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Research paper

Hemolytic and antimalarial effects of tight-binding glyoxalase 1 inhibitors on the host-parasite unit of erythrocytes infected with *Plasmodium falciparum*

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ABSTRACT

Glyoxalases prevent the formation of advanced glycation end products by converting glycolysis-derived methylglyoxal to D-lactate with the help of glutathione. Vander Jagt and colleagues previously showed that erythrocytes release about thirty times more D-lactate after infection with the human malaria parasite *Plasmodium falciparum*. Functional glyoxalases in the host-parasite unit might therefore be crucial for parasite survival. Here, we determined the antimalarial and hemolytic activity of two tight-binding glyoxalase inhibitors using infected and uninfected erythrocytes. In addition, we synthesized and analyzed a set of diester derivates of both tight-binding inhibitors resulting in up to threefold lower IC_{50} values and an altered methemoglobin formation and hemolytic activity depending on the type of ester. Inhibitor treatments of uninfected erythrocytes revealed an extremely slow inactivation of the host cell glyoxalase, irrespective of inhibitor modifications, and a potential dispensability of the host cell enzyme for parasite survival. Our study highlights the benefits and drawbacks of different esterifications of glutathione-derived inhibitors and demonstrates the suitability of glyoxalase inhibitors as a tool for deciphering the relevance and mode of action of different glyoxalase systems in a host-parasite unit.

1. Introduction

The glyoxalase system is considered to be an ancient metabolic adaptation for the efficient conversion of reactive electrophilic 2-oxoaldehydes to 2-hydroxycarboxylic acids [1]. The system consists of reduced glutathione (GSH) and two enzymes, the isomerase glyoxalase 1 (Glo1) and the thioesterase glyoxalase 2 (Glo2). Even though there are organelles and organisms with insular glyoxalases pointing to alternative functions [1–3], Glo1 and Glo2 usually act together, for example, to catalyze the formation of D-lactate from glucose-derived methylglyoxal (MG) [1–5]. The glyoxalase system in humans prevents the accumulation of MG and of MG-derived advanced glycation end products (AGEs) with implications for numerous pathophysiological conditions including diabetes and cancer [6–9]. Inhibition of Glo1 is therefore supposed to be an elegant strategy to target cancer cells with high glycolytic fluxes that necessitate a potent MG removal system [7,10,11]. An inhibition of pathogen glyoxalases might likewise result in the accumulation of harmful MG and AGEs in bacteria and parasites with high glycolytic fluxes [2,3,12–15]. Moreover, the diverse glyoxalase systems and MG removal strategies of parasitic protists are not only potential drug targets but are also suited for the identification of alternative glyoxalase functions and mechanisms [1–3,15–19].

Two D-lactate producing glyoxalase systems are found in the host-parasite unit of erythrocytes that are infected with the human malaria parasite *Plasmodium falciparum*, one in the erythrocyte and one in the parasite cytosol [2,12,19]. The apicoplast of *P. falciparum* furthermore harbors a functional Glo2-isoform and a highly mutated Glo1-like protein that is inactive in standard enzymatic assays [2,19,20]. A methylglyoxal reductase activity was found to be negligible in the host-parasite unit and infected erythrocytes were shown to release about thirty times more D-lactate [12]. Cytosolic *Pf*Glo1 from *P. falciparum* has been thoroughly analyzed *in vitro*. In contrast to its homodimeric human homologue, monomeric *Pf*Glo1 has two different, allosterically coupled active sites [2,16,19,21]. Both active sites have deviating substrate

Abbreviations: AGEs, advanced glycation end products; Glo1, glyoxalase 1; Glo2, glyoxalase 2; GSH, reduced glutathione; GSSG, glutathione disulfide; MG, methylglyoxal; *Pf*, *Plasmodium falciparum*

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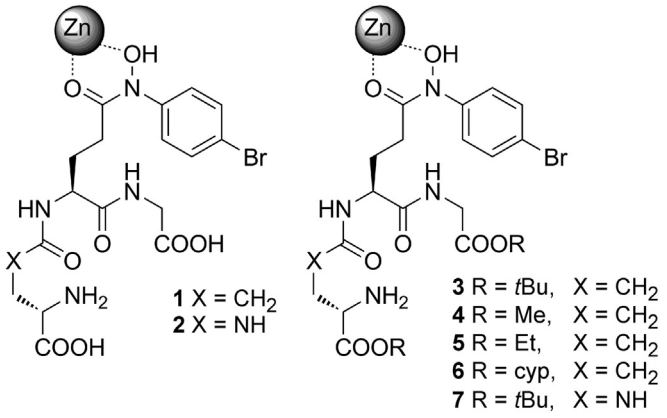

Fig. 1. Structures of Glo1 inhibitors employed in this study. The hydroxamic acid moiety mimics the transition state and interacts with the metal center of Glo1. Abbreviations used for esterifications are *t*Bu (*tert* butyl), Me (methyl), Et (ethyl), cyp (cyclopentyl).

affinities [16] and can be targeted by non-glutathione [19] and glutathione-derived inhibitors [21]. Glutathione-derived Glo1 transition-state analogues, termed compound **1** and **2** (Fig. 1), were previously shown to act as glyoxalase-specific, non-competitive tight-binding inhibitors with nanomolar K_i^{app} values for recombinant *Pf*Glo1 [21]. Here, we tested the antimalarial activity and toxicity of these inhibitors as well as a set of novel ester derivates in *P. falciparum* cell culture and on uninfected human erythrocytes. In addition, we used the inhibitors as a chemical tool to address the relevance of functional human Glo1 for parasite survival.

2. Materials and methods

2.1. Chemicals

Compounds **1** and **2** were synthesized as previously described [22]. The synthesis and validation of the ester derivates **3**–**7** is described in the Supplementary materials and methods including Scheme S1. GSH, *S*-D-lactoylglutathione, MG, and DMSO were obtained from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Growth inhibition assays and hemolysis

Direct growth inhibition of *P. falciparum* blood stage parasites was analyzed for strain 3D7 that was cultured according to standard protocols [23] at 37 °C, 5% CO_2, 5% O_2, 90% N_2 and 80% humidity in RPMI medium containing 0.45% (w/v) Albumax II, 0.2 mM hypoxanthine, 2.7 μg/mL gentamicin and human A erythrocytes at an hematocrit of 1.5–3.5%. Synchronization was carried out using the sorbitol method [24]. Inhibition of parasite growth was determined from three independent experiments by counting Giemsa-stained blood smears. Compounds **1**–**7** (50 or 25 mM stock solutions in DMSO) were diluted stepwise in culture medium in 48-well plates. Afterwards, either asynchronous parasite cultures or synchronized ring stage parasite cultures were added to the medium at a final hematocrit of 1.5% and an initial parasitemia of 0.25%. The highest final concentration of DMSO in the cultures was 0.8%. Parasites were grown for 48 h before preparation of blood smears. About 750–1500 erythrocytes were counted per Giemsa-stained blood smear and data were analyzed following the recommendations of the National Institutes of Health Chemical Genomics Center using the four parameter logistic model for the determination of IC_{50} values. As a control, hemolytic effects of the tight-binding inhibitors on unparasitized erythrocytes were analyzed in parallel. After 48 h, erythrocytes were counted in a Neubauer chamber and the release of hemoglobin into the medium was determined spectrophotometrically at 405 nm.

2.3. Inhibition of the host cell Glo1 activity

Erythrocytes from five different donors were incubated in complete RPMI medium in the presence of 10 μM compounds **1**–**3** and **7** or DMSO as a control. The activities of human Glo1 and Glo2 were measured before the addition of each compound and monitored after the addition for 96 h. Every 24 h, erythrocytes were centrifuged (5 min, 300 *g*, room temperature) and a 40 μL aliquot was removed from the cell pellet. The remaining erythrocytes were resuspended in the original medium for further incubation, whereas the 40 μl aliquot was resuspended in 500 μL of cold phosphate-buffered saline (PBS containing 1.84 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4 at 24 °C) and centrifuged again (5 min, 2000 *g*, 4 °C). The supernatant was discarded and the erythrocyte aliquot was washed two more times with PBS to remove traces of extracellular inhibitors. Washed erythrocytes were subsequently lysed by resuspension in 200 μL of ice-cold water until the solution became clear. After centrifugation (10 min, 20,000 *g*, 4 °C), the supernatant was stored on ice pending immediate enzyme assays and hemoglobin measurements. The hemoglobin content in erythrocyte lysates was determined by adding 20 μL of lysate to 980 μL of 50 mM Tris/HCl, pH 7.4 at 37 °C and measuring the absorbance at the isosbestic point at 523 nm. The concentration was calculated using the formula $[Hb] = Abs_{523nm} \cdot 50 \cdot 7.12$ [mM] or $[Hb] = Abs_{523nm} \cdot 16.1 \cdot 50 \cdot 7.12$ [g/L] [25]. Activities of human Glo1 and Glo2 were determined at 240 nm and 37 °C using a thermostatted Jasco V-550 UV–vis spectrophotometer according to standard protocols [26,27] with slight modifications. Briefly, stock solutions of 100 mM GSH and 100 mM MG were freshly prepared in cold Glo1 assay buffer (50 mM $Na_xH_yPO_4$, pH 6.6 at 37 °C) before each experiment and stored on ice. The hemithioacetal substrate was formed after mixing 955 μL of pre-warmed Glo1 assay buffer with 20 μL each of the GSH and MG stock solutions followed by an incubation at 37 °C for 9.5 min. A baseline was subsequently recorded for 30 s and the Glo1 assay was started by the addition of 5 μL of erythrocyte lysate. The absorbance was monitored for 1.5 min and the activity was calculated using $\varepsilon_{240\ nm} = 2.86\ mM^{-1}\ cm^{-1}$. For human Glo2 measurements, a stock solution of 3 mM *S*-D-lactoylglutathione was freshly prepared in cold Glo2 assay buffer (50 mMTris/HCl, pH 7.4 at 37 °C) before each experiment and stored on ice. A baseline was recorded for 30 s after mixing 890 μL of pre-warmed Glo2 assay buffer with 100 μl of 3 mM *S*-D-lactoylglutathione. The Glo2 assay was started by the addition of 10 μL of cell lysate, and the absorbance was monitored for 1.5 min. Human Glo2 activities were calculated using $\varepsilon_{240nm} = 3.1\ mM^{-1}\ cm^{-1}$. All human Glo1 and Glo2 activity measurements were performed in triplicates and were normalized to the corresponding hemoglobin concentration of the lysates. Statistical analyses were performed in SigmaPlot 12.5 using the one way ANOVA on ranks method.

2.4. Effect of the host cell Glo1 activity on P. falciparum blood stage cultures

A potential indirect growth inhibition of *P. falciparum* blood stage cultures was determined with erythrocytes that were pretreated with compounds **1**, **3** and **7** as described above. After 96 h inhibitor treatment, erythrocytes were washed three times with complete RPMI medium before adding synchronized schizont parasites (purity ≥98%) that were enriched by magnetic cell separation [28,29] using a VarioMACS™ Separator with CS columns