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Malagashanine potentiates chloroquine antimalarial activity in drug resistant *Plasmodium* malaria by modifying both its efflux and influx

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Abstract

Malagashanine (MG) is the parent compound of the new N_b-C(21) seco-curan alkaloids isolated hitherto from Madagascan *Strychnos*. On account of its promising chemosensitizing activity against chloroquine (CQ)-resistant *Plasmodium falciparum* (*Pf*) strains, we investigated its mechanism of action. One prominent result was the finding that MG significantly increased the accumulation of [^{3}H]-chloroquine in chloroquine resistant (CQR) K1 and FCM29 *Pf* strains at mid and old trophozoite stages. This effect was concentration-dependent and not observed in chloroquine sensitive (CQS) strains. Comparative monitoring of the release of [^{3}H]-CQ from pre-loaded CQR and CQS *Pf* strains revealed strong concentration-dependent inhibition of CQ release by MG from the pre-loaded CQR FCM29 strain. We also found that MG substantially inhibited the loss of pre-accumulated CQ in the resistant K1 strain after a washing procedure at 4 °C. On the other hand, we observed that the addition of glucose at the old trophozoite stage induced a rapid increase in CQ accumulation in the CQS 3D7 strain cultured in glucose-free medium, but not in the CQR FCM29 strain. Interestingly, MG considerably increased (>100%) CQ accumulation in the FCM29 strain in a glucose-free medium while the addition of glucose further significantly increased this accumulation. Our study therefore clearly demonstrates that MG prevents CQ efflux from, and stimulates CQ influx into, drug-resistant *Pf*. Overall, MG appears to be a useful lead for the design and synthesis of more powerful and effective resistance modulators.
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Keywords: *Plasmodium*; Malagashanine; Chemosensitizer; Chloroquine resistance; Efflux; Chloroquine transporter

1. Introduction

Despite massive efforts to control it, malaria still remains one of the most devastating tropical diseases, mainly afflicting poor populations in developing countries. The global morbidity and mortality due to malaria have not significantly changed over the past 50 years [1]. The continued (or rising in some areas) rate of malaria infections is mainly attributable to the spread of drug resistant strains of *Plasmodium* malaria and failure to apply existing effective drugs in areas where they would be of most benefit [2]. It is therefore likely that this disease will be with us for many decades to come.

While the search for powerful antimalarial drugs having novel mechanisms of action is underway for monotherapy or drug combination purposes, elucidation of the mechanism of CQ resistance and its reversal has also generated much interest and stimulated intense debate over the years. It is now generally accepted that *Plasmodium falciparum* (*Pf*) CQR strains accumulate much less drug than CQS strains [3]. Similar results were obtained with isolated vacuoles from CQR and CQS strains [4]. However, the mechanism involved in acquisition of the resistance phenotype remains a matter of controversy and ongoing investigation. To date, two hypotheses have been put forward to explain the mechanism of CQ resistance. According to the first hypothesis, CQ does not reach the food vacuole of malaria parasites in high enough therapeutic concentration. In this reduced

Abbreviations: CQ, chloroquine; CQR, chloroquine resistant; Pf, *Plasmodium falciparum*; CQS, chloroquine sensitive; MG, malagashanine

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uptake hypothesis, two models were proposed: the first model implied alteration of the pH gradient between the external medium (pH 7.3) and the vacuole (pH 5.4) through elevation of the vacuolar pH in CQR strains, which would prevent passive diffusion of CQ in the acidic compartments of the food vacuole [5], while the second model implied alteration of a putative CQ importer [6]. However, the role of pH in chloroquine resistance is still a hotly debated topic [7]. In the second hypothesis, CQ is believed to reach the parasite food vacuole at therapeutic concentrations but it does not exert its effect for several reasons, which are based upon three proposed models. The first model is based on the assumed existence of an active verapamil-sensitive efflux pump similar to P-glycoproteins found in multi-drug resistant (MDR) cancer cells, which would extrude CQ from the food vacuole [8]. The second model implies altered binding of CQ to ferriprotoporphyrin IX [9], while the third model is based on the assumption that an energy-dependent CQ efflux pump is the basis for CQ resistance [10].

In the pioneering work of Martin et al., the calcium channel blocker verapamil was found to reverse CQ resistance in malaria [11]. It has since been extensively used as a biochemical tool to probe the mechanism of CQ resistance and its reversal, but the exact mechanism is still a challenge for all current theories [9,12]. As part of an investigation into the traditional use of the Malagasy medicinal plant *Strychnos myrtoides* to complement CQ action, we isolated malagashanine (Fig. 1) as a new alkaloid that reverses CQ resistance [13]. In our preliminary work, we found that this alkaloid displayed a good pharmacological profile. Indeed, this molecule did selectively enhance the in vitro antimalarial activity of quinolines (chloroquine, quinine and mefloquine), aminoacridines (quinacrine and pyronaridine) and a structurally unrelated drug (halofantrine) against CQR strains, Furthermore, malagashanine (MG), at a dose of 10 mg/kg, significantly potentiated chloroquine antimalarial activity in mice infected with the rodent strain *P. yoelii* [14]. This prompted us to further investigate this compound. In the present study, we carried out several experiments aimed at elucidating its mechanism of action. Our work focused on [^{3}H]-CQ accumulation and release within the CQR and CQS *Pf* strains in the presence and absence of MG, using short (2 h) and long (24 h) incubation times starting at different trophozoite stages of the parasite, in a normal or glucose-free RPMI medium. We report here compelling data suggesting that MG reverses CQ resistance by modifying both its efflux and influx in drug-resistant malaria parasites.

2. Material and methods

2.1. Chemicals

Radio-labelled [^{3}H]-CQ diphosphate (specific activity, 26 Ci/mmol) was purchased from Amersham, and [^{3}H]-hypoxanthine (specific activity, 20 Ci/mmol) from ICN Biomedicals. MG was isolated from *S. myrtoides* Gilg & Buss and further purified by counter-current chromatography in a Craig apparatus at the *Istituto Superiore di Sanità* in Rome [13]. The purity of the compound measured by the HPLC method was found to be higher than 99% [15]. CQ diphosphate, *n*-dibutylphtalate were obtained from Sigma–Aldrich Chemical Co. (St. Louis), and RPMI 1640 was provided by Gibco. Glucose-free RPMI 1640 was a generous gift from Dr. M. Lanzer.

2.2. Biological materials

The CQR *Pf* strain K1 was kindly provided by Dr. P. Grellier (Muséum National d'Histoire Naturelle, F-Paris), while the CQR FCM29 and the CQS 3D7 strains were provided by Dr. B. Pradines (IMTSSA, F-Marseille). The Nigerian CQS strain NS was routinely cultured in the UMR 5539 laboratory. Parasites were maintained in continuous culture in O$^+$ human red blood cells according to Trager and Jensen [16]. Complete medium was composed of basic medium (RPMI 1640, 25 mM $NaHCO_3$, 25 mM Hepes buffered, pH 7.4) supplemented with 10% human AB$^+$ serum and 20 μM gentamycine sulfate. The cellular density was determined by using a Naubauer counting chamber. Parasites were synchronised by treatment twice with 5% sorbitol [17].

2.3. In vitro tests for antiplasmodial activity and drug interaction

Sensitivity of *Pf* strains to CQ and to MG either alone or in combination was determined using the isotopic semi-microtest described by Desjardins et al. and [^{3}H]-hypoxanthine incorporation to assess parasite growth [18].

Stock drug solutions were prepared in RPMI 1640 and serially diluted to the appropriate concentrations using complete medium. Assays were performed in sterile 96-well microtiter plates. Synchronized suspensions at 1% final haematocrit and 1% final parasitaemia were incubated in complete medium, either without (controls) or with the test compounds. After 24 h incubation at 37 °C, 0.5 μCi of [^{3}H]-hypoxanthine was added

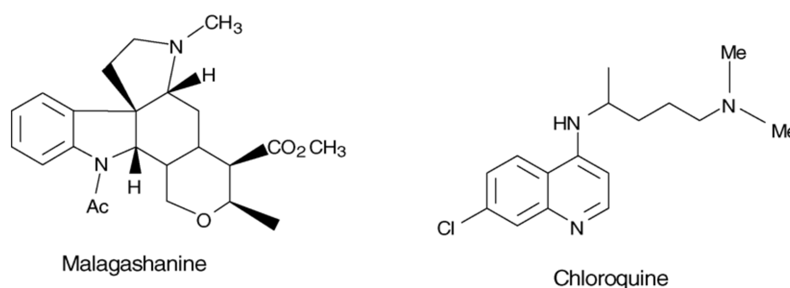

Fig. 1. Chemical structure of malagashanine and chloroquine.

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