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Andrographolide effect on both *Plasmodium falciparum* infected and non infected RBCs membranes

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ABSTRACT

Objective: To explore whether its antiplasmodium effect of andrographolide is attributed to its plausible effect on the plasma membrane of both *Plasmodium falciparum* infected and non-infected RBCs.

Methods: Anti-plasmodium effect of andrographolide against *Plasmodium falciparum* strains was screened using the conventional malaria drug sensitivity assay. The drug was incubated with uninfected RBCs to monitor its effect on their morphology, integrity and osmotic fragility. It was incubated with the plasmodium infected RBCs to monitor its effect on the parasite induced permeation pathways. Its effect on the potential of merozoites to invade new RBCs was tested using merozoite invasion assay.

Results: It showed that at andrographolide was innocuous to RBCs at concentrations approach its therapeutic level against plasmodia. Nevertheless, this inertness was dwindled at higher concentrations.

Conclusions: In spite of its success to inhibit plasmodium induced permeation pathway and the potential of merozoites to invade new RBCs, its anti-plasmodium effect can't be attributed to these functions as they were attained at concentrations higher than what is required to eradicate the parasite. Consequently, other mechanisms may be associated with its claimed actions.

1. Introduction

In spite of the achieved progress to eradicate the parasitic ailments, malaria is still a major therapeutic challenge and a significant economic burden in the developing countries due to plenty of factors, such as emergence of drug resistant strains of *Plasmodium falciparum* (*P. falciparum*), pesticide resistance among the mosquitoes vectors, the debilitating adverse effects of the conventional anti-malarials, the limited success in developing a potential vaccine and paucity of effective drugs alternatives for conventional anti-malarials [1,2].

Intra-erythrocytic ubiquity of plasmodium compromises the RBCs membrane (RBCM) integrity and enhances its permeability to biochemical entities. Moreover, RBCs aggregation and their susceptibility to hemolytic agents would be propagated as well. During the trophozoite stage (approximately after 12–15 h post invasion), PRBCs start expressing unique protein channels, known as new permeation pathways (NPP). NPPs mediate acquisition of nutrients and excretion of waste products as they mediate efflux and influx of various biochemical entities, *viz*, organic anions, amino acids, sugars, nucleotides and inorganic ions [3–5].

Andrographolide is a member of isoprenoid family of natural products and belongs to labdan diterpenoide lactones. It was first

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isolated by Boorsma from different parts of *Andrographis paniculata*. The later had been used extensively in Siddhu, Ayurvedic and tribal medicine of India as well as in other countries for many clinical applications, *viz*, respiratory tract infections, ulcerative colitis, rhematoidal arthritis, cardiovascular disorders and cancer treatment. Recently, some studies have pointed out to the antiretroviral, antibacterial pro-apoptotic, anti-inflammatory and antioxidant effects of andrographolide [6–9].

Alteration of RBCs shape or membrane properties may retard the parasite growth. Previous studies revealed that different terpenoides may adversely affect biological membrane functions through changing their permeability to different entities, induction of membrane micro-domains and ATP dependent homeostatic mechanisms that maintain cell shape and volume [10].

In this study, we assessed the anti-plasmodium potential of andrographolide and its effect on the membrane of uninfected RBCs (nRBCs), solute induced lysis of PRBCs and parasite invasiveness.

2. Materials and methods

2.1. Materials and chemicals

Human O+ erythrocytes were obtained from blood drawn from the first author. RPMI-1640 medium, albumax II, were procured from Gibco BRL (Grand Island, NY, USA). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), triton X-100, sorbitol, hypoxanthine, (100×) phosphate buffered saline (PBS), frusemide and chloroquine diphosphate (CQ) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gentamicin was purchased from Jiangxi Dongxu Chemical Technology Co., Ltd. while andrographolide was purchased from Indofine Biochemical company Inc. (Cat No.: A-003).

2.2. Parasite culturing, maintenance and synchronization

Both CQ sensitive and resistant strains of *P. falciparum* strain 3D7 were cultured in O+ red blood cells suspended in a Complete Malaria Culture Medium (cMCM) containing RPMI-1640, 25 mM HEPES (pH 7.4), 0.75 mM hypoxanthine, 0.5% albumax, 24 mM sodium bicarbonate, 11 mM glucose and 50 μg/L gentamicin. pH was maintained at 7.4 and the hematocrite level at 2%. The culture was incubated at 37 °C in a micro-aerophilic atmosphere containing 90% N_2, 5% CO_2 and 5% O_2. Furthermore, the medium was changed every 24 h and checked using Giemsa stained thin blood smears [11,12]. Before starting drug screening, the parasites were synchronized using sorbitol synchronization technique described by Vanderberg [13].

2.3. Stock solution preparation

Stock solutions of 100 mM of each of CQ and andrographolide were prepared using PBS (pH 7.4) for the first and methanol for the second (methanol was preferred over DMSO as the later is not baneful to plasmodium).

2.4. Malaria drug sensitivity assay

Malaria drug sensitivity assay was performed according to Mathias *et al*., 2010. Drug containing flat bottomed 96 well microtiter plates, featured serial dilution of CQ (1 nm–1 μM) and andrographolide (1 nm–1 mM) were incubated for 48 h at 37 °C with PRBCs (synchronized at the ring stage with parasitemia of 1% and hematocrite 1%). Control wells containing nRBCs, PRBCs and drug solution were allocated as well. Serial drug dilution was performed using cMCM as a diluent. Three plates were prepared for each drug and each dilution was done in triplicate. At the end of the incubation period, the plates had been freeze-thawed for 1 h then the well were loaded with 100 μL of SYBR green-I lysis buffer [20 mM Tris-HCL, 5 mM EDTA, 0.008% saponin and 0.008% (v/v) triton-100]. The mixture was incubated in dark at room temperature for 1 h and finally fluorescence was measured after 15 s of plate agitation twice in Victor Plate reader (Perkin Elmer, Salem, MA) at an excitation/emission wavelength of 485/535 nm. The geometric mean of the first and second pass was used to exclude any measurement error [14]. Eventually both IC_{50} and IC_{90} for CQ against *P. falciparum* 3D7 were determined according to the recommended protocol using Microsoft excel 2007 software.

2.5. Effect of andrographolide on merozoites invasiveness

Andrographolide effect on merozoite invasion was performed as previously described [4]. Briefly, non infected RBCs were treated at 37 °C for 2 h with different concentrations of the drug (1 μM/mM) dissolved in the incomplete malaria culture medium (RPMI-1640, HEPES-tris buffer 25 mmM and gentamicin 20 μg/mL). Then the exuberant andrographolide was washed twice and to each 300 μL of the treated RBCs, 100 μL of PRBCs at parasitemia >15% and rich in schizont stage (after >35 h of parasite synchronization) was added and Hct was adjusted at 4%. The mixture was incubated at the mentioned incubation conditions for 20 h, the time point at which the microscopic determination of parasitemia was done. In this test, the amount of merozoites that could have invaded the treated RBCs was compared to the positive control that contains only non treated RBCs. The percentage of parasite duplication inhibition at each concentration was calculated using the following equation

$$\%\ of\ parasitemia\ duplication\ inhibition = \frac{Pa - Pbx}{Pa} \times 100$$

wherein *Pa* and *Pbx* represents the parasitemia of the positive control and that of the culture containing RBCs treated with x concentration of andrographolide. The percentage of each concentration was plotted versus log concentration to determine the dose response curve.

2.6. Effect of andrographolide on sorbitol induced PRBCs hemolysis

Andrographolide effect on parasite induced permeability pathway was investigated as previously described [15]. Briefly, PRBCs predominated with trophozoites (after 30 h of synchronization) at 5% parasitemia were washed with cMCM and 100 μL of the washed cells were loaded into 24 well plates, featured two folds serial dilution of andrographolide or frusemide, a well-known inhibitor of plasmodium induced NPPs, with a concentration range of (500 nM–500 μM) for each at 1 mL/well. The dilutions were done using sorbitol buffer