignments were performed using the COBALT tool [41] as implemented in BLAST [42, 43] using the protein sequences taken from the Uniprot database [59]. To this end, the sequences of human (Uniprot ID: P11413, 515 residues), T. cruzi (Uniprot ID: Q4E0B2, 555 residues), M. avium (Uniprot ID: A0A0H3A0Q9, 469 residues), and L. mesenteroides (Uniprot ID: P11411, 486 residues) G6PD enzymes were used as reference, due to the availability of X-ray structures in the apo form or bound to the substrate (G6P) and/or to the NADP$^+$ cofactor (Supplementary Material Table S1). On the other hand, besides the sequence of PfG6PD–6PGL enzyme (Uniprot ID: Q8IKU0, 910 residues), the sequences of other Plasmodium G6PD–6PGL enzymes were also examined (P. reichenowi: Uniprot ID: A0A060S5P3, 907 residues; P. malariae: Uniprot ID: A0A1A8WAC7, 891 residues; P. knowlesi: Uniprot ID: A0A1A7vNJ6, 914 residues; P. vivax: Uniprot ID: A0A1G4HH51, 927 residues; P. yoelii yoelii: Uniprot ID: Q7RRD8, 949 residues; P. chabaudi chabaudi: Uniprot ID: A0A1C6XJL2, 946 residues).
Homology modeling was used to build up the 3D structure of PfG6PD. The FASTA sequence of PfG6PD was obtained from the Uniprot database with code Q8IKU0. To this end, only the ~570 residues of the PfG6PD–6PGL enzyme were used to build up the 3D structural model, in conjunction with the human enzyme (PDB code: 2BLH [27], containing D-glucose-6-phosphate), which was found to be a suitable template according to the homology modeling analysis performed with SWISSMODEL.

4.2. Molecular dynamics

Classical MD simulations were performed with the CUDA-accelerated version of the PMEMD module as implemented in AMBER12 [60] software package. The protein and substrate were simulated using the parm99SBILDN [61, 62] and glycam06 [63] force fields. Each enzyme-G6P complex was immersed in an octahedral box of TIP3P [64] water molecules, and the systems were neutralized by adding the proper number of counter ions [65] to neutralize the overall charge of the simulated system. To this end, titration calculations were previously performed with PROPKA3.1 [66] in order to verify the ionization state of the ionizable residues in the protein, and the potential effect exerted by G6P binding on the residues in the binding pocket. The results revealed that the presence of G6P had no effect on the ionization state of the residues for hG6PD. However, the pK\textsubscript{a} of His578 was increased to ~7.7 upon G6P binding to PfG6PD, an effect that may be justified by the Arg365 (hG6PD) \rightarrow Asp750 (PfG6PD) mutation, and the concomitant loss of the electrostatic influence exerted by the positive charge of Arg365 on the binding of the negative charges of the phosphate group in G6P. Accordingly, His578 was simulated in the protonated state in MD simulations of the PfG6PD-G6P complex (let us note that additional MD simulations performed for this complex with His578 in the neutral state revealed that binding of G6P was not stable, and that ligand was released from the binding pocket; data not shown).
Prior to the production runs, the energy of each system was minimized, and then the system was equilibrated. First, water molecules and counterions were refined through 3000 steps of steepest descent algorithm and 7000 steps of conjugate gradient. Then, the position of hydrogen atoms was optimized using 500 steps of steepest descent algorithm and 4500 steps of conjugate gradient. At the third stage, hydrogen atoms, water molecules, and counterions were further optimized using 2500 steps of steepest descent algorithm and 11500 steps of conjugate gradient. Thermalization of the system was performed in five steps of 50 ps, increasing the temperature from 50 to 298 K at constant volume. The residues that define the binding site and G6P were restrained during thermalization using a variable restraining force. Thus, a force constant of 10 kcal mol$^{-1}$ Å$^{-2}$ was used in the first stage of the thermalization and was subsequently decreased by increments of 2 kcal mol$^{-1}$ Å$^{-2}$ in the next stages. The density of the system was equilibrated in a subsequent 250 ps run using the NPT (298 K, 1 bar) ensemble. Finally, 100 ns production runs were performed for the two systems. SHAKE was used for those bonds containing hydrogen atoms in conjunction with a time step of 2 fs. Periodic boundary conditions were used with particle mesh Ewald for the treatment of long electrostatic interactions, and a cutoff of 10 Å for nonbonded interactions.

The solvent interaction energies (SIE) technique developed by Purisima and co-workers [52] was used to estimate the interaction free energies between G6P and both human and *P. falciparum* enzymes. In order to check the consistency of the interaction free energies, calculations were performed for three 20 ns windows along the last 40 ns of the production trajectory. For each window, a set of 200 snapshots were used in SIE computations.

4.3. *Binding mode of ML276*
Exploration of the putative binding mode of ML276 was accomplished by using an elaborate protocol that combined docking and restrained MD simulations, as shown in Scheme 3.

Briefly, the ligand was docked in the substrate-binding cavity with Glide [67]. Docking was performed using an inner/outer box of 15/30 Å, and the contact between the protonated amine of the ligand and the carboxylate group of Asp750 as pharmacophoric restraint. The best pose was then used to build the ligand-bound complex following the same procedure described above. Each system was refined by energy minimization, thermalized at 400 K, and simulated using restrained MD simulations (20 ns) at 400 K by imposing a restraint (10 kcal mol\(^{-1}\) Å\(^{-2}\)) to the distance between the ligand's amine nitrogen and the Asp750 carboxylate carbon atom. Five snapshots (taken at the end of the equilibration step, and at simulation times of 5, 10, 15, and 20 ns) were then used for
the preparation of simulation systems that were thermalized at 300 K, and subsequently subjected to gradual reduction of the restraint in 5 ns MD steps. Finally, the structural stability of the most favorable binding mode was examined using a 0.5 μs unrestrained MD simulation.

4.4. Chemistry. General methods

Melting points were determined in open capillary tubes with a MFB 595010M Gallenkamp melting point apparatus. 400 MHz 1H/ 100.6 MHz 13C NMR spectra were recorded on a Varian Mercury 400 spectrometer at the Centres Científics i Tecnologics of the University of Barcelona (CCiTUB). The chemical shifts are reported in ppm (δ scale) relative to solvent signals (CD3OD at 3.31 and 49.0 ppm in the 1H and 13C NMR spectra, respectively), and coupling constants are reported in Hertz (Hz). Assignments given for the NMR spectra of the new compounds have been carried out by comparison with the NMR data of compounds 4, 6, and 10, which in turn, were assigned on the basis of DEPT, COSY 1H/1H (standard procedures), and COSY 1H/13C (gHSQC and gHMBC sequences) experiments. IR spectra were run on a Perkin Elmer Spectrum RX I spectrophotometer. Absorption values are expressed as wavenumbers (cm⁻¹); only significant absorption bands are given. Optical rotations were measured on a PerkinElmer model 241 polarimeter. Column chromatography was performed on silica gel 60 AC.C (40–60 mesh, SDS, ref 2000027). Thin-layer chromatography was performed with aluminium-backed sheets with silica gel 60 F254 (Merck, ref 1.05554), and spots were visualized with UV light and 1% aqueous solution of KMnO4. High resolution mass spectra were carried out at the CCiTUB with a LC/MSD TOF Agilent Technologies spectrometer. Due to the hygroscopic nature of most of the target
compounds, they were stored under drying conditions in a vacuum oven before preparation of the samples for biological assays.

4.4.1. Methyl 6-[(2-aminoethyl)thio]-6-deoxy-α-D-glucopyranoside (2)

A solution of cysteamine hydrochloride (235 mg, 2.07 mmol) in anhydrous DMF (9 mL) was cooled at 0 °C with an ice bath, and then treated portionwise with NaH (60% dispersion in oil, 166 mg, 4.14 mmol). After stirring the mixture for 10 min, chloroderivative 1 (400 mg, 1.88 mmol) was added. The reaction mixture was stirred at room temperature for 48 h and evaporated under reduced pressure to give a yellow solid (877 mg), which was purified through column chromatography (40–60 μm silica gel, CH₂Cl₂ / MeOH / 50% aq. NH₄OH mixtures, gradient elution). On elution with CH₂Cl₂ / MeOH / 50% aq. NH₄OH 80:20:0 to 75:25:1, amine 2 (508 mg, quantitative yield) was isolated as a beige very hygroscopic solid; Rf 0.04 (CH₂Cl₂ / MeOH 7:3).

2·HCl: [α]²⁰D = + 74 (c 0.88, MeOH); IR (ATR) ν 3500–2500 (max at 3221, 2907, 2839, OH, ’NH, CH st) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.74 (dd, J = 14.4 Hz, J’ = 8.0 Hz, 1H, 6-Ha), 2.88 (dd, J = 14.0 Hz, J’ = 7.6 Hz, 1H, 1’-Ha), 2.92 (dd, J = 14.0 Hz, J’ = 7.6 Hz, 1H, 1’-Hb), 3.00 (dd, J = 14.4 Hz, J’ = 2.0 Hz, 1H, 6-Hb), 3.16 (t, J = 6.8 Hz, 2H, 2’-H₂), 3.25 (dd, J = J’ = 9.6 Hz, 1H, 4-H), 3.39 (dd, J = 9.6 Hz, J’ = 3.6 Hz, 1H, 2-H), 3.43 (s, 3H, 1-OCH₃), 3.59 (dd, J = J’ = 9.6 Hz, 1H, 3-H), 3.68 (ddd, J = 9.6 Hz, J’ = 8.0 Hz, J” = 2.0 Hz, 1H, 5-H), 4.66 (d, J = 3.6 Hz, 1H, 1-H), 4.85 (s, OH, ’NH₃); ¹³C NMR (100.6 MHz, CD₃OD) δ 31.1 (CH₂, C1’), 34.2 (CH₂, C6), 39.9 (CH₂, C2’), 55.7 (CH₃, 1-OCH₃), 73.50 (CH), 73.54 (CH) (C2, C5), 74.3 (CH, C4), 74.9 (CH, C3), 101.3 (CH, C1); HRMS (ESI), calcd for (C₉H₁₀NO₅S + H⁺) 254.1057, found 254.1056.

4.4.2. Methyl 6-deoxy-6-[(2-guanidinoethyl)thio]-α-D-glucopyranoside (3)
To a suspension of amine 2 (77 mg, 0.30 mmol) in acetonitrile (3 mL), Et₃N (0.04 mL, 29 mg, 0.29 mmol) and 1H-pyrazole-1-carboxamidine hydrochloride (58 mg, 0.40 mmol) were added. The reaction mixture was stirred under reflux overnight and cooled to room temperature. The solvent was poured off and the precipitate was dried in vacuo. The resulting brown sticky solid (46 mg) was taken in MeOH (5 mL), treated with methanolic HCl (0.5 N, 5 mL), and filtered through a 0.2 μm NYL filter. The filtrate was evaporated under reduced pressure, washed successively with CH₃CN (2 × 3 mL), CH₂Cl₂ (2 × 5 mL), and pentane (2 × 3 mL), and finally dried at 35 °C / 30 Torr, to provide guanidine 3·HCl (39 mg, 0.12 mmol, 39% yield) as a brownish hygroscopic solid; [α]20°D = + 72 (c 2.2, MeOH); IR (ATR) ν 3500‒2500 (max at 3303, 3173, 2918, OH, +NH, NH, CH) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.71 (dd, J = 14.4 Hz, J' = 8.0 Hz, 1H, 6-Hₐ), 2.81 (dt, J = 13.6 Hz, J' = 6.8 Hz, 1H, 1'-Hₐ), 2.86 (dt, J = 13.6 Hz, J' = 6.8 Hz, 1H, 1'-Hₐ), 3.00 (dd, J = 14.4 Hz, J' = 2.4 Hz, 1H, 6-Hₐ), 3.24 (dd, J = 9.6 Hz, J' = 9.2 Hz, 1H, 4-H), 3.39 (dd, J = 9.6 Hz, J' = 3.6 Hz, 1H, 2-H), 3.42 (t, J = 6.8 Hz, 2H, 2'-Hₐ), 3.43 (s, 3H, 1'-OCH₃), 3.59 (dd, J = 9.6 Hz, J' = 9.2 Hz, 1H, 3-H), 3.66 (ddd, J = 9.6 Hz, J' = 8.0 Hz, J'' = 2.4 Hz, 1H, 5-H), 4.65 (d, J = 3.6 Hz, 1H, 1-H), 4.84 (s, OH, NH, NH₂, +NH₂); ¹³C NMR (100.6 MHz, CD₃OD) δ 33.0 (CH₂, C1’), 34.5 (CH₂, C6), 42.1 (CH₂, C2’), 55.6 (CH₃, 1-OCH₃), 73.5 (CH, C2), 73.7 (CH, C5), 74.4 (CH, C4), 74.9 (CH, C3), 101.1 (CH; C1), 158.6 (C, guanidine C); HRMS (ESI), calcd for (C₁₀H₂₁N₃O₅S + H⁺) 296.1275, found 296.1279.

4.4.3. **Methyl 6-[(2-cyanoethyl)thio]-6-deoxy-α-D-glucopyranoside (4)**

A solution of 3-mercapto propanenitrile (646 mg, 7.41 mmol) in anhydrous DMF (12 mL) was cooled at 0 °C with an ice bath, and then treated portionwise with NaH (60% dispersion in oil, 296 mg, 7.40 mmol). After stirring the mixture for 10 min, chlorodervative 1 (700 mg, 3.29 mmol) was added. The reaction mixture was stirred at
room temperature for 72 h and evaporated under reduced pressure to give a yellow solid (2.05 g), which was purified through column chromatography (40–60 μm silica gel, CH₂Cl₂ / MeOH mixtures, gradient elution). On elution with CH₂Cl₂ / MeOH 92:8 to 90:10, nitrile 4 (891 mg, quantitative yield) was isolated as a beige solid; \( R_t \) 0.34 (CH₂Cl₂ / MeOH 9:1).

A solution of 4 (50 mg, 0.19 mmol) in MeOH (5 mL) was filtered through a 0.20 μm NYL filter and evaporated under reduced pressure. After washing the resulting solid successively with CH₂Cl₂ (2 × 5 mL) and pentane (2 × 3 mL), and drying at 35 °C / 30 Torr, the analytical sample of 4 (43 mg) was obtained as a yellowish hygroscopic solid; \([\alpha]_{D}^{20} = +143 \) (c 0.86, MeOH); IR (ATR) ν 3335, 3192 (OH st), 2251 (CN st) cm⁻¹; \(^1\)H NMR (400 MHz, CD₃OD) \( \delta \) 2.73 (dd, \( J = 14.4 \) Hz, \( J' = 8.0 \) Hz, 1H, 6-Hₐ), 2.78 (t, \( J = 7.2 \) Hz, 2H, 2′-H₂), 2.86 (dd, \( J = 13.6 \) Hz, \( J' = 7.2 \) Hz, 1H, 1′-Hₐ), 2.93 (dd, \( J = 13.6 \) Hz, \( J' = 7.2 \) Hz, 1H, 1′-Hₐ), 3.03 (dd, \( J = 14.4 \) Hz, \( J' = 2.4 \) Hz, 1H, 6-Hₐ), 3.24 (dd, \( J = J' = 9.6 \) Hz, 1H, 4-H), 3.40 (dd, \( J = 9.6 \) Hz, \( J' = 3.6 \) Hz, 1H, 2-H), 3.44 (s, 3H, 1-OCH₃), 3.58 (dd, \( J = J' = 9.6 \) Hz, 1H, 3-H), 3.66 (ddd, \( J = 9.6 \) Hz, \( J' = 8.0 \) Hz, \( J'' = 2.4 \) Hz, 1H, 5-H), 4.65 (d, \( J = 3.6 \) Hz, 1H, 1-H), 4.84 (s, OH); \(^{13}\)C NMR (100.6 MHz, CD₃OD) \( \delta \) 19.3 (CH₂, C2′), 29.9 (CH₂, C1′), 34.7 (CH₂, C6), 55.6 (CH₃, 1-OCH₃), 73.6 (CH, C2), 73.8 (CH, C5), 74.4 (CH, C4), 75.0 (CH, C3), 101.2 (CH, C1), 120.2 (C, CN); HRMS (ESI), calcd for (C₁₀H₁₇NO₅S + NH₄⁺) 281.1166, found 281.1166.

4.4.4. Methyl 6-[(3-aminopropyl)thio]-6-deoxy-α-D-glucopyranoside (5)

A mixture of nitrile 4 (213 mg, 0.81 mmol) and Raney-Ni (50% water, 300 mg) in MeOH (80 mL) was reacted with hydrogen (16 atm) at room temperature overnight in a Parr stirred reactor. The resulting suspension was filtered through Celite® and rinsed with MeOH (25 mL). The solvent was removed in vacuo to afford a brownish oil (205 mg), which was purified through column chromatography (40–60 μm silica gel, CH₂Cl₂
on elution with CH$_2$Cl$_2$ / MeOH / 50% aq. NH$_4$OH 75:25:1 to 65:35:1, amine 5 (88 mg, 41% yield) was isolated as a colourless sticky solid; $R_f$ 0.11 (CH$_2$Cl$_2$ / MeOH / 50% aq. NH$_4$OH 7:3:0.1).

A solution of 5 (40 mg, 0.15 mmol) in MeOH (5 mL) was filtered through a 0.20 μm NYL filter, treated with methanolic HCl (0.5 N, 1.35 mL) and evaporated under reduced pressure. The resulting solid was washed successively with CH$_2$Cl$_2$ (2 × 5 mL) and pentane (2 × 3 mL) to give, after drying at 35 ºC / 30 Torr, 5·HCl (44 mg) as a yellowish hygroscopic solid; $[\alpha]_{20}^{\text{D}} = +171$ (c 0.46, MeOH); IR (ATR) $\nu$ 3500–2500 (max at 3301, 2912, 2854, 2839, OH, +NH, CH st) cm$^{-1}$; $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 1.99 (tt, $J = J' = 8.0$ Hz, 2H, 2'-H$_2$), 2.66 (dd, $J = 14.0$ Hz, $J' = 8.0$ Hz, 1H), 2.68 (dd, $J = 14.0$ Hz, $J' = 8.0$ Hz, 1H), 2.73 (m, 1H) (6-H$_a$, 1'-Ha, 1'-H$_b$), 2.98 (dd, $J = 14.0$ Hz, $J' = 2.8$ Hz, 1H, 6-H$_b$), 3.13 (t, $J = 8.0$ Hz, 2H, 3'-H$_2$), 3.22 (dd, $J = 9.6$ Hz, $J' = 8.8$ Hz, 1H, 4-H), 3.38 (dd, $J = 9.6$ Hz, $J' = 3.6$ Hz, 1H, 2-H), 3.42 (s, 3H, 1-OCH$_3$), 3.58 (dd, $J = 9.6$ Hz, $J' = 8.8$ Hz, 1H, 5-H), 3.65 (br dd, $J = 9.6$ Hz, $J' = 8.0$ Hz, 1H, 5-H), 4.63 (d, $J = 3.6$ Hz, 1H, 1-H), 4.85 (s, OH, +NH$_3$); $^{13}$C NMR (100.6 MHz, CD$_3$OD) $\delta$ 27.1 (CH$_2$, C2'), 30.6 (CH$_2$, C1'), 34.4 (CH$_2$, C6), 47.9 (CH$_2$, C3'), 55.6 (CH$_3$, 1-OCH$_3$), 73.5 (CH, C2), 73.6 (CH, C5), 74.5 (CH, C4), 75.0 (CH, C3), 101.1 (CH, C1), HRMS (ESI), calcd for $\text{C}_{10}\text{H}_{21}\text{NO}_5\text{S} + \text{H}^+$ 268.1213, found 268.1214.

4.4.5. *Methyl 6-[(2-cyanoethyl)sulfonyl]-6-deoxy-α-D-glucopyranoside* (6)

A suspension of nitrile 4 (210 mg, 0.80 mmol) and NaHCO$_3$ (181 mg, 2.16 mmol) in CH$_2$Cl$_2$ (3.5 mL) was cooled at 0 ºC with an ice bath and treated with m-chloroperbenzoic acid (77% purity, 483 mg, 2.16 mmol). The reaction mixture was stirred at room temperature for 3 h and evaporated under reduced pressure to give a solid residue (726 mg), which was purified through column chromatography (40–60 μm silica gel, CH$_2$Cl$_2$ / MeOH mixtures, gradient elution). On elution with CH$_2$Cl$_2$ / MeOH / 50% aq. NH$_4$OH mixtures, gradient elution).
92:8 to 88:12, slightly impure cyanosulfone 6 (289 mg, quantitative yield) was isolated; 

$R_f$ 0.57 (CH$_2$Cl$_2$ / MeOH 8:2). A solution of 6 (21 mg, 0.07 mmol) in MeOH (5 mL) was filtered through a 0.20 μm NYL filter and evaporated under reduced pressure. The analytical sample of 6 (18 mg) was obtained by washing the resulting solid successively with CH$_2$Cl$_2$ (2 × 5 mL) and pentane (2 × 3 mL), and drying at 35 °C / 30 Torr; mp 151–153 °C; $[\alpha]^{20}_D = +127$ (c 0.38, MeOH); IR (ATR) ν 3378, 3308 (OH st), 2270 (CN st), 1295, 1137, 1115 (SO$_2$ st) cm$^{-1}$; $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 3.00 (t, $J = 7.2$ Hz, 2H, 2'-H$_2$), 3.16 (dd, $J = 9.6$ Hz, $J' = 8.8$ Hz, 1H, 4-H), 3.41 (dd, $J = 9.6$ Hz, $J' = 3.6$ Hz, 1H, 2-H), superimposed in part 3.46 (dd, $J = 14.0$ Hz, $J' = 2.4$ Hz, 1H, 6-H$_a$), 3.47 (s, 3H, 1-OCH$_3$), superimposed in part 3.51 (dd, $J = 14.0$ Hz, $J' = 9.6$ Hz, 1H, 6-H$_b$), 3.53 (m, 1 H, 1'-H$_a$), 3.58 (dd, $J = 14.0$ Hz, $J' = 7.2$ Hz, 1H, 1'-H$_b$), 3.62 (dd, $J = 9.6$ Hz, $J' = 8.8$ Hz, 1H, 3-H), 4.06 (ddd, $J = J' = 9.6$ Hz, $J'' = 2.4$ Hz, 1H, 5-H), 4.71 (d, $J = 3.6$ Hz, 1H, 1-H), 4.85 (s, OH); $^{13}$C NMR (100.6 MHz, CD$_3$OD) $\delta$ 11.5 (CH$_2$, C2'), 51.3 (CH$_2$, C1'), 56.0 (CH$_2$, C6), 56.4 (CH$_3$, 1-OCH$_3$), 68.7 (CH, C5), 73.2 (CH, C2), 74.1 (CH, C4), 74.7 (CH, C3), 101.6 (CH, C1), 118.6 (C, CN); HRMS (ESI), calcd for (C$_{15}$H$_{17}$NO$_7$S + NH$_4^+$) 313.1064, found 313.1062.

4.4.6. Methyl 6-[(3-aminopropyl)sulfonyl]-6-deoxy-$\alpha$-D-glucopyranoside (7)

A mixture of cyanosulfone 6 (289 mg, 0.98 mmol) and Raney-Ni (50% water, 300 mg) in MeOH (80 mL) was reacted with hydrogen (16 atm) at room temperature overnight in a Parr stirred reactor. The resulting suspension was filtered through Celite® and rinsed with MeOH (25 mL). The solvent was removed in vacuo to afford a yellow oil (186 mg), which was purified through column chromatography (40–60 μm silica gel, CH$_2$Cl$_2$ / MeOH mixtures, gradient elution). On elution with CH$_2$Cl$_2$ / MeOH 82:18 to 78:22, aminosulfone 7 (82 mg, 28% yield) was isolated; $R_f$ 0.09 (CH$_2$Cl$_2$ / MeOH / 50% aq. NH$_4$OH 7:3:0.1).
A solution of 7 (29 mg, 0.10 mmol) in MeOH (5 mL) was filtered through a 0.20 μm NYL filter, treated with methanolic HCl (0.5 N, 0.87 mL) and evaporated under reduced pressure. The resulting solid was washed successively with CH₂Cl₂ (2 × 5 mL) and pentane (2 × 3 mL) to give, after drying at 35 °C / 30 Torr, 7·HCl (19 mg) as a yellowish hygroscopic solid; [α]²⁰_D = + 147 (c 1.3, MeOH); IR (ATR) ν 3500‒2500 (max at 3241, 2916, 2844, OH, +NH, C₆H₆st), 1280, 1132, 1102 (SO₂st) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.19 (tt, J = J' = 7.6 Hz, 2H, 2'-H₂), 3.13 (t, J = 7.6 Hz, 2H, 3'-H₂), 3.15 (dd, J = 9.6 Hz, J' = 8.8 Hz, 1H, 4-H), superimposed in part with the solvent signal 3.22 (dt, J = 14.0 Hz, J' = 7.6 Hz, 1H, 1'-Hₐ), superimposed in part with the solvent signal 3.33 (dt, J = 14.0 Hz, J' = 7.6 Hz, 1H, 1'-Hₖ), 3.40 (dd, J = 10.0 Hz, J' = 3.6 Hz, 1H, 2-H), superimposed in part 3.45 (dd, J = 12.0 Hz, J' = 8.0 Hz, 1H, 6-Hₖ), superimposed in part 3.459 (dd, J = 12.0 Hz, J' = 4.0 Hz, 1H, 6-Hₐ), 3.460 (s, 3H, 1-OCH₃), 3.62 (dd, J = 10.0 Hz, J' = 8.8 Hz, 1H, 3-H), 4.06 (ddd, J = 9.6 Hz, J' = 8.0 Hz, J” = 4.0 Hz, 1H, 5-H), 4.69 (d, J = 3.6 Hz, 1H, 1-H), 4.85 (s, OH, +NH₃); ¹³C NMR (100.6 MHz, CD₃OD) δ 21.3 (CH₂, C2’), 39.5 (CH₂, C3’), 53.0 (CH₂, C1’), 55.9 (CH₂, C6), 56.3 (CH₃, 1-OCH₃), 68.6 (CH, C5), 73.3 (CH, C2), 74.2 (CH, C4), 74.7 (CH, C3), 101.5 (CH, C1); HRMS (ESI), calcd for (C₁₀H₁₄NO₅S + H⁺) 300.1111; found 300.1114.

4.4.7. Methyl 6-deoxy-6-[3-guanidinopropyl]sulfonyl]-α-D-glucopyranoside (8)

To a suspension of aminosulfone 7 (27 mg, 0.09 mmol) in acetonitrile (1 mL), Et₃N (0.02 mL, 15 mg, 0.14 mmol) and 1H-pyrazole-1-carboxamidine hydrochloride (16 mg, 0.11 mmol) were added. The reaction mixture was stirred under reflux for 6 h and cooled to room temperature. The solvent was poured off and the precipitate was dried in vacuo. The resulting yellow sticky solid (39 mg) was taken in MeOH (2 mL), treated with methanolic HCl (0.5 N, 2 mL), and filtered through a 0.2 μm NYL filter. The filtrate was evaporated under reduced pressure, washed successively with CH₃CN (2 × 2
mL), CH₂Cl₂ (2 × 2 mL), and pentane (2 × 2 mL), and dried at 35 ºC / 30 Torr, to provide guanidine 8·HCl (21 mg, 0.05 mmol, 62% yield) as a yellowish hygroscopic solid; [α]²₀°ᵤ = + 155 (c 0.80, MeOH); IR (ATR) ν 3500–2500 (max at a 3335, 3313, 3160, 2928, 2912, OH, NH⁺, NH, CH st), 1644, 1630 (C=N st), 1293, 1104 (SO₂ st) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.10 (t, J = J' = 7.6 Hz, 2H, 2'-H₂), 3.15 (dd, J = 9.6 Hz, J' = 9.2 Hz, 1H, 4-H), 3.24–3.35 (complex signal, 2H, 1'-Hₐ, 1'-Hₖ), 3.35 (t, J = 7.6 Hz, 2H, 3'-H₂), 4.06 (ddd, J = J' = 9.6 Hz, J'' = 5.6 Hz, 1H, 5-H), 4.85 (s, OH, NH, NH₂, +NH₂); ¹³C NMR (100.6 MHz, CD₃OD) δ 22.7 (CH₂, C2'), 40.9 (CH₂, C3'), 53.0 (CH₂, C1'), 55.9 (CH₂, C6), 56.3 (CH₃, 1-OCH₃), 68.6 (CH, C5), 73.3 (CH, C2), 74.2 (CH, C4), 74.7 (CH, C3), 101.5 (CH, C1), 158.7 (C, guanidine C); HRMS (ESI), calecd for (C₁₁H₂₁N₃O₇S + H⁺) 342.1329; found 342.1367.

4.4.8. Methyl 6-deoxy-6-(2-phthalimidoethanesulfonamido)-α-D-glucopyranoside (10)

A solution of amine 9 (500 mg, 2.59 mmol) and Et₃N (0.51 mL, 370 mg, 3.66 mmol) in anhydrous DMF (15 mL) was cooled at 0 ºC with an ice bath and treated with 2-phthalimidoethanesulfonyl chloride (744 mg, 2.72 mmol). The reaction mixture was stirred at room temperature for 6 days, treated with NH₄Cl (5 mL), and evaporated under reduced pressure to give a solid residue (2.51 g), which was purified through column chromatography (40–60 μm silica gel, CH₂Cl₂ / MeOH mixtures, gradient elution). On elution with CH₂Cl₂ / MeOH 90:10, phthalimidosulfonamide 10 (1.07 g, 96% yield) was isolated as a white solid; Rf 0.52 (CH₂Cl₂ / MeOH 9:1); mp 143–145 ºC; [α]²₀°ᵤ = + 100 (c 0.28, MeOH); IR (ATR) ν 3449, 3311 (OH, NH st), 1770, 1705 (C=O st), 1316, 1142 (SO₂ st) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ superimposed in part 3.23 (dd, J = 9.6 Hz, J' = 8.8 Hz, 1H, 4-H), 3.24 (dd, J = 14.0 Hz, J' = 6.8 Hz, 1H,
6-Ha), superimposed in part 3.42 (dd, J = 9.6 Hz, J’ = 3.6 Hz, 1H, 2-H), 3.43 (s, 3H, 1-OCH3), 3.47 (dt, J = 12.0 Hz, J’ = 6.8 Hz, 1H, 1’-Ha), 3.49 (dt, J = 12.0 Hz, J’ = 6.8 Hz, 1H, 1’-Hb), 3.52 (dd, J = 14.0 Hz, J’ = 2.8 Hz, 1H, 6-Hb), 3.59 (ddd, J = 9.6 Hz, J’ = 6.8 Hz, J” = 2.8 Hz, 1H), 3.61 (dd, J = 9.6 Hz, J’ = 8.8 Hz, 1H, 3-H), 4.12 (t, J = 6.8 Hz, 2H, 2’-H2), 4.71 (d, J = 3.6 Hz, 1H, 1-H), 4.84 (s, OH, NH), 7.80 (m, 2H, phthalimide Hmeta), 7.87 (m, 2H, phthalimide Hortho); 13C NMR (100.6 MHz, CD3OD) δ 33.8 (CH2, C2’), 45.1 (CH2, C6), 50.2 (CH2, C1’), 55.8 (CH3, 1-OCH3), 72.1 (CH, C5), 72.9 (CH, C4), 73.5 (CH, C2), 74.9 (CH, C3), 101.3 (CH, C1), 124.3 (2CH, phthalimide Cortho), 133.4 (2C, phthalimide Cipso), 135.5 (2CH, phthalimide Cmeta), 169.4 (2C, phthalimide CO); HRMS (ESI), calcd for (C17H22N2O8S + H+) 431.1119; found 431.1120.

4.4.9. Methyl 6-(2-aminooethanesulfonamido)-6-deoxy-α-D-glucopyranoside (11)

To a solution of phthalimidosulfonamide 10 (473 mg, 1.10 mmol) in MeOH (3.3 mL), hydrazine monohydrate (0.07 mL, 72 mg, 1.44 mmol) was added, and the reaction mixture was stirred under reflux overnight. The resulting mixture was evaporated under reduced pressure to give a solid residue (516 mg). After two consecutive column chromatography purifications (40–60 μm silica gel, CH2Cl2 / MeOH / 50% aq. NH4OH mixtures, gradient elution), aminosulfonamide 11 (95 mg, 29% yield) was isolated on elution with CH2Cl2 / MeOH / 50% aq. NH4OH 80:20:0.2; Rf 0.06 (CH2Cl2 / MeOH / 50% aq. NH4OH 8:2:0.05).

A solution of 11 (95 mg, 0.32 mmol) in MeOH (1 mL) was filtered through a 0.2 μm NYL filter, treated with methanolic HCl (0.5 N, 2.84 mL), and evaporated under reduced pressure. The resulting solid was washed with pentane (3 × 3 mL) and dried at 35 °C / 30 Torr, to provide 11·HCl (85 mg) as a brown highly hygroscopic solid; mp 72–76 °C; [α]20 D = + 6 (c 0.52, MeOH); IR (ATR) ν 3500–2500 (max at 3221, 3141,
2902, 2839, OH, NH⁺, NH, CH st), 1310, 1141 (SO₂ st) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 3.19 (dd, J = 9.6 Hz, J’ = 9.2 Hz, 1H, 4-H), 3.26 (dd, J = 14.0 Hz, J’ = 6.8 Hz, 1H, 6-Hₐ), 3.39 (dd, J = 9.2 Hz, J’ = 4.0 Hz, 1H, 2-H), 3.37–3.40 (complex signal, 2H), 3.43–3.48 (complex signal, 2H) (1’-Hₐ, 1’-Hₕ, 2’-H₂), 3.43 (s, 3H, 1-OCH₃), 3.53 (dd, J = 14.0 Hz, J’ = 2.8 Hz, 1H, 6-Hₐ), 3.59 (dd, J = 9.6 Hz, J’ = 6.8 Hz, J” = 2.8 Hz, 1H, 5-H), 3.61 (dd, J = J’ = 9.2 Hz, 1H, 3-H), 4.70 (d, J = 4.0 Hz, 1H, 1-H), 4.85 (s, OH, NH); ¹³C NMR (100.6 MHz, CD₃OD) δ 35.9 (CH₂, C₂’), 45.0 (CH₂, C₆), 50.2 (CH₂, C₁’), 55.8 (CH₃, 1-OCH₃), 72.1 (CH, C₅), 72.8 (CH, C₄), 73.5 (CH, C₂), 74.9 (CH, C₃), 101.3 (CH, C₁); HRMS (ESI), calcd for (C₉H₂₀N₂O₇S + H⁺) 301.1064; found 301.1060.

4.4.10. Methyl 6-deoxy-6-(2-guanidoethanesulfonamido)-α-D-glucopyranoside (12)

To a suspension of aminosulfonamide 11 (67 mg, 0.22 mmol) in acetonitrile (2 mL), Et₃N (0.06 mL, 44 mg, 0.43 mmol) and 1H-pyrazole-1-carboxamidine hydrochloride (38 mg, 0.26 mmol) were added. The reaction mixture was stirred under reflux for 6 h and cooled to room temperature. The solvent was poured off and the precipitate was dried in vacuo. The resulting brown sticky solid (65 mg) was taken in MeOH (5 mL), treated with methanolic HCl (0.5 N, 5 mL), and filtered through a 0.2 μm NYL filter. The filtrate was evaporated under reduced pressure, washed successively with CH₃CN (2 × 3 mL), CH₂Cl₂ (2 × 5 mL), and pentane (2 × 3 mL), and dried at 35 °C / 30 Torr, to provide guanidine 12·HCl (24 mg, 0.06 mmol, 29% yield) as a brownish highly hygroscopic solid; [α]²⁰_D = + 17 (c 0.35, MeOH); IR (ATR) ν 3500–2500 (max at 3340, 3174, 2907, 2844, OH, NH, NH, CH st), 1650, 1643, 1630 (C=N st), 1309, 1139 (SO₂ st) cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ superimposed in part 3.20 (dd, J = 9.6 Hz, J’ = 9.2 Hz, 1H, 4-H), 3.24 (dd, J = 14.0 Hz, J’ = 7.2 Hz, 1H, 6-Hₐ), 3.34–3.39 (complex signal, 2H, 1’-Hₐ, 1’-Hₕ), superimposed in part 3.42 (dd, J = 9.6 Hz, J’ = 4.0 Hz, 1H, 2-
H), 3.43 (s, 3H, 1-OCH₃), 3.52 (dd, J = 14.0 Hz, J’ = 2.8 Hz, 1H, 6-H₆), superimposed in part 3.59 (ddd, J = 9.6 Hz, J’ = 7.2 Hz, J” = 2.8 Hz, 1H, 5-H), 3.61 (dd, J = 9.6 Hz, J’ = 9.2 Hz, 1H, 3-H), 3.66 (t, J = 6.0 Hz, 2H, 2’-H₂), 4.69 (d, J = 4.0 Hz, 1H, 1-H), 4.85 (s, OH, NH, NH₂, NH₂); ¹³C NMR (100.6 MHz, CD₃OD) δ 37.6 (CH₂, C₂’), 44.9 (CH₂, C₆), 51.9 (CH₂, C₁’), 55.8 (CH₃, 1-OCH₃), 72.1 (CH, C₅), 72.7 (CH, C₄), 73.4 (CH, C₂), 74.8 (CH, C₃), 101.2 (CH, C₁), 158.6 (C, guanidine C); HRMS (ESI), calcd for (C₁₀H₂₂N₄O₇S + H⁺) 343.1282; found 343.1280.

4.5. In vitro culture of Plasmodium falciparum

P. falciparum strain Dd2 (clone MRA-150; Malaria Research and Reference Reagent Resource Center: http://www.mr4.org) was maintained in continuous culture following the protocol previously described [68]. The culture media consisted of standard RPMI 1640 (Sigma-Aldrich) supplemented with 0.5 % Albumax I (Gibco), 100 μM hypoxanthine (Sigma-Aldrich), 25 mM HEPES (Sigma-Aldrich), 12.5 μg/mL gentamicine (Sigma-Aldrich), and 25 mM NaHCO₃ (Sigma-Aldrich), and incubated in 5% CO₂ at 37 ºC in tissue culture flasks (Iwaki). Growth progress was monitored by microscopy in thin blood smears stained with Wright’s eosin methylene blue solution (Merck), using the freely available Plasmoscore software [69] to determine parasitaemia values. A detailed description of P. falciparum culture and synchronization methods used in this work has been reported previously [68].

4.6. Antimalarial drug activity assay

Drug activity was assayed by the PicoGreen microfluorimetric DNA-based assay monitoring parasite growth inhibition at different drug concentrations [70]. PicoGreen (P7589) was purchased from Invitrogen and diluted as indicated by the manufacturer in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Synchronized rings from stock cultures were used to test serial dilutions of the inhibitors in 96-well culture
microplates. Thus, 150 μL of parasites at 2% hematocrit and 1% parasitemia were allowed to grow for 48 h in 5% CO₂ at 37 °C. The parasites were then centrifuged at 600 × g for 10 min and resuspended in saponin (0.15%, wt/vol, in phosphate-buffered saline (PBS)) to lyse the erythrocytes and release the malaria parasites. To eliminate all traces of hemoglobin, the pellet was washed by the addition of 200 μL of PBS followed by centrifugation at 600 × g. The washing step was repeated twice to ensure complete removal of hemoglobin. Finally, pellets were resuspended in 100 μL of PBS. A 100-μL volume of PicoGreen diluted in TE was added to each well. Plates were incubated for 30 to 60 min in the dark, and the fluorescence intensity was measured at 485-nm excitation and 528-nm emission. Growth inhibition defining the half maximal inhibitory concentration (IC₅₀) was calculated as previously described [68]. Upon treatment, the parasite morphology was evaluated by microscopic analysis of thin blood smears stained with Wright's stain. Smears from drug-free cultures were used as a control.

4.7. Steady-state enzymatic assays to characterize human and parasite G6PD inhibition.

To compare the mechanism of action of the inhibitors, the kinetic parameters of human and PfG6PD were determined in a range of concentrations in the linear portion of the Lineweaver-Burk plots in the presence and absence of inhibitor for analysis and curve fitting into the Michaelis-Menten equation. These assays were run in triplicate in a 900 μL of a mixture containing 0.1 M Tris (pH 8.0) and varying concentrations of G6P (10–300 μM) or NADP⁺ (5–200 μM) at the corresponding saturating concentrations of the other substrate: NADP⁺ or G6P (200 μM) [28]. The inhibitor was added to the mixture without prior incubation at concentrations between 40 and 160 μM. In all experiments the exact concentrations of the solutions of G6P and NADP⁺ were enzymatically determined. The assays were carried out at 25 °C in a Perkin-Elmer
LS-50B spectrofluorimeter, and the linear increase in fluorescence intensity of the NADPH levels were observed at 450 nm emission (excitation at 340 nm). Care was taken to ensure that initial rates were measured along the time length of the assay (5–10 min). \(K_i, K_M\) and \(V_{max}\) values were calculated using Graphpad Prism software.

Recombinant human G6PD was obtained as previously reported [28]. \(Pf\)G6PD was partially purified from cultures at 80–90% trophozoites according to previously reported procedures [25].

4.8. In vitro cell viability assay

HEPG2 human hepatoma cells were cultured 24 h in triplicate in 96-well flat-bottomed microplates in DMEM Glutamax (GIBCO) growth medium in a final volume of 100 μL per well. Cells grown in the presence of medium alone were used as controls. To evaluate the potential cytotoxic effect of the G6PD inhibitors, one set of plates received serial dilutions from 0.2 to 2 mM of each inhibitor. Cells were seeded during 24 h and 48 h in triplicate in 96-well flat-bottomed microplates at a density of \(5 \times 10^3\) cells per well in 100 μL growth medium. Treatment and control in growth medium was performed after 24 h and during further 24 h incubation in a final volume of 200 μL (100 μL cells + 100 μL medium with or without treatment). Following treatment, cell viability was determined by means of the colorimetric EZ4U (Biomedica, Vienna, Austria) according to the manufacturers instructions, and absorbance at 490/610 nm was measured spectrophotometrically in a Cary 50 BIO Microplate Reader 50MPR (Varian).

Author contributions

The manuscript was written through contributions from all authors. All authors have given approval of the final version of the manuscript.
Conflicts of interest

The authors declare no conflict of interest about this article.

Abbreviations

G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; G6PD–6PGL, glucose-6-phosphate dehydrogenase–6-phosphogluconolactonase; hG6PD, human glucose-6-phosphate dehydrogenase; MD, molecular dynamics; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PfG6PD, Plasmodium falciparum glucose-6-phosphate dehydrogenase; PPP, pentose phosphate pathway; RBC, red blood cells.

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Supplementary material
X-ray structures available for G6PD of different species, results from homology modeling and MD simulations, and NMR spectra of compounds. Coordinates of the PfG6PD structural model are available upon request to the authors. Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech...

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