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Antimalarial activity of the terpene nerolidol

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ABSTRACT

Malaria, an infectious disease that kills more than 438,000 people per year worldwide, is a major public health problem. The emergence of strains resistant to conventional therapeutic agents necessitates the discovery of new drugs. We previously demonstrated that various substances, including terpenes, have antimalarial activity in vitro and in vivo. Nerolidol is a sesquiterpene present as an essential oil in several plants that is used in scented products and has been approved by the US Food and Drug Administration as a food-flavouring agent. In this study, the antimalarial activity of nerolidol was investigated in a mouse model of malaria. Mice were infected with *Plasmodium berghei* ANKA and were treated with 1000 mg/kg/dose nerolidol in two doses delivered by the oral or inhalation route. In mice treated with nerolidol, parasitaemia was inhibited by >99% (oral) and >80% (inhalation) until 14 days after infection ($P < 0.0001$). On Day 30 post-infection, the survival rate of orally treated mice was 90% compared with 16% in controls ($P < 0.0001$). In contrast, inhalation-treated mice showed a survival rate of 50% vs. 42% in controls ($P > 0.05$). The toxicity of nerolidol administered by either route was not significant, whilst genotoxicity was observed only at the highest dose tested. These results indicate that combined use of nerolidol and other drugs targeting different points of the same isoprenoid pathway may be an effective treatment for malaria.
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1. Introduction

Malaria, a major tropical disease caused by protozoa of the genus *Plasmodium*, affects 214 million people worldwide and causes more than 438,000 deaths per year. *Plasmodium falciparum* is responsible for the most severe form of the disease [1]. Given the increasing resistance of parasites to all drugs currently used to treat malaria, new approaches to drug design are urgently needed [2,3]. The isoprenoid metabolism pathway is a potential target for evaluating the effectiveness of candidate antimalarial drugs. The first intermediates of this pathway are synthesised by 2C-methyl-D-erythritol-4-phosphate (MEP) [4] localised in the apicoplast, an organelle present in most protozoan parasites of the subphylum Apicomplexa, including plasmodia [5].

Our group previously demonstrated that the MEP pathway is active in the intraerythrocytic stages of *P. falciparum* [6] and synthesises dolichol of the 11–12 isoprene unit [7], ubiquinone [8], isoprenylated/dolichylated proteins [9,10], carotenoids [11], tocopherol [12] and menaquinone [13]. We also showed that nerolidol interferes with the isoprenoid biosynthetic pathway by blocking the biosynthesis of dolichols and ubiquinone as well as protein isoprenylation in *P. falciparum* [14]. Nerolidol showed a half-maximal inhibitory concentration (IC_{50}) of 760 nM and a supra-additive effect against *P. falciparum* in vitro when combined with fosmidomycin or squalestatin [15]. Nerolidol is a sesquiterpene with a floral odour that is present as an essential oil in several plants. It is used in many scented products and has been approved by the US Food and Drug Administration (FDA) as a food-flavouring agent. Nerolidol has many therapeutic properties, including antitumour [16], anti-ulcer [17], antioxidant [18] and antibacterial [19] effects as well as activity against several protozoa [20]. It was recently shown that nerolidol inhibits the in vitro growth of four species of *Babesia* [21]. Furthermore, parasitaemia was reduced in mice infected with *Babesia microti* that were treated with 100 mg/kg nerolidol [21].

The Waiãpi Indians from the west side of the Amapá Amazonian forest use volatile components of *Virola surinamensi* leaves for the treatment of malaria; at the peak of fever, the infected patient is exposed to the volatile component in the vapour. Analysis of the essential oil extracted from this plant species by gas chromatography coupled with mass spectrometry (GC/MS) revealed the presence of monoterpenes, sesquiterpenes and phenylpropanoids [22]. Treatment of *P. falciparum* with *V. surinamensi* essential oil in vitro inhibited parasite development after 48 h. Nerolidol has been identified as one of the active constituents of this oil [22]. Inhalation of terpenes, which are volatile and thus easily administered, has gastroprotective effects in rodents and in patients with recurrent malignant glioma [23,24]. The present study investigated the

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in vivo antimalarial activity of nerolidol administered by oral and inhalation routes in mice infected with *Plasmodium berghei* ANKA.

2. Materials and methods

2.1. Nerolidol quantification

Nerolidol (Merck, Hohenbrunn, Germany) levels in mouse serum following administration by the inhalation route were measured as previously described for the oral route [25]. Briefly, five male BALB/c mice (5–6 weeks old, weight 20 ± 2 g) were used to quantify plasma nerolidol levels. A single dose of nerolidol (1000 mg/kg) was administered by inhalation as described below (Section 2.6). Blood samples were collected at 0, 30, 180, 360 and 720 min. Plasma was separated from blood cells by centrifugation at 3000 × *g* for 10 min at 4 °C and was stored at –20 °C for 72 h before analysis by GC/MS [25]. Nerolidol bioavailability or the area under the concentration–time curve in the 12 h after administration (AUC_{0-12}) was calculated according to the trapezoidal rule.

2.2. Assessment of nerolidol toxicity

To assess the acute toxicity of nerolidol, levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine were measured by colorimetric assays after 15 days of treatment with 2000 mg/kg/day terpene administered as two doses at 12-h intervals. Following treatment, five animals were sedated with ketamine and xylazine (60 mg/kg and 8 mg/kg by intraperitoneal injection, respectively). Plasma was collected by cardiac puncture and transaminase (ALT and AST) and creatinine levels were evaluated using Bioclin kits (Belo Horizonte, MG, Brazil).

2.3. Assessment of nerolidol genotoxicity

The comet assay was carried out as previously described [26,27]. Nerolidol was diluted in 70% ethanol to obtain a 50 mM stock solution. Blood samples were obtained by cardiac puncture of uninfected BALB/c mice and were diluted 1:1 in RPMI medium. Lymphocytes were isolated using Ficoll-Paque PLUS and were centrifuged at 800 × *g* for 20 min. Then, 5×10^4 cells were diluted in 3.2, 6.5 or 13 μM nerolidol or 800 μM H_2O_2 as a positive control (to verify the sensitivity of the test) and were incubated at 37 °C for 3 h. After treatment, cells were centrifuged at 800 × *g* for 5 min and an aliquot was used in the trypan blue viability test.

The extent and distribution of DNA damage determined by the comet test was examined by epifluorescence microscopy (1000× magnification) in cells stained with 20 μg/mL ethidium bromide. Randomly selected, non-overlapping cells on slides (50 per coded slide) were scored in a blinded fashion according to comet tail length as follows: class 0, no tail; class 1, tail shorter than the diameter of the head (nucleus); class 2, tail length one to two times the diameter of the head; and class 3, tail longer than twice the diameter of the head. The amount of DNA in the tail was determined using ImageJ software (National Institutes of Health, Bethesda, MD). The damage index (DI) was calculated as a value from 0 (no tail: 100 cells × 0) to 300 (with maximum migration: 100 cells × 3) [28].

2.4. Experimental animals

BALB/c mice were bred and housed under specific pathogen-free conditions at the vivarium sector of the Institute of Biomedical Sciences, University of São Paulo (São Paulo, Brazil). All animals were fed a regular diet. All procedures were carried out in accordance with national regulations on animal experimentation adopted by the Brazilian Society of Laboratory Animal Science and were authorised by the Ethics Committee in Animal Experimentation.

2.5. Mouse infection with P. berghei ANKA

Infection experiments were performed in male BALB/c mice (5–6 weeks old, weight 20 ± 2 g). *P. berghei* ANKA was propagated in the mice; in each experiment, a fresh blood sample was obtained and a suspension of 1×10^6 parasitised red blood cells in 200 μL was injected intraperitoneally into each mouse [29]. Mice were randomly divided into the following four groups: group 1, infected animals treated with vehicle by inhalation ($n = 7$); group 2, infected animals treated with vehicle by oral administration ($n = 7$); group 3, infected animals treated with nerolidol by oral administration ($n = 10$); and group 4, infected animals treated with nerolidol by inhalation ($n = 10$). Our laboratory previously established this parasite burden as the 50% lethal dose at 12 days after inoculation.

2.6. Nerolidol administration by oral and inhalation routes

BALB/c mice were treated with 2000 mg/kg/day nerolidol administered as two daily doses at 12-h intervals. Treatment was initiated 2 h after infection with *P. berghei* ANKA and was continued for 15 consecutive days. Nerolidol was mixed in corn oil and mice were administered 40 μL by the oral route. The control group received only corn oil. Nerolidol was delivered by inhalation via a commercial nebuliser (SoniClear, São Paulo, Brazil) at a rate of 0.5 mL/min by generating 0.5–5.0 μm particles. The head of each mouse was exposed for 5 min to a nebulised solution of nerolidol diluted in buffered saline (0.15 M NaCl, 0.007 M Na_2HPO_4, 0.01 M NaH_2PO_4; pH 7.4) in 2% Tween 20; the control group was exposed to nebulised buffered saline solution in 2% Tween 20. Brief and repeated exposure of the head was used since it limits exposure and prevents particle ingestion by the animal [30].

2.7. Assessment of parasitaemia

All mice were examined at 3-day intervals and parasitaemia was detected by panoptic staining of blood smears. The blood sample was collected through a small incision at the tip of the tail and the percentage of parasitised erythrocytes among 1000 that were counted was determined by optical microscopy at 1000× magnification. Inhibition of parasite growth in drug-treated groups was calculated with respect to control (untreated) animals as follows: [(average parasitaemia in the control group) – (parasitaemia in the test group)]/parasitaemia in the control group. Results are expressed as a percent reduction of parasitaemia [31].

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was applied to normally distributed data, followed by the Tukey's post-hoc test for multiple comparisons. The effect of treatment on survival was determined by Kaplan–Meier curves, with statistical differences evaluated by the log-rank method. Analyses were performed using GraphPad Prism v.5.3 software (GraphPad Inc., La Jolla, CA).

3. Results and discussion

3.1. Nerolidol quantification by gas chromatography–mass spectrometry

The plasma nerolidol concentration following a single inhaled dose of 1000 mg/kg was measured as previously described for administration by the oral route, for which the maximum concentration (1.57 ± 0.22 μM) was observed after 6 h [25]. In contrast, the maximum concentration by inhalation (2.7 ± 0.5 μM) was detected soon after administration was complete, i.e. within 5 min (Fig. 1). The plasma concentration of nerolidol decreased from

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