Inhibition of hexose transport and abrogation of pH homeostasis in the intraerythrocytic malaria parasite by an O-3-hexose derivative

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Abstract An O-3-hexose derivative, shown previously to inhibit a malaria parasite hexose transporter expressed in Xenopus oocytes as well as to suppress the multiplication of parasites, both in vitro and in vivo, was shown here to block the uptake of hexose sugars into isolated blood-stage parasites. This led to a decline in ATP levels and the loss of intracellular pH control.

The results are consistent with those obtained with the cloned transporter. They support the notion that the transporter mediates uptake of glucose into the intraerythrocytic parasite and provide further support for the view that it is a suitable antimalarial drug target.

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1. Introduction

The intraerythrocytic malaria parasite relies on anaerobic metabolism (glycolysis) to maintain its intracellular ATP concentration. Glucose uptake into the most virulent human malaria parasite, Plasmodium falciparum, is a saturable, equilibrative process [1,2] and has been attributed to PiHT (P. falciparum hexose transporter; [3–6]). PiHT is encoded by a single copy gene belonging to the Major Facilitator Superfamily of transporters, with orthologues in mammalian cells. It is localised to the parasite plasma membrane and, when expressed in Xenopus oocytes, transports both glucose and fructose [3,4]. A recent study in which a range of O-3-hexose derivatives were tested for their effects on PiHT expressed in Xenopus oocytes identified a number of effective inhibitors of the transporter [6]. One of these, a long chain O-3-hexose derivative (compound 3361; 3-O-((undec-10-en)-yl)-d-glucose), which showed selective inhibition of PiHT relative to several mammalian sugar transporters, inhibited both the growth of P. falciparum parasites in vitro and the proliferation of the murine parasite, Plasmodium berghei, in vivo [6]. It was postulated that the antiplasmodial effect of this compound was due to inhibition of PiHT and the consequent deprivation of the parasite of d-glucose. Glucose and fructose antagonised the inhibition of parasite growth by compound 3361, consistent with the compound targeting hexose utilisation rather than unrelated pathways. However, direct inhibition by 3361 of hexose uptake by the parasite and the consequent disruption of parasite energetics have not been demonstrated.

In this study, we have investigated the effect of compound 3361 on the uptake of hexose sugars into mature, trophozoite-stage parasites ‘isolated’ from their host cells using a saponin-permeabilisation technique. We show that compound 3361 is an effective inhibitor of hexose transport across the parasite plasma membrane and that its addition to a parasite suspension leads to a rapid decline in ATP and a consequent loss of pH control in both the parasite cytosol and the parasite’s internal ‘digestive vacuole’, a lysosomal compartment which plays a key role in the metabolism of the parasite.

2. Materials and methods

2.1. Parasite culture and isolation

All experiments were carried out using the 3D7 strain of P. falciparum at the mature trophozoite-stage (36–40 h post-invasion). The parasites were cultured in Group O, Rh+ erythrocytes and synchronised as described elsewhere [7]. Parasites were ‘isolated’ from their host erythrocytes using saponin as described elsewhere [8]. Saponin renders the erythrocyte and parasitophorous vacuole membranes permeable to macromolecules [9] but leaves the parasite plasma membrane intact and able to maintain transmembrane ion gradients [8,10] and a substantial membrane potential [7]. For the purpose of all the experiments reported here, the isolated parasites were suspended in a HEPES-buffered saline containing 125 mM NaCl, 5 mM KCl, 1 mM MgCl2 and 25 mM HEPES, pH 7.1, at 37 °C. Unless specified otherwise, the medium contained 5 mM glucose.

2.2. Hexose uptake measurements

Uptake of [14C]3-O-methylglucose ([14C]3OMG) and [14C]2-deoxyglucose ([14C]2DOG) into isolated parasites and the phosphorylation of [14C]2DOG in parasite lysates were monitored using the methods described previously for pantothenic acid [8,11]. ‘Distribution ratios’ (i.e., the ratio of the estimated intracellular concentration of radiolabel relative to the extracellular concentration) were calculated as described previously [8] using a value of 28 fl for the H2O volume of an individual parasite.

2.3. Measurement of intracellular ATP

ATP levels in isolated parasites were measured using the luciferin/luciferase assay as described previously [1,12].

2.4. Measurement of cytosolic and digestive vacuole pH

The pH of the parasite cytosol (pHcyt) was measured using the ratiometric pH-sensitive fluorescent indicator 2′,7′-bis-(2-carboxyethyl)-
5-(and-6)-carboxyfluorescein (BCECF) in conjunction with a spectrofluorometer, as described previously [12]. Changes in the pH of the parasite’s acidic digestive vacuole were monitored using a membrane-impermeant dextran-linked form of the fluorescent pH indicator fluorescein, loaded into the digestive vacuole by its incorporation into uninfected erythrocytes prior to their invasion by the parasite as described previously [13].

2.5. Reagents Compound 3361 [6] was obtained from Dr. Christophe Morin (Laboratoire d’Etudes Dynamiques et Structurales de la Selectivité, Université Joseph-Fourier Grenoble, France). It was added to cell suspensions as a methanol solution, with appropriate solvent controls carried out in each case. [14C]3OMG was from Amersham, [14C]2DOG was from NEN, and BCECF and dextran-linked fluorescein were from Molecular Probes. All other reagents were of analytical grade.

3. Results

In initial measurements of the uptake of the non-metabolizable hexose [14C]3OMG by isolated parasites in glucose-free medium, it was found that the sugar equilibrated rapidly across the parasite plasma membrane, reaching an estimated distribution ratio of approximately 1.2 within 20 s (Fig. 1A). Addition of compound 3361 at a concentration of 200 μM (some four-fold higher than the Ki for inhibition of glucose transport via PfHTT, expressed in oocytes; [6]) caused a marked reduction in the rate of equilibration (Fig. 1A); the estimated distribution ratios at 20 s and 1 min were significantly lower in the presence of compound 3361 than under control conditions (P < 0.001 and P = 0.012, respectively; paired t-test). However, quantitation of the degree of the inhibition of the transport rate was made difficult by the very fast transport under the conditions of the experiment, and the technical problems associated with measuring the uptake of a solute which equilibrates between the intra- and extracellular solutions under conditions in which up to two-thirds of the volume of the cell pellet was in the extracellular solution.

Another hexose analogue, [14C]2DOG, offers significant advantages as a probe of hexose uptake into the parasite as, on entering the cell, it is phosphorylated and thereby trapped in the cytosol, causing the accumulation of radiolabel to concentrations well above that in the extracellular medium [1]. Fig. 1B shows time-courses for the uptake of [14C]2DOG by isolated parasites in the presence and absence of compound 3361. In the presence of the inhibitor, the radiolabel accumulated to an intracellular concentration >25-fold higher than the extracellular concentration within 3 min. In parasites treated with compound 3361 (200 μM), the initial rate of accumulation was decreased by >95%.

The pronounced inhibition of [14C]2DOG uptake by compound 3361 may be due to an effect of the reagent on the transport mechanism and/or on the phosphorylation of [14C]2DOG by hexokinase. The effect of compound 3361 on the phosphorylation of [14C]2DOG was tested directly using parasite lysates. As shown in Fig. 1C, [14C]2DOG phosphorylation by lysate prepared from isolated P. falciparum trophozoites was not affected significantly by 200 μM of compound 3361. By contrast, the initial rate of phosphorylation was reduced by >95% by the addition to the medium of unlabelled glucose (10 mM) which acts as a competitive inhibitor of [14C]2DOG phosphorylation.

The failure of compound 3361 to inhibit [14C]2DOG phosphorylation indicates that the decreased uptake of [14C]2DOG into isolated parasites in the presence of compound 3361 is attributable to the inhibition of the transport of the hexose by the compound across the parasite plasma membrane.

Fig. 2 illustrates the biochemical consequences of the addition of the hexose transport inhibitor for the parasite. As shown in Fig. 2A, on addition of 200 μM of compound 3361 to isolated parasites suspended in a medium containing 5 mM glucose, the [ATP] decreased to <20% of its normal resting value within 10 min. The inhibitor also caused a rapid decrease in pHcyt (Fig. 2B) and a slower increase in the pH of the parasite’s digestive vacuole (Fig. 2C). As shown in Fig. 3, the rate of acidification of the parasite cytosol, immediately following the addition of the inhibitor, increased with increasing concentration of compound 3361 (Fig. 3A) and de-
creased with increasing concentration of glucose in the medium (Fig. 3B).

4. Discussion

The intraerythrocytic malaria parasite is reliant on the uptake of hexose sugar from the external medium to fuel glycolysis and thereby generates ATP. Glucose traverses the host erythrocyte membrane rapidly via the endogenous erythrocyte glucose transporter as well as, much more slowly, via the ‘new permeability pathways’ induced by the parasite in the host cell membrane [1,2,14,15]. From the red cell cytosol it crosses the ‘parasitophorous vacuole membrane’ enclosing the intracellular parasite, presumably via the high-capacity pores that are thought to render this membrane freely permeable to low molecular weight solutes [16]. Having gained access to the parasitophorous vacuole, it is taken up via transporters in the parasite plasma membrane.

Previous measurements on the transport of hexose sugars in intact P. falciparum-infected erythrocytes yielded data consistent with the view that the transport of sugars into the intracellular parasite is an equilibrative (rather than a concentrative) process [1], mediated via a saturable carrier [2]. The subsequent cloning and characterisation of a PfHT localised to the vicinity of the intraerythrocytic parasite surface [3] and showing saturation kinetics but little dependence on either the extracellular Na⁺ concentration or extracellular pH [5] lent support for this view, as well as providing the first detailed characterisation of a Plasmodium transporter [3,4].

In this study, the transport of hexose sugar across the parasite plasma membrane was monitored directly using parasites isolated from their host erythrocyte by saponin-permeabilisation of the erythrocyte and parasitophorous vacuole membranes; the analysis is therefore free of the complexities associated with that of data derived from the intact parasitised erythrocyte and its multiple membrane systems [1,2]. The transport of the non-metabolisable glucose analogue [14C]3OMG into the parasite was extremely fast, equilibrating within 20 s (Fig. 1A). The observation that, at equilibrium, its distribution ratio was close to 1 confirms the conclusions from the earlier study on intact parasitised cells that glucose transport into the intracellular parasite is equilibrative rather than accumulative in nature [1]. In the case of the phosphorylatable glucose analogue [14C]2DOG, the observation that the concentration of radiolabel in isolated parasites was well in excess
of that in the external medium within 22 s of combining the parasites and [14C]2DOG (i.e., by the first timepoint in Fig. 1B) is consistent with transport having been sufficiently fast for the hexose sugar to have equilibrated between the intra- and extracellular solutions within this time period. Addition of compound 3361 caused a significant inhibition of [14C]3OMG transport and a marked inhibition of [14C]2DOG accumulation, via a mechanism that was unrelated to an effect on phosphorylation (Fig. 1C) and which can therefore be attributed to an inhibition of the transporter. Compound 3361 has already been shown to be an effective inhibitor of PfHT expressed in Xenopus oocytes. These data therefore lend support to the hypothesis [5] that PfHT is the major route of entry of glucose into the intracellular parasite.

Compound 3361 has been shown previously to inhibit the in vitro growth of P. falciparum, measured over a 48-h period, as well as inhibiting the propagation of P. berghei parasites in mice [6]. The data of Fig. 2 provide insights into the earliest effects of the compound on the parasite. Within seconds of its addition, there is a decrease in the intracellular ATP concentration (Fig. 2A). The parasite uses ATP to fuel H+$^+$-pumping ATPases on both the parasite plasma membrane [12,17] and the membrane of its internal digestive vacuole [13]. The decline in [ATP] following the addition of compound 3361 resulted in a loss of pH control in both the cytosolic and digestive vacuole compartments: there was a rapid acidification of the parasite cytosol (Fig. 2B) and a slower alkalinisation of the digestive vacuole (Fig. 2C). The cytosolic pH decreased to a value of approximately 6.95 slightly below that in the extracellular solution (7.1). The likely explanation for this is that under the conditions of the experiment, the inhibitor did not completely abolish glucose uptake and that although ATP levels decreased sufficiently to slow the plasma membrane and digestive vacuole H+$^+$-ATPases there was still sufficient metabolism occurring to generate a significant acid load within the parasite. The finding in Fig. 1A that hexose transport was not inhibited fully by compound 3361 and in Fig. 2A that the parasite still retained a significant [ATP] in the presence of inhibitor are both consistent with this interpretation.

Acidification of the cytosol will interfere with the plethora of metabolic processes taking place in this compartment, while alkalinisation of the digestive vacuole will impede the action of the various acid-dependent proteases that are needed to break down the host cell haemoglobin in this compartment [18].

The inhibitor-induced acidification of the parasite cytosol was concentration-dependent (Fig. 3A) and reversed by increasing concentration of glucose (Fig. 3B). The latter observation is consistent with inhibition of glucose permeation rather than to unrelated effects (e.g., direct inhibition of the H+$^+$ pumps or disruption of the membranes enclosing the relevant compartments), and with the inhibition of the parasite’s hexose transporter being competitive in nature.

The results of this study highlight the potential of the malaria parasite’s hexose transporter as an antimalarial drug target. Patients with severe malaria commonly become hypoglycaemic [19]. The lower the extracellular glucose concentration, the more effective the inhibitor will be at perturbing ion homeostasis in the parasite (Fig. 3B); the efficacy of such compounds might therefore be expected to be enhanced in patients with hypoglycaemia. Furthermore, selective inhibitors of PfHT should not only kill the parasite but, by causing an immediate inhibition of glucose uptake into the parasite while leaving the GLUT1 transporter of the blood–brain barrier unaffected [6], should prevent sequestered parasites from competing with the brain for glucose, as may occur in cerebral malaria [20].

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