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Encapsulation of metalloporphyrins improves their capacity to block the viability of the human malaria parasite *Plasmodium falciparum*

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Abstract

Several synthetic metallated protoporphyrins (M-PPIX) were tested for their ability to block the cell cycle of the lethal human malaria parasite *Plasmodium falciparum*. After encapsulating the porphyrin derivatives in micro- and nanocapsules of marine atelocollagen, their effects on cultures of red blood cells infected (RBC) with *P. falciparum* were verified. RBCs infected with synchronized *P. falciparum* incubated for 48 h with different concentrations of metalloporphyrin showed a toxic effect over a micromolar range. Strikingly, the IC_{50} of encapsulated metalloporphyrins reached nanomolar concentrations, where Zn-PPIX showed the best antimalarial effect, with an IC_{50} = 330 nM. This value is an 80-fold increase in the antimalarial activity compared to the antimalarial effect of non-encapsulated Zn-PPIX. These findings reveal that the incubation of *P. falciparum* infected-RBCs with 20 μM Zn-PPIX reduced the size of hemozoin crystal by 34%, whereas a 28% reduction was noticed with chloroquine, confirming the importance of heme detoxification pathway in drug therapy.

Key words: *Plasmodium falciparum*; Malaria; Nanoparticle encapsulation; Metalloporphyrins

Introduction

The increasing incidence of malaria worldwide makes the discovery of new targets and methods to combat the parasite an urgent research topic. Studies to investigate the biology of the malaria parasite *Plasmodium falciparum* are fundamental, as they provide the basis for the development of new antimalarials. The *P. falciparum* cycle in red blood cells (RBCs) proceeds through a series of developmental and proliferative stages and is followed by cell lysis after 48 h and the release of free merozoites that subsequently invade other RBCs and reinitiate the cycle.

During the RBC phase, the parasite utilizes the host's hemoglobin as a source of amino acids through a specialized and efficient process.[1] However, hemoglobin degradation generates iron protoporphyrin-IX (heme) as a toxic by-product that damages membranes through lipid peroxidation and other mechanisms and inhibits a wide range of enzymes, such as proteases and DNA polymerase.[2–5] The most important heme detoxification mechanism found in *Plasmodium* is the conversion of heme to hemozoin (β-hematin).[6] In fact, half of the heme in the host's total hemoglobin is converted to hemozoin.[7]

Micromolar concentrations of metal-free protoporphyrins and metalloporphyrins have been found to be toxic to *P. falciparum*, decreasing the viability and heme polymerization of *P. falciparum*.[8,9] Heme degradation to biliverdin (BV) is a critical step in the primary pathway of hemoprotein catabolism in mammals,

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and is also a means of nullifying heme toxicity.[10] Heme is first converted to BV by the enzyme heme oxygenase (HO).

Recently the expression of a heme oxygenase enzyme by *P. falciparum* (*Pf*HO) was also demonstrated.[11,12] However, as the recombinant *Pf*HO enzyme has been shown to have low activity, it is thought that *Pf*HO is inefficient in responding to heme toxicity in *P. falciparum*. In this study, we examined the ability of several metalloporphyrins to block the intraerythrocytic cell cycle of the human malaria parasite *P. falciparum*. Porphyrins are compounds constituted by a macrocycle called porphine, consisting of four pyrrole rings linked together through methenyl bridge. Different metal ions can be coordinated to this macrocycle by the removal of H^+ ions of the internal NH groups, giving rise to metalloporphyrins.[13] In biological systems, metalloporphyrins appear conjugated to protein forming structures of great importance such as hemoglobin, myoglobin and cytochromes.

To address the low activity of the metalloporphyrins,[9,14] we encapsulated the metalloporphyrins in non-toxic polymeric micro- and nanocapsules of marine atelocollagen. Such atelocollagen capsules have been shown to cross cell membranes and act as shuttles for the internalization of the molecules dissolved in their core.[15,16] Accordingly, in this study we show that the antimalarial activity of the metallated protoporphyrin IX derivatives is greatly improved by their incorporation in the non-toxic polymeric micro- and nanocapsules, decreasing the IC_{50} to the nanomolar range. Using confocal microscopy, a marked decrease in hemozoin formation in *P. falciparum*-infected RBCs was also observed clearly indicating the possibility of developing antimalarial drugs that would disrupt the parasite's heme metabolism.

Materials and methods

P. falciparum parasites (3D7 strain) were maintained in culture as described by Trager and Jensen.[17]

Metalloporphyrin synthesis

The 3,7,12,17-tetramethyl-8,13-divinyl-2,18-porphynedipropionic acid, protoporphyrin IX (Sigma Aldrich, USA), was metallated with Zn(II), Ni(II), Cu(II) and Co(III) using the acetate method, as described in Supplementary Material (Method and Figures S1 and S2). The results were compared with those of heme (iron(III) protoporphyrin IX chloride; Fe-PPIX) purchased from Sigma Aldrich, USA.

Encapsulation of metallated protoporphyrins

The free base and six (Fe, Co, Cu, Mn, Ni and Zn) metallated protoporphyrin-IX (M-PPIX) derivatives were encapsulated using the coacervation method[18] (see Figure S3 in Supplementary Material), which was previously described for the encapsulation of *meso*-phenyl(pyridyl)porphyrin derivatives.[15] Typically, the dye was dissolved in dimethylsulfoxide (DMSO) and added to an oil phase (20% of total formulation) constituted by a mixture of isopropyl myristate (42.5%), almond oil (42.5%), propylene glycol (10%) and Tween 20 (5%), poured into an aqueous phase (80% of total formulation) constituted of aqueous xanthan gum (1%), then marine atelocollagen (1%) and sodium sulfate (0.5%) were added under vigorous stirring. For more particle size reduction, ultrasonication was performed using an ultrasonic tip (VibraCell from Sonics) for 10 min, energy set at 100 kJ. Malvern Zetasizer Model Nano ZS was used for particle size measurements and zeta potential determination. A control formulation with the same composition as above free from the M-PPIX derivative was also prepared.

Effect of metalloporphyrins/encapsulated metalloporphyrins on P. falciparum viability

Erythrocytes infected with desynchronized *P. falciparum* (3D7 strain) at 2% parasitemia were incubated for 48 h in flat bottom 48-well ELISA plates containing different concentrations (0.1, 1, 10, 25 and 50 μM) of heme, protoporphyrin IX and metalloporphyrin (Zn-PPIX, Ni-PPIX, Mn-PPIX Cu-PPIX and Co-PPIX). The controls were infected RBCs with no treatment and infected RBCs with DMSO. The metalloporphyrins were solubilized in DMSO. Encapsulated heme, PPIX, Zn-PPIX, Ni-PPIX, Mn-PPIX Cu-PPIX and Co-PPIX (at 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 nM) were suspended in RPMI (Roswell Park Memorial Institute) 1640 culture medium (Gibco). Nanocapsules treated with pure DMSO were used as control. After all incubations, the cells were centrifuged for 7 min at 800 × *g* at room temperature and fixed overnight with phosphate buffer solution (PBS) pH 7.4 containing 2% formaldehyde (v/v) (Labysynth). The fixed cells were resuspended in PBS pH 7.4 containing 0.1% Triton X-100 (v/v) (Sigma) and 5 nM of oxazole yellow homodimer (YOYO-1-labeled DNA, Molecular Probes) and incubated at 37 °C for 30 min. Parasitemia was determined from dot plots (side scatter versus fluorescence) of 10^5 cells acquired on a FACSCalibur flow cytometer using CELLQUEST software (Becton Dickinson). Fluorescence was excited with an Argon laser at 488 nm, and the fluorescence emission was collected at 520-530 nm. Initial gating was carried out with unstained, uninfected erythrocytes to account for the erythrocyte autofluorescence.

Hemozoin area measurements in P. falciparum-infected RBCs after Zn-PPIX uptake

Erythrocytes infected with *P. falciparum* in the trophozoite stage (considered initial time T_0) were split in three flasks with complete medium, one containing 25 μM of Zn-PPIX, one containing 1 μM chloroquine and one containing a 0.05% final volume of DMSO. The parasite-infected cells were incubated for 2 h at 37 °C, washed once with PBS and then placed in a plate that had been previously pre-treated for 15 min with poly-L-lysine to ensure adhesion of the cells to the plate. The images from the plate were captured on an LSM 510 confocal microscope (Zeiss) using the Argon laser (wavelength 488) and a 488 primary dichroic mirror (HFT 488). The lens was used with an apochromatic objective with a magnification of ×63 and a numerical aperture of 1.4. All images were captured with the same resolution. The images were analyzed and the hemozoin crystal size was obtained using the Image J software. The sizes of the hemozoin culture of T_0 and the culture that was pre-treated with 25 μM of Zn-PPIX and 1 μM chloroquine for 2 h were compared with the size of the control culture that received DMSO only.