Potential role of *Plasmodium falciparum* exported protein 1 in the chloroquine mode of action

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**ABSTRACT**

In the human malaria parasite *Plasmodium falciparum*, membrane glutathione S-transferases (GST) have recently emerged as potential cellular detoxifying units and as drug target candidates with the artemisinin (ART) class of antimalarials inhibiting their activity at single-digit nanomolar potency when activated by iron sources such as cytotoxic hematin. Here we put forward the hypothesis that the membrane GST *Plasmodium falciparum* exported protein 1 (PfEXP1, PF3D7_1121600) might be directly involved in the mode of action of the unrelated antimalarial 4-aminoquinoline drug chloroquine (CQ). Along this line we report potent biochemical inhibition of membrane glutathione S-transferase activity in recombinant PfEXP1 through CQ at half maximal inhibitory CQ concentrations of 9.02 nM and 19.33 nM when using hematin and the iron deficient 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, respectively. Thus, in contrast to ART, CQ may not require activation through an iron source such as hematin for a potent inhibition of membrane GST activity. Arguably, these data represent the first instance of low nanomolar inhibition of an essential *Plasmodium falciparum* enzyme through a 4-aminoquinoline and might encourage further investigation of PfEXP1 as a potential CQ target candidate.

1. Introduction

The molecular and biochemical mechanisms of action for the 4-aminoquinoline chloroquine (CQ) remain to be fully understood, with one leading antimalarial effect depending on reducing the buildup of non-toxic hemozoin (Hz) biocrystals originating with the toxic heme that is liberated inside the malaria parasite's food vacuole (FV) upon catabolism of the host red blood cell (RBC) hemoglobin during the asexual blood stages of *Plasmodium falciparum* malaria (Ecker et al., 2012). However, even under normal conditions without CQ, a significant portion of heme, which upon liberation from hemoglobin inside the FV is rapidly oxidized to form cytotoxic hematin, evades conversion into hemozoin: estimates (Egan et al., 2002; Ginsburg et al., 1998) vary between 5% and 70% of the total heme/hematin stored at several mM concentrations inside the FV (Loria et al., 1999). But heme/hematin is long known to directly disrupt membrane structure and kill malaria parasites already at low micromolar concentrations (Orjih et al., 1981). Thus until it is shown that at any point during the asexual intraerythrocytic stages of malaria at least 99.9% of the free heme/hematin is readily transformed into Hz before its toxic and lethal effects on parasite membranes can take place other heme/hematin protection, sequestration or degradation mechanisms must be necessarily considered. For example, heme/hematin may also be degraded through a glutathione-dependent mechanism that complements Hz formation (Ecker et al., 2012; Ginsburg et al., 1998; Lisewski et al., 2014) in which initially the thiol group of reduced GSH spontaneously (i.e., without additional enzyme action) forms adducts with the iron inside the porphyrin ring (see, Fig. 1A for a model reaction). Such glutathione...

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mediated degradation pathways might also constitute potential anti-malarial drug targets and play a role in drug resistance (Muller, 2015). We have recently identified (Lisewski et al., 2014) a more efficient enzymatic hematin reaction based on the functional characterization of the parasite's essential gene product Plasmodium falciparum exported protein 1 (PfEXP1, also referred to as antigen 5.1, Ag5.1, or circumsporozoite related antigen, CRA, with gene identifier PF3D7_1121600). In contrast to the other known soluble, cytosolic Plasmodium falciparum glutathione S-transferase (PfGST, PF3D7_1419300) the evolutionarily unrelated PfEXP1 is from the parasitophorous vacuolar and early FV

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**Fig. 1.** Schematic overview of glutathione S-transferase (GST) mediated degradation reaction of the substrates hematin (A) and 1-chloro-2,4-dinitrobenzene (B). In (B), GST enzymatically generates a thiolate anion $G{S}^{-}$ by proton removal from GSH. The thiolate then conjugates with CDNB, producing an intermediate Meisenheimer complex which, after chloride dissociation, gives the final glutathionyl-dinitrobenzene (GS-DNB) conjugate. In (A), a possible conjugate of a single glutathione molecule with the iron is not depicted. (C) CQ potently inhibits the GST activity of recombinant and bacterially (E. coli) expressed PfEXP1 toward hematin with half maximal inhibitory concentration ($IC_{50}$) of $9.02 \pm 0.69 \text{nM}$ and a Hill coefficient $n_H = 0.53 \pm 0.02$. Inset: Inhibition kinetics from absorbance spectrophotometry at 395 nm at zero (red, solid line) and at 100 nM CQ concentration (orange, dashed line). (D) CQ also potently inhibits the GST activity of PfEXP1 toward the standard GST substrate CDNB with an $IC_{50}$ of $19.33 \pm 2.67 \text{nM}$ and $n_H = 0.51 \pm 0.04$. Inset: Inhibition kinetics from absorbance spectrophotometry at 340 nm at zero (red, solid line) and at 100 nM CQ concentration (orange, dashed line). The corresponding uninhibited (at 0 nM CQ concentration) GST specific activity toward CDNB was estimated at $7.3 \pm 2.6 \text{μmol/min/mg}$. Fractional velocities calculated from slopes to linear least square fits of the kinetics data. $IC_{50}$ and Hill coefficient values $n_H$ are least square fits to the standard dose response curve $1-(1 + 10^{n_H \cdot (\log IC_{50} - \log [CQ])})^{-1}$. Error bars represent standard errors from at least three measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Fig. 2. (A) CQ inhibition of membrane GST activity in recombinant PfEXP1, expressed in the yeast Pichia pastoris, toward the substrate hematin with a half maximal inhibitory concentration (IC_{50}) of 11.82 ± 2.33 nM, and (B) of 11.12 ± 4.12 nM with CDNB as substrate. In contrast, the other quinoline antimalarial drug tested, the quinoline-4-methanol quinine, displays a more than tenfold weaker inhibition with an IC_{50} of 233.03 ± 41.19 nM and of 179.04 ± 52.14 nM, respectively.

2. Results

Our enzyme kinetics data indicate that CQ is a potent inhibitor of PfEXP1-mediated hematin degradation, with an estimated IC_{50} of 9.02 ± 0.69 nM, after using the same spectrophotometry assay as described in Lisewski et al. (2014) with purified PfEXP1 after its recombinant bacterial expression in Escherichia coli (Fig. 1C). A PfEXP1 purity level of 90% or more in the prepared protein solution used for our inhibition studies was also confirmed through monoclonal antibodies to PfEXP1, which inhibited GST activity of the PfEXP1 extract with an estimated IC_{50} of 0.6 ± 0.2 ng/ml (Supplementary Information Table S1). In the drug inhibition assay (see Experimental Methods), hematin degradation over time was measured by tracing the height of its characteristic Soret absorption peak at 395 nm. The inhibition of GSH-mediated hematin degradation through CQ was also observed through liquid chromatography mass spectrometry of the reaction product, i.e. by monitoring the yield of glutathione-hematin complexes. Increasing CQ concentrations lowered GSH-hematin levels below the level of spontaneous GSH-hematin formation (Supplementary Information, Fig. S1A). In contrast, inhibition of GSH-hematin formation through ART did not reach below this point of spontaneous formation. These data suggest that at higher drug concentrations above the micromolar range CQ, but not ART, binds to hematin and prevents its association with GSH, which recalls earlier observations about CQ-heme/hematin associations (Atamma and Ginsburg, 1995 and Ginsburg et al., 1998).

The low nanomolar potency at which CQ displays PfEXP1 inhibition is not due to its association with a potential iron carrying activator such as hematin because CQ also inhibits PfEXP1 activity without hematin, e.g. in our case when hematin was replaced with the standard GST assay substrate 1-chloro-2,4-dinitrobenzene (CDNB, see also Fig. 1B and D), with an estimated IC_{50} of 19.33 ± 2.67 nM. Thus CQ has similar potency in PfEXP1 GST inhibition toward hematin (IC_{50} of 9.02 ± 0.69 nM, see above) as toward CDNB. This mode of inhibition probably does not depend on a potential formation of tight CQ-CDNB complexes: in the concentration range between 1 nM and 10^5 nM liquid chromatography mass spectrometry did not detect any CQ concentration dependent formation of these adducts (Supplementary Information, Fig. S1B), indicating that CQ is inhibiting PfEXP1 directly and not through a pre-formation of covalently bound CQ-CDNB complexes. Because PfEXP1 units form homo-oligomers inside the membrane (Spelmann et al., 2006), we further analyzed whether cooperativity plays a role during PfEXP1 inhibition. For CQ, a Hill equation analysis resulted in n_H = 0.53 ± 0.02 for hematin, and in n_H = 0.51 ± 0.04 for CDNB, which, under the assumption that this indicator of negative cooperative binding is not due to a mixture of nonidentical binding sites, suggest binding to at least 1/n_H = 2 identical sites (Abeliovich, 2005). A similar analysis for ART resulted in n_H = 0.56 which points to a similar level of cooperativity.

To further assess the robustness and specificity of our results, we tested inhibition of recombinant PfEXP1 protein this time produced in a eukaryotic expression system (the yeast Pichia pastoris, see Experimental Methods). In this setting, the potent, low nanomolar inhibition of GST activity from highly purified protein through CQ was confirmed (Fig. 2A and B) with estimated IC_{50} of 11.82 ± 2.33 nM for hematin and 11.12 ± 4.12 nM for CDNB. Inhibition specificity among quinoline antimalarial drugs was also tested by choosing the main quinoline-4-methanol representative quinine, one of the oldest known antimalarials. Quinine inhibition of PfEXP1 was markedly weaker for both substrates hematin and CDNB with IC_{50} of 233.03 ± 41.19 nM.
and of 179.04 ± 52.14 nM, respectively. This result suggests that in Plasmodium falciparum 4-aminquinolines might be about an order of magnitude more potent inhibitors of membrane GST than quinolone-4-methanols.

3. Discussion

When the antimalarial drug CQ accumulates by a factor > 1000 inside the FV (Ecker et al., 2012), hemozoin formation is thought to be blocked by the association of chloroquine with heme into complexes (Chou et al., 1980; Xu Kelly et al., 2001). These adducts have a dissociation constant near 5 μM (Egan et al., 1997; Xu Kelly et al., 2001) and are toxic to the parasite (see the more recent result in Xu Kelly et al., 2001), with direct isothermal titration calorimetry measurements of CQ-hematin associations, for the apparent disparity to earlier indirect equilibrium measurements by Chou et al. (1980). If, as here suggested at the purely molecular level in vitro, potent, low nanomolar inhibition of PfEXP1 through CQ would occur also inside the parasite in vivo, then PfEXP1 might be further considered as a potential early target of CQ. This is because unlike the other target heme/hematin this mode of action would not require a > 1000 fold drug accumulation to reach CQ concentrations sufficiently high for its direct antimalarial effects.

We note that our measured IC50 quite precisely match growth inhibition IC50 in CQ sensitive falciparum strains reported at 10 nM (strain NF54) and 8 nM (strain D6), see Delves et al. (2012). In comparison to these sensitive strains, PfEXP1 differential expression upon CQ challenge in CQ resistant strains is significantly enhanced (Koncarevic et al., 2007), a pattern also more recently observed for mefloquine (Segura et al., 2014), and for artesunate (Lisewski et al., 2014).

Only during the parasite’s main growth phase, i.e. during the asexual blood stages of Plasmodium falciparum, the exp1 locus and its function are likely essential (Maier et al., 2008). This is further underscored by the dose-dependent parasite growth inhibition through PfEXP1 specific monoclonal antibodies (Kara et al., 1988), the inhibition of GST activity in recombinant PfEXP1 through these antibodies (Supplementary Information, Table S1), and by the significantly reduced exp1 expression levels outside of the asexual erythrocytic stages (see, for example, the compiled life cycle expression data in the PlasmoDB database http://plasmodb.org/PlasmoDB/app/record/gene/PF3D7.1121600#ExpressionGraphs). Given the relative lack of free heme/hematin outside of infected erythrocytes, CQ main action against GST PfEXP1 mediated hematin degradation would be therefore confined to the parasite’s main growth stages inside the host’s RBCs (Delves et al., 2012).

Furthermore, this potentially essential enzymatic activity of PfEXP1 might help to explain why CQ resistant strains, which overall present lower basal PfEXP1 levels than in sensitive strains (Koncarevic et al., 2007), are less fit than CQ sensitive strains (Ecker et al., 2012; Muller, 2015). During the RBC growth stages of the parasite, lowering membrane GST mediated heme/hematin degradation activity would lead to a lower overall fitness of the parasite.

Our data also support a consideration of PfEXP1 as a potential antimalarial multi-drug target. This is underscored by the observation that both CQ and ART were discovered independently with obviously dissimilar molecular structures: chemical similarity based on the Tanimoto score is 0.074 and without statistical significance (Baldi and Nasr, 2010), yet both target PfEXP1 with high potency. This either means that ART and CQ have many high potency protein targets (Ismail et al., 2016a,b; Wang et al., 2015; Zhou et al., 2016) of which PfEXP1 may be one of many that both drugs have in common; or that such common targets are rare and that this drug promiscuity of PfEXP1 makes it an outstanding element within the entire drug target space of the human malaria parasite.

4. Experimental methods

4.1. Cloning, expression, and purification of P. falciparum EXP1

Experimental protocols were chosen closely after the methods described in Lisewski et al. (2014) as follows. P. falciparum exp1 cDNA was PCR amplified using the plasmid DNA clone pHRepExpGF (we thank Dr. Kasturi Haldar for its deposition at the Malaria Research and Reference Reagent Resource Center, MR4, Manassas, VA, USA). The amplified exp1 product was sequence verified and cloned between Ndel and HindIII restriction sites in the Kan PET28a (+) vector to enable expression of N-terminal His-tagged PfEXP1 protein (OL617, PET28a-His/PfEXP1). The exp1 plasmid was transformed into BL21 (DE3) E. coli cells. 1 L cultures in LB broth were grown at 37 °C to an OD600 of 0.5, and protein expression was induced with 0.1 mM IPTG. Induced cultures were grown for 14 h at 30 °C and cells were harvested by centrifugation and stored at −80 °C, until further processing. To extract the membrane-bound proteins, cell pellets were thawed and lysed with bacterial lysis reagent with additional detergent (octyl thioglucoside, 60 mM final concentration). His-tagged PfEXP1 from the total lysate was purified using Ni-NTA agarose affinity column chromatography. In Western blot analyses the bacterially expressed recombinant His-tagged PfEXP1 protein were resolved by SDS-PAGE using 12% gels and immunoblotted with anti-EXP1 monoclonal antibody diluted 1:5000 (kindly provided by the European Malaria repository at http://www.malarialaresearch.eu/reagents/monoclonal-antibody/S1-anti-exp1 where it was originally deposited by Dr. Jana McBride). Chemiluminescence was detected by western blotting and autoradiographed. The PfEXP1 protein was determined to be greater than 90% pure using silver-stained SDS-PAGE gels. Recombinant PfEXP1 were aliquoted and stored in GST assay buffer (pH 7.3) containing 20% glycerol and 10% Triton X-100 at −80 °C.

The P. falciparum exp1 gene was subcloned in pPICZA expression vector and transformed into yeast Pichia pastoris competent cells using the Pichia EasyComp Transformation kit (Invitrogen). The expression and solubilization of the protein were conducted as previously described for leukotriene C4 synthase (Martinez Molina et al., 2007). To purify the protein, a Ni-Sepharose column was packed with 5 mL of Ni-Sepharose Fast Flow (GE Healthcare Biosciences) by gravity flow. The column with the sample loaded was washed with 3 column volumes of buffer A (25 mM Tris (pH 7.5), 0.15 M NaCl, 5% glycerol and 0.3% DM) followed by an equal volume of buffer A containing 20 and 40 mM imidazole. PfEXP1 was eluted with buffer A containing 300 mM imidazole. The purity of the protein was checked by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS – PAGE). PfEXP1 was further purified by size exclusion chromatography using S200 10/300 (GE Healthcare) using buffer B (25 mM Tris (pH 7.5), 100 mM NaCl and 0.3% DM) and the protein was stored frozen at −20 °C The enzyme solution was always prepared without glutathione.

4.2. Glutathione S-Transferase enzyme assay

Glutathione S-transferase activity in the purified recombinant PfEXP1 toward 1-chloro-2,4-dinitrobenzene (CDNB) was assayed using the commercial GST assay kit. Assays were conducted with buffer at pH 6.5. PfEXP1 protein was preincubated with 0.1% Triton X-100 and 2 mM GSH on ice for 30 min, followed by the addition of CDNB to initiate the enzymatic reaction. The absorbance of the reaction was monitored at 340 nm, every 15 s for a period of 5 min. Assays were repeated at least three times independently for each sample, and the GST-specific activity toward CDNB was calculated as per the GST kit protocol (with a 1 ml cuvette and 1 cm path length). GST activity toward hematin was assayed by preincubating 100 nM protein with 0.1% Triton X-100, 2 mM GSH in pH 6.5 assay buffer for 30 min on ice followed by the addition of hematin to initiate hematin degradation. Absorbance was monitored at 395 nm every 15 s for 5 min.
4.3. PfEXP1 drug inhibition assay

Varying amounts of chloroquine and quinine were dissolved in water and 100% ethanol respectively and added to PfEXP1-CDNB and PfEXP1-hematin reactions to determine GSH conjugation and hematin degradation inhibition, respectively. PfEXP1 was pre-incubated in GST assay buffer pH 6.5 with 0.1% Triton X-100 and inhibitor(s) on ice for 30 min followed by the addition of 2 mM GSH and CDNB/hematin. The reaction was monitored for 3 min at 340 nm for formation of CDNB-GSH adduct and at 395 nm to monitor the loss of hematin over time.

4.4. Reaction product assay with liquid chromatography mass spectrometry (LC-MS)

The formation of GSH-hematin conjugate ions with mass-to-charge ratio m/z 923 was optimized with low fragmentation voltage; collision induced dissociation mass spectra were recorded with the parent adduct ion m/z 923 display and the m/z 615 ions that formed after loss of reduced glutathione. Samples were examined in positive ionization modes using an electrospray ionization source. Single reaction monitoring (SRM) experiments were performed using an electrospray ionization triple quadrupole mass spectrometer. The RP separation in-line with QQQ mass Agilent Technologies spectrometers. The SRP separation was carried out using an analytical Zorbax Eclipse XDB-C18 column (50 × 4.6 mm id.; 1.8 μm) that was used for SRM analysis. Reaction times were controlled for and solvent blank readings were subtracted. LC-MS monitoring of GSH-CDNB adducts was conducted in a similar way at m/z 522.

Contributions

J.P.Q., N.P. and M.M. conducted the experiments. A.M.L., J.P.Q., N.P. and M.M. processed and represented the experimental data. A.M.L., A.S. and O.L. designed the study and wrote the manuscript, with the contributions from J.P.Q., N.P., M.M. and J.Z.H.

Competing interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2017.12.003.

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