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A non-radiolabeled heme–GSH interaction test for the screening of antimalarial compounds

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Abstract

Intraerythrocytic *Plasmodium* produces large amounts of toxic heme during the digestion of hemoglobin, a parasite specific pathway. Heme is then partially biocrystallized into hemozoin and mostly detoxified by reduced glutathione. We proposed an *in vitro* micro assay to test the ability of drugs to inhibit heme-glutathione dependent degradation. As glutathione and o-phthalaldehyde form a fluorescent adduct, we followed the extinction of the fluorescent signal when heme was added with or without antimalarial compounds. In this assay, 50 μ M of amodiaquine, arthemether, chloroquine, methylene blue, mefloquine and quinine inhibited the interaction between glutathione (50 μ M) and heme (50 μ M), while atovaquone did not. Consequently, this test could detect drugs that can inhibit heme–GSH degradation in a fast, simple and specific way, making it suitable for high throughput screening of potential antimalarials.

Index Descriptors and Abbreviations: *Plasmodium falciparum*; Glutathione; Heme; Antimalarials; Screening; GSH, Reduced glutathione; OPA, o-phthalaldehyde; HPLC, high performance liquid chromatography; AMO, amodiaquine; ART, arthemether; ATO, atovaquone; CQ, chloroquine; MB, methylene blue; MEF, mefloquine; Q, quinine

1. Introduction

Drug-resistant parasites and insecticide-resistant mosquito vectors have made treatment and control of malaria more difficult. Therefore safe, affordable, and effective new drugs are urgently needed (WHO-TDR The World Health Organization, 2006). The search for new antimalarials is generally based on *in vitro* tests against cultivated *Plasmodium falciparum* and/or *in vivo* against rodent malaria models. Apart from being expensive and raising ethical issues, these tests are also time-consuming, results being obtained in 3–5 days, which is not suitable for high throughput screening. A faster and cheaper way to detect antimalarial activities is to study the impact of a drug on a *Plasmodium*

specific metabolic event or a “target”, reproduced in microtiterplates. An improved understanding of the biochemistry of malaria parasites has made it possible to identify many potential targets for new drugs, the degradation of hemoglobin being one of them. The malaria parasite breaks up hemoglobin inside the red blood cells, as a major source of production of amino acids, releasing free toxic heme. *Plasmodium* protects itself against the toxicity of heme by biocrystallizing around 30% of it into insoluble hemozoin. Many tests to detect potential antimalarials were based on this pathway (Baelmans et al., 2000; Basilico et al., 1998; Deharo et al., 2002; Kurosawa et al., 2000). Interestingly, the major degradation pathway of heme is not the biocrystallization, since around 70% of non-crystallized heme exits the food vacuole and is subsequently catabolized by GSH, leading to the formation of oxidized glutathione (Ginsburg et al., 1998). Consequently a drug that can inhibit the interaction between GSH and heme should have even better

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potential antimalarial properties than one that acts on bio-crystallization. Previous studies (Cohn and Lyle, 1966; Hissin and Hilf, 1976) have shown that GSH reacted with OPA in a highly specific manner, giving a strong fluorescent GSH–OPA adduct with excitation/emission spectra reaching 350 and 420 nm, respectively (Neuschwander-Tetri and Roll, 1989; Hissin and Hilf, 1976). On this basis we developed a new *in vitro* assay to follow heme–GSH dependent degradation by fluorescence spectroscopy. For this purpose we first determined the optimal conditions of the test by HPLC, and we subsequently measured the activity of AMO, ART, ATO, CQ, MB, MEF, and Q in this model.

2. Materials and methods

2.1. Chemicals

All chemicals were from Sigma–Aldrich (L’Isle d’Abeau Chesnes, France) except: acetonitrile (HPLC grade) from SDS (Peypin, France), ART from Cambrex (Verviers, Belgium), ATO was a gift of GlaxoSmithKline (Marly-le-Roi, France) and MEF from Hoffmann-La Roche (Basel, Switzerland).

GSH (Sigma–Aldrich G4251) is manufactured by extraction from yeast and does not contain materials of animal origin.

2.2. HPLC study for the assessing of optimal conditions

The OPA–GSH adduct detection was optimized by HPLC according to Cereser et al. (2001). A Waters HPLC system was used (Waters 600 solvent delivery system, Waters 717 plus auto sampler and Waters 474 scanning fluorescence detector). Analysis were monitored with $\lambda_{\text{ex}} = 320 \text{ nm}$ and $\lambda_{\text{em}} = 450 \text{ nm}$ (Hissin and Hilf, 1976; Scaduto, 1988). Data were acquired and processed in a Waters Millennium workstation. An Inertsil, ODS-2, C18 column ($5 \mu\text{m}$, $250 \times 4.6 \text{ mm}$) (AIT, Mesnil Le Roi, France) was used to separate the GSH, OPA, and OPA–GSH adduct. Column temperature was kept at 37°C . The mobile phase (isocratic mode) was a mixture of solvents 35% and 65% $\text{CH}_3\text{CN}/\text{sodium acetate buffer}$, 50 mM, pH = 6.2, respectively. The flow rate was 0.7 ml/min and 20 μl of sample were injected. After the optimization of the OPA–GSH adduct detection by liquid chromatography, the heme–GSH interaction was also studied (same experimental conditions). The main experimental parameters of both studied reactions (GSH derivation and heme–GSH interaction) were: incubation temperature, atmosphere (O_2 , N_2), pH, OPA/GSH, and heme/GSH ratio values.

2.3. Heme-glutathione interaction study

The OPA–GSH fluorescent adduct was detected with a 96-well plate reader (BGM, Polarstar) at 37°C in 0.2 M Hepes buffer, pH = 7. Standard solutions of GSH (300 μM) in HCl (0.1 M), OPA (17.5 mM) in a mixture of methanol/

sodium tetraborate and of heme (300 μM) in NaOH (0.2 M) were freshly prepared. Drugs, 10 mM in MeOH or in Hepes buffer (pH = 7), were diluted with HEPES buffer to 300 μM . These solutions were kept in darkness. Final concentrations in the plate were 50 μM except for OPA, whose concentration was 2.5 mM. Drugs (25 μl) were incubated with GSH (25 μl) and heme (25 μl) for 30 min and fluorescence intensities measured at $t = 0$ and at $t = 30 \text{ min}$. Fluorescence emission started with the addition of 25 μl of OPA (17.5 mM). Plates were shaken for 10 s with a shaking width of 1 mm. OPA was added just before the fluorescence measurement at 0 and 30 min incubation times. The following mixtures were used as control to determine potential interactions resulting into a fluorescence emission: drug/GSH (50 $\mu\text{M}/50 \mu\text{M}$), drug/heme (50 $\mu\text{M}/50 \mu\text{M}$), drug/OPA (50 $\mu\text{M}/2.5 \text{ mM}$), heme/OPA (50 $\mu\text{M}/2.5 \text{ mM}$), GSH/OPA (50 $\mu\text{M}/2.5 \text{ mM}$) and HEPES buffer/OPA (0.2 M/2.5 mM). Fluorescence reduction obtained with heme–GSH between $t = 0$ and $t = 30 \text{ min}$ was the “positive” control. Results were expressed in the average % of GSH after a 30 min incubation in the media. The fluorescence intensity at $t = 0 \text{ min}$ corresponds to 100% of GSH. The % of remaining GSH was defined as $(\text{Fluorescence}_{(t=30\text{min})}/\text{Fluorescence}_{(t=0\text{min})}) \times 100$. A statistical analysis was performed with the STATA 7.0 software. A Student’s *t*-test was used to compare the % GSH remaining values.

3. Results and discussion

We first assessed the best experimental conditions to carry out the assay, starting from experimental data reported by Ginsburg (Ginsburg et al., 1998). The HPLC study of GSH derivation by OPA indicated a GSH/OPA optimal ratio of 1/50 and an OPA derivation time of 5 min. As shown in Table 1, the following conditions were also established: GSH/heme: 1/1; drug/GSH/heme: 1/1/1 and incubation time: 30 min. Since under nitrogen the heme–GSH dependent degradation was inhibited, assays were carried out in a normally oxygenated environment, at 37°C .

As shown in Fig. 1, the percentage of remaining GSH after 30 min of incubation of GSH + heme with AMO, ART, CQ, MB, MEF, and Q was significantly higher than the positive control (GSH + heme + solvent), AMO being the most active. This is in line with the fact that AMO, CQ, and MEF are known to competitively inhibit the degradation

Table 1
Optimum conditions to perform the test

GSH/OPA optimal ratio	1/50
OPA derivation time	5 min
GSH/heme optimal ratio	1/1
Drug/GSH/heme optimal ratio	1/1/1
Incubation time	30 min
Atmosphere	Room
Temperature	37°C
pH	7

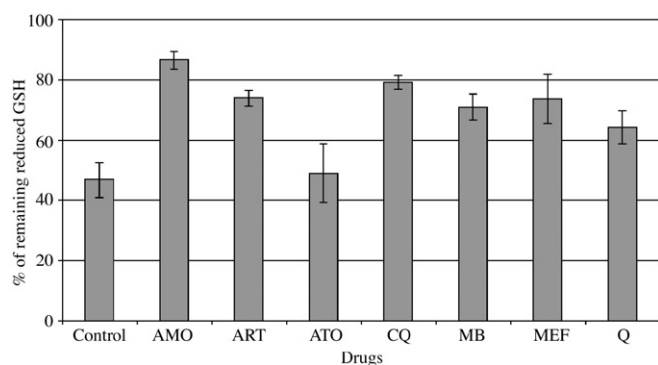


Fig. 1. Impact of tested drugs on the percentage of remaining reduced glutathione after 30 min of incubation. Percentage of remaining reduced GSH = $(\text{Fluorescence}_{(t=30\text{min})}/\text{Fluorescence}_{(t=0\text{min})} \times 100)$, from 3 independent experiments.

of heme by GSH (Ginsburg et al., 1998). It is also known that MB links itself to heme and inhibits β -hematin formation much more effectively than Q, which is the least active drug in our test (Deharo et al., 2002; Wainwright and Amaral, 2005). Interestingly, in our ferriprotoporphyrin IX biomineralization inhibition test (Deharo et al., 2002) Q was more than 10 times less active than CQ and MB, while in the present study the difference was not so obvious. This means that Q is more active on the heme–GSH interaction than on the hemozoin formation. In a previous study based on scanning spectrophotometry of heme–GSH interaction at 360 nm, Steele et al. (2002) also showed the inhibitory effect of AMO, CQ, and Q but claimed that ART was inactive. This sounds strange because even if ART has been recently shown to target the sarco/endoplasmic reticulum calcium-dependent ATPase of *Plasmodium* (Eckstein-Ludwig et al., 2003), free radicals generated from ART are known to form adducts with a variety of biological macromolecules, including heme (Meshnick, 2002), which is in line with our results. On the contrary ATO did not interfere with the heme–GSH interaction, the remaining GSH being similar to control. This was expected as ATO does not act on heme detoxification pathways but on the electron transport through the parasite mitochondrial cytochrome bc1 complex and affects the mitochondrial membrane potential (Mather et al., 2005). These results prove the capacity of the assay to differentiate compounds that do not act on the heme–GSH dependent degradation pathway.

In conclusion, the technique reported herein is simple, fast and specific to detect molecules that can inhibit heme–GSH dependent degradation.

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