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Dihydroxyacetone and methylglyoxal as permeants of the *Plasmodium* aquaglyceroporin inhibit parasite proliferation

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Abstract

The aquaglyceroporin of *Plasmodium falciparum* (PfAQP) is a bi-functional channel with permeability for water and solutes. Its functions supposedly are in osmotic protection of parasites and in facilitation of glycerol permeation for glycerolipid biosynthesis. Here, we show PfAQP permeability for the glycolysis-related metabolites methylglyoxal, a cytotoxic byproduct, and dihydroxyacetone, a ketotriose. AQP3, the red cell aquaglyceroporin, also passed dihydroxacetone but excluded methylglyoxal. Proliferation of malaria parasites was inhibited by methylglyoxal with an IC_{50} around 200 μM. Surprisingly, also dihydroxyacetone, which is an energy source in human cells, was antiproliferative in chloroquine-sensitive and resistant strains with an IC_{50} around 3 mM. We expressed *P. falciparum* glyceraldehyde 3-phosphate dehydrogenase (PfGAPDH) to examine whether it is inhibited by either carbonyl compound. Methylglyoxal did not affect PfGAPDH on incubation with 2.5 mM for 20 h. Treatment with 2.5 mM dihydroxyacetone, however, abolished PfGAPDH activity within 6 h. Aquaglyceroporin permeability for glycolytic metabolites may thus be of physiological significance.

Keywords: Aquaglyceroporin; Dihydroxyacetone; Methylglyoxal; Glyceraldehyde 3-phosphate dehydrogenase; Malaria; Plasmodia

1. Introduction

In the *Plasmodium* genome, only a single aquaglyceroporin is encoded and genes for water-specific aquaporins are absent [1]. We have identified and cloned the aquaglyceroporin from *Plasmodium falciparum* (PfAQP), which causes the most severe form of malaria in humans [2]. Functional studies have established that PfAQP has excellent permeability for both, water and glycerol [3]. Therefore, we concluded that physiological roles of PfAQP may reside in (i) the protection of the parasites from osmotic stress during kidney passages or during transmission between a human and an insect host, (ii) glycerol uptake as a precursor for membrane lipid biosynthesis, and (iii) mitigation of oxidative stress by increasing the $NADH/NAD^+$ ratio [2].

Apart from water and glycerol, the PfAQP permeability profile included other uncharged solutes, such as polyols of up to five carbon-hydroxyls in length, because the layout of the inner pore surface allows compounds with different stereochemical configuration to pass [3]. Small compounds that contain carbonyl groups, such as glyceraldehyde, are also permeants of aquaglyceroporins [4]. This aldotriose and its ketotriose isomer dihydroxyacetone (DHA) are metabolized via glycolysis after phosphorylation to glyceraldehyde 3-phosphate and DHA phosphate, respectively. Non-phosphorylated DHA, however, is cytotoxic as shown in growth inhibition experiments using a yeast deletion strain that lacks both endogenous DHA kinase genes [5]. The exact mode of DHA toxicity is unknown but it is assumed that DHA covalently modifies DNA or proteins in a non-enzymatic Maillard reaction [6]. In the *Plasmodium* genome, DHA kinase genes are missing suggesting that malaria parasites could be similarly sensitive to DHA as the yeast knockout strain [5].

Another glycolysis-related carbonyl compound, methylglyoxal (MG), is toxic. The toxicity is based on the chemical reactivity of two vicinal carbonyl groups that give rise to covalent modification of protein molecules [7]. MG is derived

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from glyceraldehyde 3-phosphate as a toxic byproduct of the glycolytic pathway [8]. Since malaria parasites rely on glycolysis as the sole energy source, the intracellular buildup of MG is substantial and requires an efficient detoxification mechanism [8].

Here, we examined whether DHA and MG are permeants of PfAQP and whether incubation with these compounds affects *P. falciparum*. We report that PfAQP permitted passage of both, DHA and MG. Treatment with either compound attenuated in vitro proliferation of *P. falciparum*. We further observed inactivation of *Plasmodium* glyceraldehyde 3-phosphate dehydrogenase (PfGAPDH) by DHA treatment thus identifying this glycolytic enzyme as a potential target of DHA in plasmodia.

2. Materials and Methods

2.1. Expression of AQP1, AQP3 and PfAQP in oocytes and swelling assays

Cloning and cRNA transcription of rat AQP1, rat AQP3 and PfAQP was as described [2]. *Xenopus laevis* oocytes of stages V and VI were defolliculated with collagenase A (Roche) and injected with 5 ng of rat AQP1, 2.5 ng of PfAQP+2.5 ng of rat AQP1, and 2.5 ng of rat AQP3 + 2.5 ng of rat AQP1 cRNA. Co-expression of an aquaglyceroporin with a water-specific aquaporin is required because solute induced oocyte swelling is dependent on unhindered secondary influx of water [2]. Oocytes were kept at 15 °C for 3 days in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4). To assay solute permeability, oocytes were abruptly transferred into isosmotic ND96, in which 65 mM NaCl were replaced by 130 mM of the test solute (glycerol, DHA, MG) and were video monitored for 1 min. The coefficient of solute permeability, P_s [$\mu m\ s^{-1}$], was calculated from $P_s=[osm_{total}\cdot V_0\cdot d(V/V_0)/dt]/[S\cdot(sol_{out}-sol_{in})]$, with $osm_{total}=300$ mosM (total osmolarity of the system), $V_0=9\cdot10^{-4}$ cm^3 (initial oocyte volume), $d(V/V_0)/dt$ (relative volume increase in s^{-1}), $S=0.045$ cm^2 (oocyte surface area), and $(sol_{out}-sol_{in})=130$ mosM (osmotic solute gradient).

2.2. Culture of blood stage P. falciparum and purification of genomic DNA

P. falciparum parasites (strains Binh1 [9] and 3D7 [10]) were cultivated at 5% hematocrit (O^+ blood type) in RPMI 1640 medium supplemented with 25 mM HEPES, 20 μg/ml gentamicin sulphate, 2 mM glutamine, 200 mM hypoxanthine, and 0.5% Albumax II (Invitrogen) at 37 °C in 90% N_2, 5% O_2 and 5% CO_2. Synchronization was done twice within 6 h using 5% sorbitol solution [11]. For staining, thin blood films on glass slides were fixed in methanol for 30 s, air-dried, and stained in 10% Giemsa solution (Sigma) for 20 min. The slides were washed under running tap water and parasitemia was determined from 5000 to 7000 erythrocytes per sample at 100× magnification. *P. falciparum* genomic DNA was purified using the QIAamp DNA Blood Mini kit (Qiagen).

2.3. Inhibition of P. falciparum proliferation by dihydroxyacetone and methylglyoxal

An ELISA based on the quantitation of the *P. falciparum* histidine rich protein 2 (HRP2) was used (Malaria Ag CELISA, Cellabs) [12]. Briefly, 96-well plates were pre-dosed with 10 μl of DHA or MG dilutions in complete RPMI 1640 medium. Synchronized parasite cultures (80% rings) were diluted to 0.05% of parasitemia at a hematocrit of 1.5%. Then, 90 μl of the diluted cultures were added to each well of the pre-dosed plates. After 3 days at 37 °C in 90% N_2, 5% O_2 and 5% CO_2 the plates freeze–thawed twice. The samples were transferred to ELISA plates and analyzed according to the Cellabs protocol using an ELISA plate reader at 450 nm (Elx808 bio-tek instruments).

2.4. Cloning, expression and purification of PfGAPDH

The open reading frame of the PfGAPDH gene was amplified from *P. falciparum* genomic DNA (Binh1 strain), checked by DNA-sequencing, and subcloned into pQE30 adding an N-terminal RGS-His_6 tag (Qiagen). Protein expression in *E. coli* BL21 [rep4] cells was induced at an OD_{600} of 0.6 with 1 mM isopropyl thiogalactoside. After 14 h at 37 °C cells were harvested at 3000×*g* for 10 min, resuspended in lysis buffer (300 mM NaCl, 50 mM Tris–HCl, pH 8.0) and lysed using a French press. The particulate fraction was removed by centrifugation at 27,000×*g* and the soluble PfGAPDH protein was bound to Ni-NTA agarose (Qiagen) for 2 h on ice. After washing with lysis buffer supplemented with 10 mM and 30 mM imidazole, the PfGAPDH protein was eluted (300 mM NaCl, 150 mM imidazole, 10 mM DTT, 1 mM NAD^+, 50 mM Tris–HCl, pH 8.0) and dialyzed against assay buffer (300 mM NaCl, 1 mM NAD^+, 50 mM Na_2HPO_4, 10 mM DTT, 50 mM Tris–HCl pH 8.0). Purified PfGAPDH protein was stored at room temperature at 3 mg/ml.

2.5. GAPDH enzyme assay

Recombinant PfGAPDH or commercial rabbit GAPDH (Roche) was diluted to 5 μg/ml in assay buffer at room temperature. The reaction was started by adding 360 μM DL-glyceraldehyde 3-phosphate and NADH was monitored at 340 nm for 1 min. One unit is defined as the amount of GAPDH that generates 1 μmol of NADH per min at 25 °C. Inhibition of PfGAPDH and rabbit GAPDH was assayed after incubation at 37 °C for up to 20 h with 2.5 mM DHA or MG.

3. Results

First, we expressed PfAQP and the mammalian aquaglyceroporin AQP3 in *Xenopus* oocytes and determined the permeability for glycerol, DHA and MG (Fig. 1A and B). Oocytes expressing water-specific AQP1 were practically impermeable for all three test solutes. The solute permeability coefficients, P_s (Fig. 1C), were calculated from the initial 30 s of the swelling curves. At 0.49 $\mu m\ s^{-1}$, PfAQP glycerol permeability was almost double that of AQP3 (0.29 $\mu m\ s^{-1}$) confirming former results [2]. Both aquaglyceroporins passed DHA equally well, i.e., 0.32 $\mu m\ s^{-1}$ (PfAQP) and 0.28 $\mu m\ s^{-1}$ (AQP3), whereas MG permeability was different, i.e., 0.29 $\mu m\ s^{-1}$ (PfAQP) and 0 $\mu m\ s^{-1}$ (AQP3; Fig. 1B and C).

Second, we asked whether DHA is toxic for plasmodia. We infected human red cells with *P. falciparum* Binh1 and treated the culture for 4 days with 0–10 mM DHA. Survival and proliferation was determined by counting the number of infected red cells after Giemsa staining of the parasites (sample images are shown in Fig. 2A). 1 mM DHA was well tolerated by the parasites, whereas 2.5 mM DHA attenuated proliferation by 20%. At 5–10 mM DHA, the parasites died within 1–2 days (Fig. 2B). Using the parasitemia values after 4 days, we determined an IC_{50} of 3.3 mM DHA (Fig. 2C).

To confirm the DHA effect on parasite proliferation independently, we used an established ELISA method based on the quantification of a constitutively expressed plasmodial histidine-rich protein [12]. Again, parasite proliferation was arrested by 5 or 10 mM of DHA (Fig. 3A, closed symbols). The IC_{50} of 3.0 mM DHA corresponded well to that obtained by parasite counts. We then applied this assay to *P. falciparum* strain 3D7 [10] which, contrary to Binh1, is sensitive to chloroquine. The IC_{50} of 2.6 mM DHA for the 3D7 strain was essentially identical to that for Binh1 (Fig. 3A, open symbols).

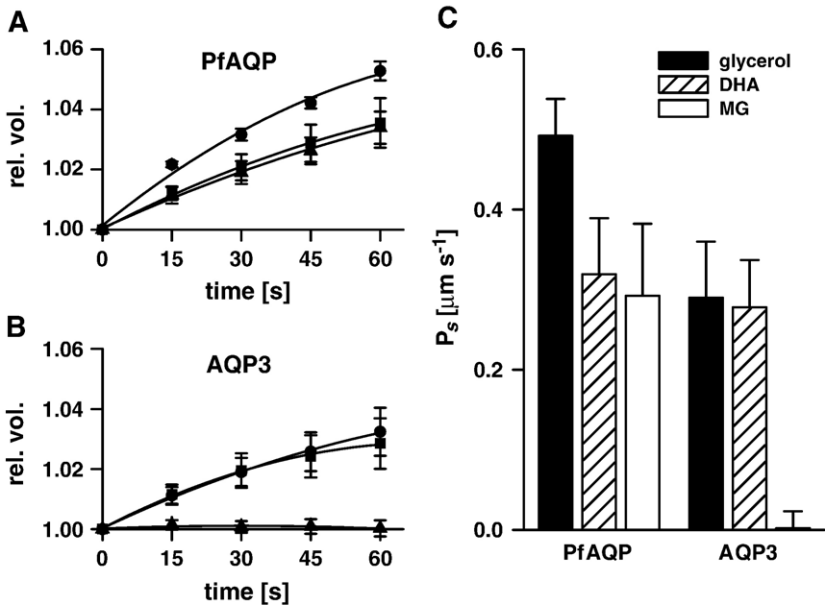

Fig. 1. Permeability of PfAQP and AQP3 for glycerol, dihydroxyacetone and methylglyoxal. (A) Swelling curves of oocytes expressing PfAQP upon abrupt exposure to an isomotic chemical gradient (130 mM) of glycerol (circles), dihydroxyacetone (squares) and methylglyoxal (triangles). (B) Isosmotic swelling of oocytes expressing AQP3 in a 130 mM solute gradient (symbols as in A). (C) Solute permeability coefficients (P values) as determined from the initial slope of the oocyte swelling curves (n=4–10±S.E.M.).

We further tested the effect of MG on both, Binh1 and 3D7 (Fig. 3B). It turned out that MG inhibited parasite proliferation with 10- to 20-fold higher potency irrespective of the strain used (IC_{50} of 223 μM MG for Binh1 and 128 μM MG for 3D7; Fig. 3B). Combinations of DHA or MG with chloroquine were neither competitive nor synergistic in 3D7 (data not shown), indicating that the mode of DHA and MG toxicity as well as detoxification mechanisms are independent from those of chloroquine.

We excluded the possibility that the above effects were due to effects of DHA and MG on erythrocyte membranes (invasion of *P. falciparum*) or gross metabolism (intracellular proliferation of *P. falciparum*). A 4-day treatment of uninfected erythrocytes with up to 10 mM DHA and up to 2 mM MG neither caused any morphological abnormalities nor cell lysis (data not shown). Concerning DHA, this was not surprising because it is used as an energy source due to triose kinase activity and subsequent metabolism of DHA phosphate in glycolysis [13]. Further, we used red cells which were treated with 10 mM DHA and 2 mM MG for 4 days prior to infection with *P. falciparum*. After removal of the metabolites these pretreated erythrocytes were infected by plasmodia. Parasite development was exactly as in

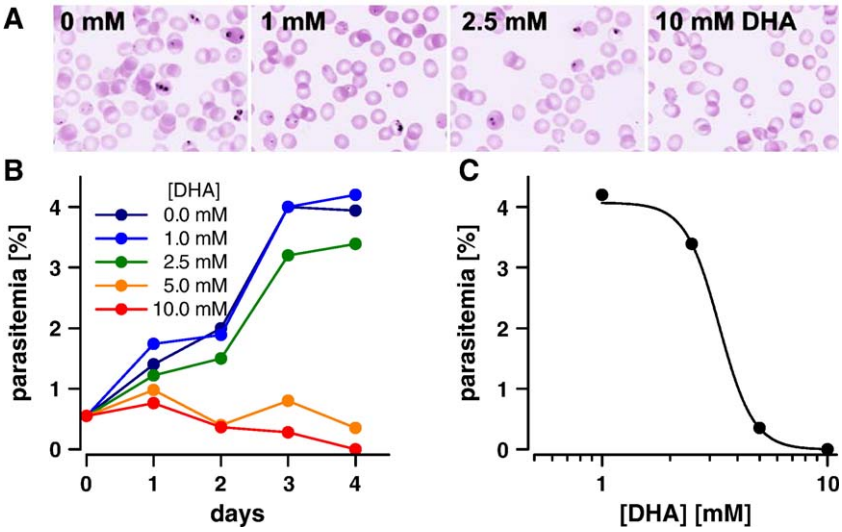

Fig. 2. Effect of dihydroxyacetone on the proliferation of *P. falciparum* malaria parasites in vitro. (A) Sample images showing Giemsa stained parasites (Binh1) after 4 days of culturing with increasing concentrations of dihydroxyacetone. (B) Growth curves of *P. falciparum* parasites cultured with dihydroxyacetone as determined from Giemsa stained blood smears. (C) Parasitemia data after 4 days of culturing with dihydroxyacetone plotted in a growth inhibition curve. The IC_{50} calculated from this curve is 3.3 mM DHA.

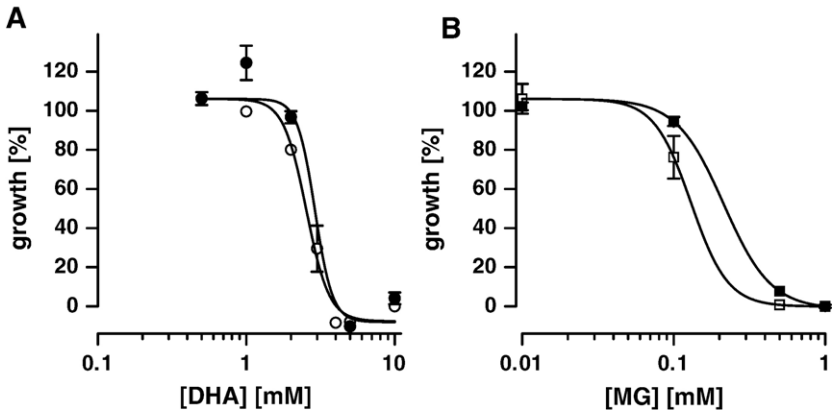

Fig. 3. Growth inhibition of two *P. falciparum* strains treated with dihydroxyacetone and methylglyoxal determined by ELISA. (A) Shown is the inhibitory effect of dihydroxyacetone on plasmodia of Binh1 (filled symbols) and 3D7 (open symbols). IC_{50} values based on these curves are 3.0 mM DHA for Binh1 and 2.6 mM DHA for 3D7. (B) Inhibition curves of Binh1 (filled symbols) and 3D7 parasites (open symbols) treated with methylglyoxal. IC_{50} values are 223 μM MG for Binh1 and 128 μM MG for 3D7 (n=2–3±S.E.M.).

untreated red cells (data not shown). Therefore, we reasonably argue, that the effect of DHA and MG on plasmodia proliferation is direct and not via modifications of the host erythrocyte.

Reactive carbonyl compounds covalently bind to and inhibit GAPDH [14,15], which oxidizes glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate using NAD^+ as a cofactor. *P. falciparum* GAPDH was cloned and expressed before [16]. We amplified by PCR the corresponding open reading frame using genomic DNA from Binh1 as a template. DNA sequencing of the PCR product revealed a single, probably strain-specific nucleotide variation ($C_{418}A$) compared to the published genome (www.plasmoDB.org; gene_ID 140598) resulting in a $Q_{140}K$ exchange. A lysine at this position is quite common, e.g. in the human, rabbit and E. coli GAPDH enzymes, and is thus not expected to affect catalysis. We expressed PfGAPDH in *E. coli* and purified the enzyme via an N-terminal RGS-His_6-tag. About 3 mg of enzyme/l culture with a specific enzyme activity of 9.8 U/mg was obtained. The specific activity was comparable to that of a commercial rabbit GAPDH (7.8 U/mg).

For GAPDH inhibition assays, proteins were pre-incubated at 37 °C with DHA or MG (Fig. 4). Incubation with 200 μM MG, i.e., the IC_{50} concentration of parasite proliferation, had no effect on rabbit and Plasmodium GAPDH (data not shown). With rabbit GAPDH, 20 h with 2.5 mM MG were necessary to abolish enzyme activity (Fig. 4A, squares). Surprisingly, despite the chemical reactivity of vicinal carbonyl groups, 2.5 mM MG, i.e., a concentration 10-fold higher than the IC_{50} for MG inhibition of parasite proliferation, had no effect on PfGAPDH activity (Fig. 4B, squares). This indicated that inhibition of PfGAPDH by MG is unrelated to its cytotoxic effects.

Under similar conditions, 2.5 mM DHA inhibited the GAPDH from *P. falciparum* and rabbit fully. Partial inhibition of the rabbit GAPDH enzyme by DHA was almost instantaneous (Fig. 4A, circles). The PfGAPDH enzyme was fully inhibited after 6 h (Fig. 4B, circles). Considering its central role in the glycolytic pathway, PfGAPDH is a reasonable target that may at least partially explain DHA toxicity in plasmodia.

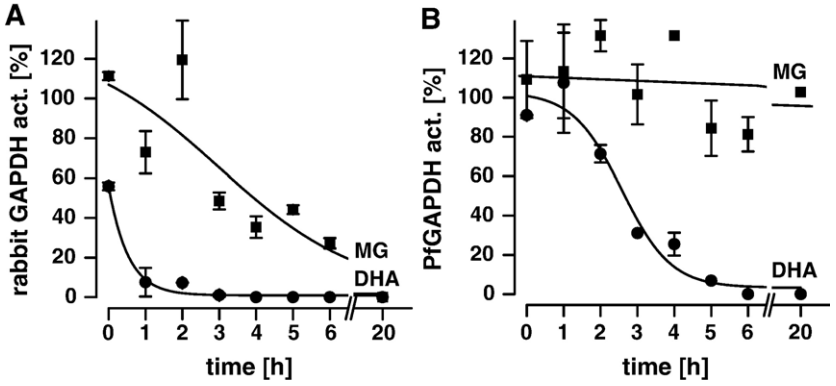

Fig. 4. Effect of dihydroxyacetone and methylglyoxal on GAPDH. The decline in activity of rabbit GAPDH (A) and *P. falciparum* GAPDH (B) after treatment with 2.5 mM dihydroxyacetone (circles) or methylglyoxal (squares) is plotted. Specific activities of the untreated GAPDH enzymes at 0 h are 7.8 U/mg (rabbit) and 9.8 U/mg (*P. falciparum*) (n=2±S.E.M.).

4. Discussion

Aquaporin water and solute permeability has been linked to various physiological functions, most prominently to body water homeostasis [17], but also to lipid metabolism [18], and to carbon dioxide [19] or ammonia facilitation in plants [20]. Here, we describe aquaporin permeability for glycolysis-related compounds, i.e., a ketotriose sugar molecule, DHA, and a cytotoxic byproduct of glycolysis, MG.

Despite a resemblance regarding molecule size and composition, DHA and MG exhibit specific structural properties. For instance, MG is more hydrophobic and less flexible than DHA due to the lack of one hydroxyl group and the presence of two vicinal sp^2-hybridized carbonyl carbons, making MG a strict hydrogen-bond acceptor whereas DHA can act as both, donor and acceptor. Such distinctions may account for the observed differences in MG permeability of the PfAQP and AQP3 pores.

In view of the high energy demand of rapidly proliferating plasmodia, the glycolytic pathway, which is used exclusively for energy generation, is considered as a suitable target for antimalarial chemotherapy [21]. The rate of glucose consumption of plasmodia infected red cells exceeds that of uninfected red cells by a factor of 100 [22]. Along with the massive increase of substrate flow through glycolysis, the formation of cytotoxic MG from the intermediate glyceraldehyde 3-phosphate as a spontaneous side-reaction becomes physiologically relevant [23]. For detoxification, the glyoxalase I and II enzymes convert MG to D-lactate. In fact, D-lactate formation is 30 times higher in plasmodia infected red cells than in uninfected control cells [23]. Thus, an external application of MG may overwhelm the capacity of the glyoxalase system and raise intracellular MG to toxic levels.

MG has been shown to covalently modify arginine, lysine and cysteine residues of numerous proteins [24]. Hence, its mode of toxicity is expected to be rather pleiotropic than specific and selectivity for the malaria parasite in vivo is unlikely. Of immediate physiological relevance may be the inhibition of mammalian GAPDH after prolonged incubation with MG at micro-molar concentrations [14]. It is not known which amino acid residues in the GAPDH protein are modified by carbonyl compounds. A cysteine in the active center (Cys_{149} in rabbit GAPDH, Cys_{153} in PfGAPDH) involved in transition state stabilization is a potential site for modification [25]. Nevertheless, the susceptibility of rabbit GAPDH and PfGAPDH to MG treatment is markedly dissimilar indicating differences in the accessibility of the catalytic center through the substrate channels of these GAPDH enzymes. Comparison of the rabbit GAPDH [25] and PfGAPDH [26] crystal structures indeed shows a peculiar two-amino acid insertion Lys–Gly at positions 194–195 of PfGAPDH that modifies the fold of a loop close to the catalytic center and co-factor binding site.

More surprising than the anticipated toxic effect of MG is the antiproliferative activity of DHA. In principle, this triose can easily be metabolized via glycolysis and human red cells can proliferate with DHA as the sole energy source [13]. Plasmodia, however, apparently cannot effectively utilize DHA probably due to the missing kinase which is not identifiable in the genome. Therefore, inhibition of GAPDH may be the key effect in plasmodia that explains its toxicity although various other target proteins may be modified by DHA [6]. Obviously, inhibition of glucose utilization in a cell with no other means of ATP generation will severely hamper parasite proliferation. In addition, when GAPDH is inhibited, glyceraldehyde 3-phosphate will accumulate, thus increasing the spontaneous formation of cytotoxic MG. Albeit the high concentration of DHA required for inhibition of parasite proliferation most likely precludes any use as an antimalarial in patients, our experiments demonstrate that PfGAPDH is an attractive drug target provided for selective compounds can be found.

The passage of MG and DHA through PfAQP adds two compounds to the list of potentially cytotoxic permeants of aquaglyceroporins, which so far consisted of ammonia, hydroxyurea, and arsenite [20,27,28]. These examples stress the multi-functionality of aquaglyceroporins and may be indicative of additional functions in the disposal of unwanted metabolites from the cytosol.

Acknowledgments

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