**Exploring inhibition of Pdx1, a component of the PLP synthase complex of the human malaria parasite *Plasmodium falciparum***

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Malaria tropica is a devastating infectious disease caused by *Plasmodium falciparum*. This parasite synthesizes vitamin B₆ *de novo* via the PLP (pyridoxal 5'-phosphate) synthase enzymatic complex consisting of PfPdx1 and PfPdx2 proteins. Biosynthesis of PLP is largely performed by PfPdx1, ammonia provided by PfPdx2 subunits is condensed together with RSP (D-ribose 5-phosphate) and G3P (D-ribose 5-phosphate). PfPdx1 accommodates both the RSP and G3P substrates and intricately co-ordinates the reaction mechanism, which is composed of a series of imine bond formations, leading to the production of PLP. We demonstrate that E4P (D-erythrose 4-phosphate) inhibits PfPdx1 in a dose-dependent manner. We propose that the acyclic phospho-sugar E4P, with a C1 aldehyde group similar to acyclic RSP, could interfere with RSP imine bond formations in the PfPdx1 reaction mechanism. Molecular docking and subsequent screening identified the E4P hydrazide analogue 4PEHz (4-phospho-D-erythronhydrazide), which selectively inhibited PfPdx1 with an IC₅₀ of 43 μM. PfPdx1 contained in the heteromeric PLP synthase complex was shown to be more sensitive to 4PEHz and was inhibited with an IC₅₀ of 16 μM. Moreover, the compound had an IC₅₀ value of 10 μM against cultured *P. falciparum* intraerythrocytic parasites. To analyse further the selectivity of 4PEHz, transgenic cell lines overexpressing PfPdx1 and PfPdx2 showed that additional copies of the protein complex conferred protection against 4PEHz, indicating that the PLP synthase is directly affected by 4PEHz in vivo. These PfPdx1 inhibitors represent novel lead scaffolds which are capable of targeting PLP biosynthesis, and we propose this as a viable strategy for the development of new therapeutics against malaria.

**Key words:** D-erythrose 4-phosphate (E4P), Pdx1, *Plasmodium falciparum*, pyridoxal 5'-phosphate (PLP), PLP synthase, vitamin B₆.

**INTRODUCTION**

The parasitic disease malaria, caused by members of the genus *Plasmodium*, represents one of the most serious threats to humans worldwide. More than 2 billion people are exposed to the parasites leading to an estimated 500 million clinical cases and more than 1 million deaths per annum, mostly young children in Africa. An effective vaccine is not available, and prophylaxis as well as chemotherapy depends solely on the administration of a small number of drugs [1,2]. There are fewer than 30 different small-molecule scaffolds or chemotypes that are effective against *Plasmodium falciparum* parasites, and the clinically employed repertoire of antimalarials consists of only approximately ten different scaffolds [3]. At the current rate of parasite drug-resistance development, this handful of antimalarials is under threat of rapidly becoming ineffective. For this reason, the discovery of newer chemical scaffolds and the exploitation of novel drug targets in this parasite are of utmost importance.

PLP (pyridoxal 5'-phosphate) is the catalytic active form of vitamin B₆, and a vital prosthetic group to a wide range of enzymes primarily involving amino acid biosynthesis [4]. The cofactor is extremely versatile, and partakes in enzyme-catalysed racemizations, transaminations, decarboxylations and substitution reactions [4]. During catalysis, PLP functions to stabilize the negative charge at the C= of a PLP–amino acid intermediate [4]. It has become apparent that the function of PLP in *P. falciparum* parasites is not restricted to its action as a prosthetic group, as the molecule is also required in response towards oxidative stress caused by the generation of singlet oxygen during haemoglobin digestion [5,6]. Currently, two known PLP-biosynthetic pathways exist: the DXP (1-deoxy-D-xylulose 5-phosphate)-dependent pathway and the DXP-independent route. The DXP-dependent pathway is present in *Escherichia coli* and some proteobacteria [7]. The pathway involves several enzymes which condense E4P (D-erythrose 4-phosphate), DXP and glutamate to form PNP (pyridoxine 5'-phosphate), which is subsequently converted into PLP [8]. The DXP-independent pathway, consisting of the enzymes Pdx1 and Pdx2, is found in plants and fungi, as well as the human pathogens *Plasmodium falciparum*, *Toxoplasma gondii* and *Mycobacterium tuberculosis* [5,9]. The pathway is independent of DXP, and instead R5P (D-ribose 5-phosphate), G3P (D-ribose 5-phosphate) and L-glutamine are used to form PLP. *P. falciparum* parasites have a functional DXP-independent pathway consisting

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**Abbreviations used:** AHT, anhydrotetracycline; DHAP, dihydroxyacetone phosphate; DRSP, 2-deoxy-o-ribose 5-phosphate; DXP, 1-deoxy-o-xylulose 5-phosphate; E4P, o-erythrose 4-phosphate; EHz, o-erythronhydrazide; F6P, o-fructose 6-phosphate; G3P, o-glyceraldehyde 3-phosphate; GPI, glucose-6-phosphate isomerase; LB, Luria-Bertani; LB-amp, LB agar plates containing 50 μg/ml ampicillin; PfPdx1, *Plasmodium falciparum* Pdx1; PfPdx2, *Plasmodium falciparum* Pdx2; PLP, pyridoxal 5-phosphate; R5P, o-ribose 5-phosphate; RMS, root mean square; RMSD, RMS deviation; RPI, R5P isomerase; Ru5P, o-ribulose 5-phosphate; ScPdx1, *Saccharomyces cerevisiae* Pdx1; TIM, triose phosphate isomerase; TrnPdx1, *Thermoplasma maritima* Pdx1; TpPdx1, *Thermus thermophilus* Pdx1.

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of a PLP synthase, which is made up of \( P/Pdx1 \) and \( P/Pdx2 \). Human hosts of the malaria parasite lack the biosynthetic enzymes and are unable to produce PLP, making this parasite-exclusive pathway an attractive target for the development of therapeutics.

The catalytic centre of the PLP synthase is Pdx1, where R5P, G3P and ammonia are combined through an intricately complex biochemical process [10]. Pdx1 proteins have been shown to alternatively use Ru5P (D-ribulose 5-phosphate) and DHAP (dihydroxyacetone phosphate) as substitute pentose and triose substrates respectively [11–13]. The protein is therefore thought to contain RPI (R5P isomerase) and TIM (triose phosphate isomerase) activities in order to convert these alternative substrates. The reaction mechanism of PLP formation in Pdx1 has been extensively elucidated. The reaction in Pdx1 is initiated by RSP ring opening and the formation of an imine adduct between RSP and an internal Lys83 [10]. Initial binding of R5P to the internal lysine residue occurs via the Cl aldehyde group [10,14]. Binding of R5P is followed by isomerization into an Ru5P intermediate, which eventually leads to the formation of an internal chromophore with characteristic absorbance at 320 nm, termed \( \lambda_{320} \) [10,14,15]. The addition of G3P to Pdx1 with bound \( \lambda_{320} \) forms leads to the formation and release of PLP [10]. It is thought that the furanose form of R5P is bound by Pdx1; however, the exact mechanism of RSP ring opening remains unclear [15].

In the present paper, we report the identification of \( P/Pdx1 \) inhibitors capable of interfering with PLP production. Analogues of R5P and G3P, in which stereochemical arrangement and hydrolysable phosphate groups were retained, were found to inhibit \( P/Pdx1 \) more effectively than their non-phosphorylated counterparts. The Cl aldehyde reactivity found on some of these inhibitors was also an important feature. The present study revealed inhibitors with appreciable inhibitory effect against \( P. falciparum \) parasites \textit{in vitro} and we demonstrated target specificity against \( P/Pdx1 \) \textit{in vivo}.

**EXPERIMENTAL**

**Materials**

EH2 (d-erythrohydrozide) was synthesized by iThemba Pharmaceuticals and supplied as an HPLC-purified product, which was structurally confirmed by \( ^1\)H-NMR and MS. All other chemical compounds including R5P, G3P, E4P and d-erythrose were purchased from Sigma–Aldrich unless stated otherwise. Albumax II and gentamycin were obtained from Invitrogen. \( [\text{H}] \)Hypoxanthine (specific activity 30 Ci/mmol; 0.03 mM) was purchased from American Radiolabeled Chemicals and Ultima Gold\textsuperscript{TM} scintillation fluid was from PerkinElmer. Restriction enzymes and ligases were purchased from New England Biolabs. Oligonucleotides were obtained from Sigma–Aldrich. The cloning vector pASK-IBA3, Strep-Tactin\textsuperscript{®} Sepharose, AHT (anhydrotretracycline) and desthiobiotin were obtained from IBA (Institut für Bioanalytik).

**In silico modelling and ligand screening**

Molecular modelling was performed using the Discovery Studio suite (version 3.0, Accelrys Software) unless specified otherwise. \( P/Pdx1 \) homology models were generated using \textit{Thermotoga maritima} (\( Tm\)Pdx1, PDB code 2ISS) and \textit{Thermus thermophilus} (\( T\)Pdx1, PDB code 2ZZB) Pdx1 as templates. At the time the present study was initiated, the crystal structure of \textit{Plasmodium berghei} Pdx1 (\( Pb\)Pdx1, PDB code 4ADU) [16] was not available. Several homology models were created, each incorporating a different ligand from the template structures, including Ru5P and 2-methyl-2,4-pentanediol, as well as surrounding water molecules. The quality of the structures was assessed using model-derived pseudo-energy terms as well as stereochemical pseudo-energy terms and Ramachandran plots were generated using PDBsum [17,18]. Analysis with PROCHECK showed a slight decrease in the average G factors between the crystal structure (0.39–0.41) and the homology models (0.00); scores above −0.5 are considered acceptable [17,18]. The homology models were subjected to 200 steps of steepest-descent energy minimization using the CHARMM forcefield with an RMS (root mean square) gradient tolerance of 0.1, followed by 200 steps of conjugate gradient minimization. R5P was selected from the optimized protein–substrate complex to define a binding cavity and assigned a sphere radius of 8 Å (1 Å = 0.1 nm). The defined binding cavity was used to extract pharmacophore features from the different \( P/Pdx1 \) models, followed by clustering and high-throughput virtual screening of 2 million compounds of the ZINC database drug-like subset [19]. The database was generated as a multi-conformer composite database using catDB with a maximum of 250 representative conformations (Accelrys Software). The drug-like database was screened against five pharmacophore models, and the 300 best-hit compounds were identified on the basis of their pharmacophore fit-values. These compounds were subjected to molecular docking simulations using LigandFit employing iterative rigid-body minimization in a DREIDING energy forcefield with 1500 Monte Carlo trials [20,21]. The ligand poses were ranked according to the docking score (DockScore). In addition, ligand poses were evaluated using LigScore empirical scoring functions [22]. R5P was used as control in cross-docking to validate ligand docking and scoring. Graphical displays were generated with VMD [23].

Following the release of the \( Pb/Pdx1 \) structure in 2012 [16], additional docking was performed on the \( Pb/Pdx1 \) homologue. The \( Pb/Pdx1 \) structure was prepared by removing the covalent bond between Lys\textsuperscript{44} and R5P, followed by minimization as described above. Post-minimization, R5P was selected to assign a binding site and sphere radius, similar to the docking simulations described above. Lys\textsuperscript{44} was maintained in a protonated state during the docking simulations (predicted \( pK_a \) of 12.1). Ligands were docked and scored using LigandFit and LigScore, as described above.

**Mutagenesis of \( P/Pdx1 \)**

Deletion of the C-terminal region of \( P/Pdx1 \) was performed on \( P/Pdx1 \) cloned previously in the expression plasmid pASK-IBA3 (IBA) [5]. Multiple deletion mutagenesis PCRs were set up, and contained 35 ng of expression plasmid with 30 pmol each of the sense primer (\( 5’-\text{GGCGCGGGTCTCAATGAGATCAATCTGC} \)) and antisense primer (\( 5’-\text{GGCCGCCTCTCGCAATCTCAGC} \)) (the site of C-terminally truncated region is indicated with an asterisk) in a final volume of 50 μl with PCR Supermix (Invitrogen) containing recombinant Taq DNA polymerase. The cycling parameters were 95°C for 3 min, then 35 cycles of 95°C for 30 s, 41°C for 90 s and 60°C for 2 min. Parent template was removed from the reactions by incubation with 20 units of DpnI (New England Biolabs) for 2 h at 37°C. Following Invitrogen PureLink\textsuperscript{®} PCR purification, 50 μl of the eluted solutions were digested with 10 units of BsaI (New England Biolabs) for 3 h at 37°C, purified and ligated (16 μl) into similarly digested pASK-IBA3 expression plasmids at an insert/vector ratio of 3:1 using T4 DNA ligase (New England Biolabs). Ligation reactions (20 μl volume) were transformed directly into 200 μl of XL10 Gold competent.
E. coli cells (Stratagene) were transformed by heat-shock transformation for 60 s at 42°C, cooling on ice, followed by the addition of 800 μl of LB (Luria–Bertani) medium. Cells were plated on to LB-amp (LB agar plates containing 50 μg/ml ampicillin) and grown for 16 h at 37°C. Single colonies were selected and used to inoculate 2 ml of LB-amp medium, and grown for 16 h at 37°C. Plasmids were extracted using thepeqGOLD Plasmid Miniprep Kit I (Peqlab). Recombinant plasmids (10 μl) were confirmed through XbaI (5 units) and HindIII (5 units) (New England Biolabs) restriction enzyme digestions and automated sequencing (Seqlab). The P/Pdx1 R164A mutation was created with sense primer 5′-GCTATTTACATATAGCAACTGTTAAATAAAGA3′ and antisense primer 5′-TTCAATTATTTACATGTGCTATATGGTTAAATAGC-3′ (mutated residues are indicated in bold and underlined) as described previously [24].

Expression and purification of P/Pdx1 and P/Pdx2

P/Pdx1 and P/Pdx2 clones in pASK-IBA3 were transformed into E. coli BLR (DE3) [5]. Selected single colonies were used to inoculate LB-amp and were grown overnight at 37°C. Cultures were diluted 1:100 into LB-amp medium and grown to a D600 of 0.5. Expression was induced using 200 ng/ml AHT and grown for 4 h at 37°C with agitation. Cells were collected by centrifugation at 2000 g for 10 min, and the pellets were resuspended in 100 mM Tris/HCl and 150 mM NaCl at pH 8.0. PMSF (0.1 mM) and lysozyme (5 mg/10 ml reconstituted pellet) were added, and the pellets were sonicated using pulsing at output 5, duty cycle 40 with 1 min resting intervals on ice (Branson Sonifier 250) and clarified by centrifugation at 50000 g for 50 min at 4°C. Strep-Tag®-fusion proteins were purified according to the manufacturer’s recommendations (IBA).

Enzyme activity measurements

P/Pdx1 assays were performed using the 100 mM Tris/HCl and 150 mM NaCl buffer (pH 8.0) as described previously [25]. The P/Pdx1 assay contained 0.5 mM R5P, 0.5 mM G3P and 20 mM NH4Cl in a final assay volume of 250 μl with 200 μg (6 nM) of purified P/Pdx1. Initially, compounds were only screened against P/Pdx1 to discount molecules that could have interfered with P/Pdx1 and P/Pdx2 heterodimerization. In assays containing both P/Pdx1 and P/Pdx2, 20 mM L-glutamine was used instead of NH4Cl. Reaction mixtures were incubated at 37°C for 2.5 h and the PLP–Tris Schiff base was detected at 414 nm. Spectrophotometric measurements, including wavelength scans, were performed with a Uvikon 933B spectrophotometer (Bio-Tek Kontron) in 70 μl Plastibrand® cuvettes (Brand) or using a NanoDrop 1000 spectrophotometer (NanoDrop Products).

Culturing of P. falciparum and IC50 assays

P. falciparum (3D7) parasites were maintained in continuous culture using the method of Trager and Jensen [26], as modified by Das Gupta et al. [27]. The RPMI 1640 culture medium contained 25 mM Hepes, 10 mM glucose, 20 mM sodium bicarbonate, 25 mM hypoxanthine and 0.5% AlbuMAX II (Invitrogen) at pH 7.4. The RPMI 1640 medium as supplied by AppliChem contained 0.001 g/l or 4.86 μM pyridoxine hydrochloride. Parasite cultures were maintained at 4% haematocrit using human O+ erythrocytes in flask (Nunc) with shaking at 200 rev./min at 37°C. Parasite cultures (50 ml) consisting of ring-stage parasites (1–5% parasitaemia) were synchronized using 5% D-sorbitol every 48 h [28]. [3H]Hypoxanthine incorporation assays were performed as described previously [27] on ring-stage parasites (2% haematocrit and 1% parasitaemia) exposed to serial dilutions of test compound in 96-well plates at 37°C. After 24 h, 100 nCi of [3H]hypoxanthine was added, and parasite cultures were incubated for an additional 24 h, followed by harvesting (using an Inotech cell harvesting system) and collection on filter mats. Filter mats were washed four times using distilled water, dried at 60°C for 10 min and suspended in 4 ml of Ultima Gold™ scintillation fluid and counted using a TriLux liquid-scintillation counter (Wallac). The IC50 values were calculated from sigmoid dose–response curves generated using Prism 5 (GraphPad Software).

Long-term growth assays

Asynchronous P. falciparum parasites, co-transfected with P/Pdx1 and P/Pdx2 pARL1α vectors with blasticidin S and WR99210 antibiotic resistance, together with a mock cell line, containing vector with only antibiotic resistance, were maintained in culture, as described previously [6]. Blasticidin S-hydrochloride (Sigma–Aldrich) and WR99210 (Jacobus Pharmaceuticals) were maintained at final concentrations of 2.1 mM and 5 nM respectively. Long-term growth assays were performed, as described previously [29], using 12-well plates (Nunc) at 4% haematocrit, with 1% starting parasitaemia. Both P/Pdx1/P/Pdx2-overexpressing and mock parasites were maintained in culture for 7 days. Spent medium was removed from suspended erythrocytes by gently tilting the plates, and replaced with fresh medium containing both antibiotics. Fresh 4PEHeE (4-phospho-D-erythronhydrazide) was added to both treated P/Pdx1/P/Pdx2 as well as treated mock parasites every day at a final concentration of 1 μM. The growth rate was assessed by morphological monitoring and counting of Giemsa-stained thin smears using oil-immersion light microscopy at ×100 magnification, counting >1000 erythrocytes cells from each slide. The cumulative parasitaemia was calculated using the determined parasitaemia by factoring in the dilution. From the logarithmic-transformed data, the slope of the regression line was used to calculate the growth rate constant (K), using the equation K = ln(10)/ln(α).

Results were analysed using GraphPad Prism 5, in which the null hypothesis of equal regression lines slopes was tested at a 99% confidence interval (P = 0.01). ANCOVA was conducted (JMP, version 9, SAS Institute), testing whether the slopes significantly differed from each other with α = 0.05.

RESULTS

In silico docking and screening of P/Pdx1 inhibitors

Homology models of P/Pdx1 were generated from TmnPdx1 and TnPdx1 templates that share 60 and 61% sequence identity respectively with P/Pdx1. The resulting models showed an average RMSD (RMS deviation) of 3.7 and 2.4 Å compared with the Cα backbone of the TmnPdx1 and TnPdx1 templates respectively. Comparing the active-site residues (residues within 5 Å of the substrates) revealed a high degree of structural conservation with RMSD values of 0.7 and 1.0 Å respectively. Moreover, a structural comparison of our models to the recently released PbPdx1, which shares a 85% sequence identity with P/Pdx1, showed a highly conserved Cα backbone RMSD ranging between 1.2 and 1.3 Å. Moreover, the active-site residues of the P/Pdx1 models showed RMSD values of between 0.6 and 0.7 Å, confirming the quality and validity of the models. Five
diverse pharmacophore models descriptive of the PfPdx1 RSP-binding site were derived from the protein models and screened against the drug-like subset of the ZINC database. Using the 300 best-fitting compounds, additional molecular docking was used to refine and determine binding capabilities in the RSP active site (Table 1). Following docking, a total of 19 compounds were selected for in vitro testing against purified PfPdx1 whereby PLP formation was monitored at a wavelength of 414 nm using the method of Wrenger et al. [5] (Table 1). Compounds 1–4 contained sulfonate moieties and showed docking scores similar to that of RSP; however, they had no appreciable activity against PfPdx1 at 3 mM (Table 1). Four polyhydroxylated compounds were identified from the PfPdx1 pharmacophores (compounds 5–8). Compound 8 which had a terminal ketone-ester arrangement was the most effective of the polyhydroxylated compounds tested, with a 12% reduction of PfPdx1 activity at 0.5 mM, although not statistically significant (Table 1). Compounds 9, 13 and 16, which share a pyrimidine sulfonamide moiety, were not effective at inhibiting PfPdx1 (Table 1). Flomidomycin (compound 17), which was predicted to have a high docking score, had no inhibitory effect on PfPdx1 activity at 20 mM (Table 1).

We investigated whether molecules closely related to the PfPdx1 substrate RSP were capable of inhibiting the protein. The RSP analogues DRSP (2-deoxy-D-ribose 5-phosphate; compound 18) and DXP (compound 19), had docking scores similar to that of RSP (Table 1). This suggested that DRSP could have favourable binding interactions in the RSP-binding site. When tested, DRSP was a weak inhibitor of PfPdx1 even at concentrations as high as 12 mM with 56% (P < 0.10) of the enzyme activity remaining (Figure 1A). DXP decreased PfPdx1 activity to 88% at 5 mM (Table 1). Considering that initial binding of RSP to Pdx1 entails imine formation with Lys84, DRSP could compete for this interaction [15]. Spectrophotometric UV–visible absorbance spectra of PfPdx1 incubated with DRSP revealed minor decreases in I320 formation with increasing concentrations of DRSP (Figure 1A). Decreased PLP production suggested that the molecule could potentially interfere with RSP–Lys84 imine adduct formation and/or PLP formation.

The specificity and stringency of PfPdx1 for other RSP analogues that could be either incorporated into the PLP ring structure and/or interfere with RSP imine adduct formation, was tested next. Several sugars including E4P, F6P (D-fructose 6-phosphate) and DRSP (Figure 1B), and DXP (results not shown), did not support the formation of PLP, confirming the strict substrate specificity of PfPdx1. Interestingly, we noted that substitution of E4P for RSP resulted in the formation of the single I320 chromophore similar to PfPdx1 incubated with RSP in the absence of G3P (Figure 1B). The structural resemblance between E4P and RSP suggests that the former could compete with RSP for Schiff base formation with the internal Lys84. Molecular docking simulations predicted similar LigScores for E4P and RSP in PfPdx1 models (Table 2). Similarly, docking of E4P into the PbPdx1 was comparable with the RSP substrate (Figure 1C and Table 2). E4P was orientated with the aldehyde group in close proximity to Lys84 (Figure 1D). These predicted interactions were investigated in more detail, as described below.

### Intensive analysis of E4P as PfPdx1 inhibitor

The activity of PfPdx1 was assayed in the presence of various concentrations of E4P, and a dose-dependent decline in PLP synthesis was observed (Figure 2A). A concomitant increase in absorbance at 320 nm was observed for increasing concentrations of E4P (Figure 2A). To discard potential conjugation or adduct formation of PLP by E4P, calibration curves of increasing concentrations of PLP were used to show that 1 mM E4P did not significantly affect detection of PLP at 414 nm (P = 0.975, n = 4; Supplementary Figure S1 at http://www.biochemj.org/bj/449/bj4490175add.htm). This confirmed that E4P was responsible for inhibiting the production of PLP by PfPdx1. E4P inhibited the formation of PLP by PfPdx1 with an IC50 value of 3.7 ± 0.9 mM (Figure 2B and Table 2). The non-phosphorylated analogue of E4P, i.e. δ-erythro, similarly interfered with PLP formation, but was more than 40-fold weaker than E4P (Figure 2B and Table 2). This emphasized the role of such hydrolysable phosphate groups with the additional negative charge potentially facilitating entry into the PfPdx1 active site.

### Residues involved in I320 formation

Previous studies have revealed several important residues involved in I320 formation that include Asp58, Lys83 and Lys151 [24] as well as residues from the C-terminus [30]. We sought to verify the importance of these residues during PfPdx1 formation. Incubation of RSP with both wild-type Pdx1 and ERR (E136A, R139A and R140A) mutant PfPdx1 resulted in I320 formation; however, the DKK (D26A, K83A and K151A) mutant was unable to form this species (Figure 3A). This corroborates previous findings and excludes the involvement of the ERR residues located in the PLP-binding site in I320 formation [31]. An RHE (R85A, H88A and E91A) mutant PfPdx1 was shown previously to prevent dodecamer assembly, and to be incapable of activating PfPdx2 subunits [24]. These residues are therefore believed to co-ordinate Pdx1–Pdx1 subunit cross-talk. Additional experimentation with RHE PfPdx1 mutants and ΔC Pdx1, lacking the 30 C-terminal residues, revealed that both proteins were unable to form I320 (Figure 3B). These observations are in agreement with functions assigned to the C-terminal region and moreover emphasized the importance of the RHE residues for R5P binding and imine adduct formation [30]. Reduced formation of I320 in R167A PfPdx1 suggested that this residue, which is part of the G3P-binding site, indirectly influences the way R5P is processed (Figure 3C). In contrast with observations elsewhere [13], this mutation decreased enzyme activity by only ~50% (Figure 3C).

### E4P analogues show inhibitory activity against PfPdx1

From the E4P lead structure, a closely related hydrazide analogue 4PEHz [32] and the unphosphorylated form of 4PEHz, termed EHz, were selected and tested against PfPdx1. Previously, 4PEHz was shown to have weak inhibitory activity against spinach RPI [32]. Molecular docking simulations predicted favourable ligand poses for 4PEHz in the R5P cavity of PfPdx1, as well as in PbPdx1 (Table 2). The EHz molecule was predicted to have less favourable binding interactions in PfPdx1 and had smaller LigScore binding affinities compared with 4PEHz (Table 2). 4PEHz could be docked into the PbPdx1 and had hydrazide groups orientated in close proximity to Lys84, with hydrogen bonds predicted to form between the ketone substituent of 4PEHz and Lys84 (Figure 4A). Enzyme preparations of PfPdx1 with 4PEHz revealed a concentration-dependent decrease in PLP formation, with visible increases in a 320 nm chromophoric species (Figure 4B). Kinetic assays revealed 4PEHz as an effective inhibitor with an IC50 of 43 ± 8 µM, whereas EHHz was more than 20-fold weaker (Figure 4C and Table 2). Interestingly, when tested on the PfPdx1 component of the gluammine-dependent PLP synthase complex, containing PfPdx2, 4PEHz had an IC50 of 16 ± 4 µM (Figure 4D).
Table 1  *In silico*-identified compounds tested against PfPdx1

Several pharmacophores generated for PfPdx1 were screened to obtain best-fitting ligands which were, together with rationally selected compounds, subjected to additional docking into PfPdx1 homology models. R5P was used as a control during docking simulations. Resultant compounds were tested on purified PfPdx1 (see the Experimental section). Values in parentheses indicate scores calculated for ligands docked into PbPdx1. LigScore1 and LigScore2, with units of $p \text{K}_i$ ($- \log K_i$), refer to predicted receptor–ligand binding affinities. DockScore refers to the unitless rigid-body minimization energy of the final ligand pose calculated during Monte Carlo trials. Inhibition results are means ± S.E.M. from three or more independent experiments performed in duplicate.

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<th>LigScore2</th>
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<th>DockScore</th>
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<th>PfPdx1 inhibition (% residual activity)</th>
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*Absorbance of compound interfered with detection of PLP.

** P < 0.10 in a two-tailed Student's t test.

Effect of 4PEHz and EH2 at the cellular level

The effect of 4PEHz and EH2 was determined on *P. falciparum* parasite proliferation. After 48 h of incubation of intra-erythrocytic *P. falciparum* parasite cultures with 4PEHz, an IC$_{50}$ of $10.4 \pm 1.2 \mu M$ was determined (Figure 5A). The unphosphorylated EH2 was 13-fold weaker than 4PEHz, with an IC$_{50}$ of $138 \pm 9 \mu M$ (Figure 5A). To elucidate further the mode of action of 4PEHz in vivo, the compound was tested on *P/Pdx1/P/Pdx2*-overexpressing *P. falciparum* parasites [6]. Both mock control and *P/Pdx1/P/Pdx2*-overexpressing parasite proliferation was monitored over a 7-day period in the presence of $1 \mu M$ 4PEHz. The proliferation of mock parasites treated with 4PEHz significantly decreased over the course of 7 days (Figure 5B). Relative to control mock parasites, the proliferation was significantly ($P < 0.05$) affected by 4PEHz only after 48 h, and the parasite growth diminished to 10% of control parasites after 7 days. In contrast, parasites overexpressing *P/Pdx1* and *P/Pdx2* were not significantly affected by 4PEHz treatment, and showed a relatively unchanged growth profile up until 6 days. After 7 days, the growth of the *P/Pdx1/P/Pdx2*-overexpressing parasite growth was not significantly different from untreated control parasites. This suggested that complementation of PLP biosynthesis through overexpression of *P/Pdx1* and *P/Pdx2* protects the parasites from the effects of 4PEHz.

Additionally, the growth rate constants of mock and *P/Pdx1/P/Pdx2*-overexpressing parasites were compared using logarithmic growth curves (Supplementary Figure S2 at http://www.biochemj.org/bj/449/bj4490175add.htm). The slopes of the fitted lines were used to calculate the growth rate constant which represents the increase in parasite growth per unit time. The growth rate constant of the mock parasites treated with 4PEHz was smaller than that of untreated mock parasites. Statistical analyses comparing the slopes of the treated mock and untreated mock fitted lines revealed a significant difference with accompanying ANCOVA confirming this observation. This indicates that 4PEHz had an effect on parasite growth. In contrast, *P/Pdx1/P/Pdx2*-overexpressing parasites had similar growth rate constants, and the slope of the fitted lines were not statistically different, indicating that the growth rate of the *P/Pdx1/P/Pdx2*-overexpressing parasites was unaffected by 4PEHz treatment. The effect of 4PEHz on the parasite life-stage composition was also investigated (Supplementary Figures S3 and S4 at http://www.biochemj.org/bj/449/bj4490175add.htm). During the 7-day treatment period with 4PEHz, the ring-stage and trophozoite parasites from treated mock parasites had significantly different growth compositions on a single day when compared...
Inhibition of Plasmodium falciparum PLP synthase

Figure 1 Effect of alternative sugar substrates on PfPdx1

(A) Inhibition of PfPdx1 by DR5P. Representative UV-visible absorbance spectra of PfPdx1 incubated with increasing concentrations of DR5P. Decreased levels of PLP, visible as diminished 414 nm PLP Schiff base absorbance, correlated with increased concentrations of DR5P. AU, absorbance units.

(B) Potential utilization of alternative sugars by PfPdx1. Representative UV-visible absorbance spectra of PfPdx1 incubated with 3 mM R5P or E4P; and 12 mM F6P or DR5P in the presence of G3P. Only R5P supported the formation of PLP, visible at 414 nm, and in the absence of G3P (−G3P), the I320 species could be formed from R5P. A 320 nm maximum absorbance peak was observed for E4P. (C) R5P docked into PfPdx1 using LigandFit. The ligand poses or receptor-ligand binding affinities were calculated using LigScore1, with units of pKᵢ (−logKᵢ), in which R5P could be docked back into the structure with a score of 4.32. (D) E4P docked into the R5P-binding site of PfPdx1. The molecule was predicted to hydrogen bond with Lys83, mainly involving the aldehyde and hydroxy groups, similarly interacting with Asp27. E4P was calculated to have a LigScore1 of 3.00.

with untreated mock parasites (Supplementary Figure S3). In contrast, the P/Pdx1/P/Pdx2 parasite life-stage compositions remained relative unchanged and no significant differences in parasite stage growth was observed (Supplementary Figure S4). The complementation of PLP biosynthesis rescued the parasites from the effects of 4PEHz, and also suggests that 4PEHz affects P/Pdx1-related processes within the parasites.

DISCUSSION

With antimalarial drugs being rendered ineffective at alarming rates, newer chemical leads aimed at targeting diverse metabolic pathways are required. The malaria parasite depends on de novo biosynthesis of PLP by PLP synthase and the cofactor plays an essential role during amino acid metabolism and folate biosynthesis [7]. PLP-dependent enzymes involved in amino acid biosynthesis principally bind PLP via an imine bond or Schiff base through utilizing an active-site lysine residue [33]. PLP has recently been ascribed potent antioxidant roles and has been proposed to aid in reducing parasite oxidative stress associated with haem and free-iron release during digestion of host erythrocyte haemoglobin [6,34]. The components of the PLP synthase are up-regulated when parasites are exposed to singlet molecular oxygen (¹O₂), and, through protein complementation, P/Pdx1 and P/Pdx2 were associated directly with detoxification of ¹O₂ [6]. PLP biosynthesis is indispensable to the parasites and this pathway is also absent from humans, making it an ideal target for the development of chemotherapeutics.

Three distinct sites for binding of the reaction substrates and products have been identified on Pdx1. Crystallographic evidence for the R5P-binding site was provided from TmPdx1 with Ru5P bound via an imine to the Lys⁶² residue (equivalent to PfPdx1 Lys⁸³) [31]. In the Saccharomyces cerevisiae Pdx1 ScPdx1 structure, an additional site, observed previously to contain a phosphate ion, was shown to accommodate PLP [12,13,31]. Residues of the PLP-binding site in P/Pdx1 include the highly conserved ERR triad comprising Glu¹³⁶, Arg¹³⁹ and...
Arg\textsuperscript{130}, which interact mainly with the phosphate groups of PLP \[13,24\]. Moreover, a site which contained G3P was identified and involved Arg\textsuperscript{164} (equivalent to Arg\textsuperscript{167} in P/Pdx1) [13]. The R5P-, PLP- and G3P-binding sites are located more than 10 Å from each other, and, even though much is known regarding the reaction mechanism, some questions regarding substrate mobility and dynamics from these distant binding sites remains to be uncovered.

The Pdx1 protein of PLP synthase is highly conserved, and could imply evolutionary maintenance of specialized function [34,35]. Mechanistic elucidation of the Pdx1 reaction co-ordinate has highlighted the multitude of complex mechanistic steps involved in forming PLP [10,11,15]. The reaction mechanism consists of several imine bond formations [10]. Initial binding of R5P entails formation of an imine with a Pdx1 lysine residue (Figure 6) followed by isomerization into a Ru5P imine adduct. The bound intermediate is subsequently converted into the I\textsubscript{320} chromophore, which forms an imine bond with G3P and undergoes ring closure to ultimately form PLP [10] (Figure 6). Both substrates R5P (acyclic-forms) and G3P have reactive C1 aldehyde groups and are phosphorylated. The α-hydroxy or C1 aldehyde group on both R5P and G3P is essential for initial substrate binding and I\textsubscript{320} formation, as well as G3P binding. The phosphate group of G3P forms part of the final PLP molecule, whereas the phosphate group of R5P is eliminated during the reaction mechanism [10] (Figure 6). In TmPdx1, the phosphate group of R5P was shown to form hydrogen bonds with three glycine amide nitrogen atoms and could function to facilitate orientation of the molecule during catalytic conversion [13].

We sought to identify molecules which could be accommodated within the R5P active site of P/Pdx1, possibly affecting PLP formation, which would make attractive starting points for the development of novel antimalarials. Virtual screening of the P/Pdx1 R5P-binding site pharmacophores identified compounds with diverse chemical scaffolds. Several polyhydroxylated compounds with sugar-like backbones similar to that of R5P were identified; however, these were not effective at inhibiting P/Pdx1.

Compounds with terminal sulfonate groups, which were predicted to mimic phosphate groups of R5P in the R5P-binding cavity were ineffective at inhibiting P/Pdx1. The reasons for this became clear when we tested closely related analogues of R5P, which were more effective at inhibiting P/Pdx1. Initial binding to P/Pdx1 requires Schiff-base adduct or imine formation with internal lysine residues, and computationally predicted compounds were not capable of this owing to the lack of reactive functional groups.

DR5P inhibited P/Pdx1, and marginally reduced formation of I\textsubscript{320}, suggesting that DR5P may compete with R5P, albeit weakly. Following the currently proposed reaction mechanism of P/Pdx1, DR5P could be accommodated by P/Pdx1 and potentially bind to Lys\textsuperscript{83} [10]. The DR5P adduct might not undergo conversion owing to the lack of the C2 hydroxy substituent, which could affect the resident time within the active site, possibly explaining the lack of potency of DR5P. E4P effectively inhibited P/Pdx1. Structurally, E4P is similar to R5P, with one fewer carbon and hydroxyl substituent. We speculated that E4P could interfere with R5P binding in P/Pdx1. Docking simulations predicted that E4P could occupy the R5P-binding site and that the sugar-derived hydroxy substituents as well as phosphate moiety have favourable hydrogen-bonding interactions within this site. E4P could outcompete R5P for Lys\textsuperscript{83} imine formation, and potentially also undergo conversion similar to R5P (Figure 6). This would affect binding of R5P and lead to disrupted PLP formation. The E4P analogue DXP, with a terminal ketone group, was not an effective inhibitor. This suggested the C1 aldehyde reactivity, as found on E4P, was important for inhibition, and this feature could possibly determine entry into the R5P active site. D-Erythrose was almost 50-fold weaker than E4P, suggesting that the phosphate group is also essential for efficacy. Similar to R5P, the phosphate group of E4P might facilitate orientation of the molecule in the R5P-binding site. The structure–activity relationship of E4P and its analogues suggested that both the terminal α-hydroxy C1 aldehyde- and hydrolysable phosphate-group arrangements were important for effective inhibition of P/Pdx1.

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Table 2  E4P analogues and their efficacy on P/Pdx1

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<th>LigScore2</th>
<th>DockScore</th>
<th>IC\textsubscript{50} on P/Pdx1</th>
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<td>85.17 (120.89)</td>
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<td><img src="image" alt="4PEHz Structure" /></td>
<td>6.55 (3.73)</td>
<td>6.43 (1.46)</td>
<td>98.96 (117.01)</td>
<td>43 ± 8 μM</td>
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Compounds were docked into a PfPdx1 homology model and poses were evaluated using LigScore ligand pose scoring functions. R5P was used as a control during docking simulations, with LigScore and DockScore values listed in Table 1. LigScore1 and LigScore2, with units of pK\textsubscript{i}, (−logK\textsubscript{i}), refer to predicted receptor–ligand binding affinities. DockScore refers to the unitless rigid-body minimization energy of the final ligand pose calculated during Monte Carlo trials. Values in parentheses indicate scores calculated for ligands in PfPdx1. IC\textsubscript{50} values were calculated from dose–response curves of the respective compounds incubated with purified P/Pdx1, as reported in the Experimental section. IC\textsubscript{50} values are means ± S.E.M. from three or more independent experiments performed in duplicate.
Spinach RPI is inhibited by E4P, and more so by 4-phosphoerythronic acid [36]. The weak RPI activity of \( \text{PfPdx1} \) could therefore similarly be targeted by such molecules. GPIs (glucose-6-phosphate isomerases) are inhibited by acyclic phospho-sugars which have free carbonyl groups, like E4P [37–39]. Moreover, the GPI active site resembles that of TIMs, a function that has been implicated in Pdx1 proteins [40,41]. Even though no definitive site for DHAP isomerization has been found, the implicated TIM function of \( \text{PfPdx1} \), albeit weak, could be affected by E4P [13,14]. Therefore an alternative mode of action of E4P could involve binding to the postulated G3P-binding site [13]. E4P might interact with Lys\(_{120}\) (equivalent to ScPdx1 Lys\(_{117}\)), whereby E4P would mimic G3P, potentially interfering with downstream PLP ring-closure steps. E4P could therefore affect either the RPI or the TIM activity in \( \text{PfPdx1} \).

The present study suggests for the first time that the naturally occurring E4P metabolite could regulate PLP production in \( \text{P. falciparum} \) parasites through inhibition of \( \text{PfPdx1} \). Considering that the DXP-dependent pathway in \( \text{E. coli} \) utilizes the E4P precursor for the production of PLP [42], it is interesting from an evolutionary point of view that this molecule inhibits the plasmodial Pdx1 in the DXP-independent pathway. E4P is a vital precursor for the production of chorismate, and downstream phenylalanine, tyrosine and \( p \)-aminobenzoic acid, used for the
production of folates [43]. The pathway for the production of E4P from sedoheptulose 7-phosphate by transaldolases has not been identified in *P. falciparum* [44]; however, transketolases are known to produce E4P from F6P and G3P [45]. E4P is an indispensable metabolite to the parasites, and future studies might aim to explore the in vivo effects of E4P fluctuations on PLP production.

From the *Tn*Pdx1 structure, it was shown that Asp26 and Lys83 constitute the R5P-binding site, therefore could be directly involved in I320 formation [31,46]. Our observations for the DKK P/Pdx1 mutant confirmed previous results, and the residues involved in I320 formation are most probably Asp26 and Lys83 in *P*/Pdx1 [15,31]. Moreover, our findings support previous results which showed that the ΔC Pdx1 mutant is incapable of forming I320 [30]. Arg190 and Arg140 of the ERR motif form part of the PLP-binding site [13,31,46]. Mutation of these residues was shown to abolish P/Pdx1 activity, underscoring their catalytic importance most likely during PLP binding and release [24]. Our observations revealed that the ERR P/Pdx1 variant was still able to form I320. This suggested that these residues were not directly involved in R5P isomerization leading to formation of the chromophore. The highly conserved Arg85, His88 and Glu91 of the RHE motif of *P*/Pdx1, located on the loop between β3 and α3, are essential for Pdx1 hexamer assembly and subsequent Pdx2 binding and activation [24]. These residues were suggested to facilitate Pdx1 hexameric pre-assembly, which is co-ordinated and a prerequisite for PLP synthase activation [24]. In the absence of *P*/Pdx2, the RHE triple mutant was also incapable of forming the I320 species. Lack of I320 formation in the RHE mutant indicates that these residues are intricately involved in R5P binding, additionally taking part in active-site reorganization during dodecamer assembly. A glycine residue (Gly84) located on the same loop region as these RHE residues was shown to form hydrogen bonds with Asp110 and a water molecule which forms part of the G3P-binding site [13]. This suggests that the RHE-containing loop region is vital for both assembly and enzyme activity and emphasizes that Pdx1–Pdx1 interactions, involving these RHE residues, could serve as a target site for the development of novel target-based inhibitors.

The moderate inhibitory efficacy of E4P against *P*/Pdx1 motivated testing of E4P analogues against the enzyme. 4PEHz inhibited *P*/Pdx1 and the entire PLP synthase complex with low
the phosphate group, suggests that the enzyme preferentially allows the entry of more negatively charged molecules such as 4PEHz. Similar observations for E4P compared with d-erythrose also support this hypothesis. In both E4P and 4PEHz, the presence of hydrolysable phosphate groups improved inhibitory activity and suggests that these two molecules could similarly occupy the R5P-binding site on the protein, which is also supported by in silico docking results. As outlined in Figure 6, 4PEHz shares chemical similarities to both E4P and R5P, suggesting a similar mode of action compared with E4P.

4PEHz inhibited parasite growth at low micromolar concentrations, compared with the unphosphorylated EH2 analogue, which was at least 12-fold weaker. PLP is an essential cofactor, and continuously recycled within the parasite, therefore, expectedly, attenuation of PLP biosynthesis has a dramatic effect on parasite proliferation. The complementation of P/Pdx1 and P/Pdx2 in P. falciparum parasites in vivo was shown to increase tolerance to cercosporin-induced oxidative stress [6,48]. In the present study, we similarly observed that parasites which overexpress P/Pdx1 and P/Pdx2 were not affected by the P/Pdx1 inhibitor 4PEHz. In contrast, parasites harbouring the same expression plasmid, without P/Pdx1 and P/Pdx2, had reduced growth rates and were significantly attenuated by 4PEHz. Almost 3-fold greater PLP levels were reported previously in P/Pdx1/P/Pdx2 complemented parasites [6]. This complementation of PLP synthesis in vivo counteracts the effects of 4PEHz, and suggests that 4PEHz interferes primarily with this part of the parasite metabolism. These results support 4PEHz as a novel lead compound for targeting vitamin B6 biosynthesis within the malaria parasite.

Endogenous pyridoxal/pyridoxine-salvage pathways might be capable of complementing the PLP pool in the parasites and could diminish the effects of P/Pdx1 inhibitors. Indeed, the parasite possesses a functional pyridoxal kinase (PdxK), which activates salvaged B6 vitamers. This pathway has successfully been exploited using prodrugs which are trapped in the parasite upon phosphorylation by PdxK [29]. Once phosphorylated, these PLP analogues were shown to disrupt PLP-dependent processes and thus kill the parasite selectively [29]. However, pyridoxal is bound to haemoglobin and so is not available for uptake. Furthermore, the parasite shows no growth defect when cultured in vitamin B6-free medium [7]. Since 4PEHz inhibits parasite proliferation in culture medium containing approximately 5 μM pyridoxine, this suggests that 4PEHz is effective irrespective of endogenous vitamin B6-salvage pathways. Nevertheless, in combination with this strategy, compounds that target P/Pdx1 directly may contribute even more effectively by additionally starving the parasites of PLP and thus leading to parasite death. Novel lead scaffolds were identified against P/Pdx1, and were capable of targeting PLP biosynthesis within the P. falciparum parasites. Lead compounds should ideally have greater potency, and, undoubtedly, these compounds will have to undergo further optimization to improve their efficacy. These relatively simplistic low-molecular-mass compounds are well suited for additional molecular mass contributions from chemical modifications or functionalization during scaffold modifications. These compounds had structural complementarity to R5P and more noticeably contained relatively reactive C1 modifications. These compounds had structural complementarity to R5P and more noticeably contained relatively reactive C1 modifications. These compounds had structural complementarity to R5P and more noticeably contained relatively reactive C1 modifications. These compounds had structural complementarity to R5P and more noticeably contained relatively reactive C1 modifications. These compounds had structural complementarity to R5P and more noticeably contained relatively reactive C1 modifications. These compounds had structural complementarity to R5P and more noticeably contained relatively reactive C1 modifications.

Figure 5 Efficacy of 4PEHz on the proliferation of intra-erythrocytic P. falciparum parasites

(A) Parasite proliferation was assessed by monitoring the [3H]hypoxanthine incorporation. After 48 h, parasite proliferation was reported as the percentage of [3H]hypoxanthine incorporation compared with that for uninhibited control parasites. The IC50 for 4PEHz was 10.4 ± 1.2 μM, compared with 138 ± 9 μM for EH2. Results are means ± S.E.M. from more than three independent experiments performed in triplicate. Broken lines indicate 95% confidence intervals. (B) The growth of P/Pdx1/P/Pdx2-complemented and mock control parasites was determined in the presence of 1 μM 4PEHz. The parasitaemia (percentage of infected erythrocytes per 1000 erythrocytes) of the treated parasites was compared with untreated parasites and expressed as a percentage growth relative to the untreated control. Results are means ± S.E.M. from two independent experiments performed in triplicate. Comparisons between treated and untreated parasites were made using an unpaired two-tailed Student’s t test. **P < 0.01. The mock parasites were significantly affected after 48 h, and had diminished growth over the 7 day period. Parasites complemented with P/Pdx1 and P/Pdx2 were not significantly affected by 4PEHz treatment, suggesting that 4PEHz affects PLP-related processes within the parasites, and complementation of PLP biosynthesis protects these parasites from the P/Pdx1 inhibitor.
Figure 6  Schematic illustration of the formation of PLP from R5P, G3P and L-glutamine

Initial binding of R5P in Pdx1 entails imine formation of the C1 of R5P with Lys83. Following binding, R5P undergoes isomerization, imine formation with ammonia and a C1 to C5 lysine migration. Loss of phosphate results in the formation of I320, followed by imine formation with G3P, which leads to ring closure and formation of PLP. E4P and 4PEHz both inhibit PfPdx1 and share polyhydroxy stereochemical arrangement and contain hydrolysable phosphate groups similar to R5P. E4P contains a C1 aldehyde group, which is proposed to functionally mimic similar groups in R5P, and could facilitate entry into the PfPdx1 R5P active site. 4PEHz, with a terminal hydrazide group, may similarly enter the active site, and interfere with PLP formation. Original pathway data taken from [10].

mechanism also needs to be considered in order to achieve better inhibitor efficacy. Additionally, the mode and site of binding in PfPdx1 will need to be determined, and may provide insights into the mode of action of these inhibitors.

AUTHOR CONTRIBUTION

Experiments were performed by Shaun Reeksting, Ingrid Müller and Carsten Wrenger. Materials and reagents were provided by Emmanuel Burgos and Laurent Salmon. Data were analysed by Shaun Reeksting, Ingrid Müller, Pieter Burger, Abraham Louw, Lyn-Marie Birkohtz and Carsten Wrenger. The paper was written by Shaun Reeksting, Ingrid Müller, Pieter Burger, Abraham Louw, Lyn-Marie Birkohtz and Carsten Wrenger.

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S.B.R. conducted part of this work in fulfilment of the requirements for a Ph.D. from the University of Pretoria.

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Inhibition of *Plasmodium falciparum* PLP synthase


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SUPPLEMENTARY ONLINE DATA

Exploring inhibition of Pdx1, a component of the PLP synthase complex of the human malaria parasite Plasmodium falciparum

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Figure S1 Detection of PLP is not affected by E4P

Standard curves of PLP, containing concentrations ranging from 10 to 200 μM PLP with no E4P (A), or containing 1 mM (50 nmol) E4P (B), in Tris/HCl buffer conditions including 0.5 mM R5P and G3P with 20 mM NH₂Cl. Reactions were incubated at 37 °C for 1.5 h. Statistical analyses of the linear regression slope and intercepts revealed no significant differences in the slopes of the two different regression lines (P = 0.975). Similarly, ANCOVA showed no significant difference (P = 0.401) between the two regression lines. Results represent data from four independent experiments performed in triplicate. This verifies that molar excess concentrations of E4P did not affect detection of the PLP Schiff base formed at 414 nm.

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Figure S2 Effect of 4PEHz on mock and PfPdx1/PfPdx2-overexpressing transgenic parasites

Mock control cells (A) and PfPdx1/PfPdx2-overexpressing cells (B) were treated with 1 μM 4PEHz in continuous culture for 7 days. The cumulative parasitaemia (cum. parasitemia) was calculated from the observed parasitaemia taking the dilution factor into account. Results are means ± S.E.M. from two independent experiments performed in triplicate. Statistical analyses using GraphPad Prism tested a null hypothesis that the lines were the same with a 99% confidence interval (P = 0.01, F = 0.01). Mock-UT and Mock-T (B) had significantly different slopes (P < 0.0001), whereas PfPdx1/PfPdx2-UT and PfPdx1/PfPdx2-T (C) did not (P = 0.0138). Similarly ANCOVA analyses comparing Mock-UT with Mock-T revealed that the lines differed significantly (F-ratio probability <0.0001), whereas Pdx1/2-UT did not significantly differ from PfPdx1/PfPdx2-T (F-ratio probability = 0.0986). T, treated; UT, untreated.
Figure S3  Composition of parasite stages in mock control parasites during long-term exposure to 4PEHz

The parasite life-stage composition (percentage of rings, trophozoites or schizonts) of untreated (UT) (A) and treated (T) (B) *P. falciparum* mock cells was determined microscopically during long-term growth assays. Results are means ± S.E.M. from two independent experiments performed in triplicate. Comparisons between treated and untreated mock parasites were made using an unpaired two-tailed Student’s t test. **P < 0.05. The treated mock parasites had a significantly different ring and trophozoite life-stage composition compared with the untreated controls parasites at 120 h (5 days), whereas all other time points did not reveal significant differences in the life-stage compositions.
Figure S4 Composition of parasite stages in *PfPdx1/PfPdx2*-complemented parasites during long-term exposure to 4PEHz

The parasite life-stage composition (percentage of rings, trophozoites or schizonts) of untreated (UT) (A) and treated (T) (B) *PfPdx1/PfPdx2*-overexpressing parasites was determined microscopically during long-term growth assays. Results are means ± S.E.M. from two independent experiments performed in triplicate. Comparisons between treated and untreated mock parasites were made using an unpaired two-tailed Student’s t test. **P < 0.05. There were no significant differences in the life-stage compositions between untreated and treated *PfPdx1/PfPdx2* parasites at any of the time points. This confirmed that complementation of PLP biosynthesis protected the parasites from the effects of 4PEHz, and the parasites grew normally compared with untreated cells.