Terpenes Arrest Parasite Development and Inhibit Biosynthesis of Isoprenoids in Plasmodium falciparum

Herbert Rodrigues Goulart,¹ Emília A. Kimura,¹ Valnice J. Peres,¹ Alicia S. Couto,² Fulgencio A. Aquino Duarte,³ and Alejandro M. Katzin*

Departamento de Parasitologia, Instituto de Ciências Biomédicas, ¹ and Departamento de Engenharia Mecânica, Escola Politécnica,³ Universidade de São Paulo, São Paulo, Brazil, and CIHIDECAR, Departamento de Química Orgânica. Pabellón II, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires 1428, Argentina²

Received 19 November 2003/Returned for modification 31 December 2003/Accepted 8 March 2004

Development of new drugs is one of the strategies for malaria control. The biosynthesis of several isoprenoids in Plasmodium falciparum was recently described. Interestingly, some intermediates and final products biosynthesized by this pathway in mammals differ from those biosynthesized in P. falciparum. These facts prompted us to evaluate various terpenes, molecules with a similar chemical structure to the intermediates of the isoprenoid pathway, as potential antimalarial drugs. Different terpenes and S-farnesylthiosalicylic acid were tested on cultures of the intraerythrocytic stages of P. falciparum, and the 50% inhibitory concentrations for each one were found: farnesol, 64 μM; nerolidol, 760 nM; limonene, 1.22 mM; linalool, 0.28 mM; and S-farnesylthiosalicylic acid, 14 μM.

All the terpenes tested inhibited dolichol biosynthesis in the trophozoite and schizont stages when [1-¹³C]farnesyl pyrophosphate triammonium salt ([¹³C]FPP) was used as precursor. Farnesol, nerolidol, and linalool showed stronger inhibitory activity on the biosynthesis of the isoprenic side chain of the benzoquinone ring of ubiquinones in the schizont stage. Treatment of schizont stages with S-farnesylthiosalicylic acid led to a decrease in intensity of the band corresponding a p21ras protein. The inhibitory effect of terpenes and S-farnesylthiosalicylic acid on the biosynthesis of both dolichol and the isoprenic side chain of ubiquinones and the isoprenylation of proteins in the intraerythrocytic stages of P. falciparum appears to be specific, because overall protein biosynthesis was not affected. Combinations of some terpenes or S-farnesylthiosalicylic acid tested in this work with other antimalarial drugs, like fosmidomycin, could be a new strategy for the treatment of malaria.

Despite attempts at eradication made since the 1950s, malaria is still the most important parasitic disease of humans. As a consequence of increasing parasite resistance to virtually all reagents used for malaria therapy, new approaches to drug design are urgently needed (38).

Isoprenoids play important roles as components of structural cholesterol, steroid hormones in mammals, carotenoids in plants, and ubiquinones in all living organisms (34, 35).

Recently, the identification of two genes encoding the enzymes 1-deoxy-d-xylulose-5-P (DOXP) synthase and DOXP reductoisomerase suggested that isoprenoid biosynthesis in Plasmodium falciparum depends on the DOXP/2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (21). In contrast, in humans, isoprenoids are synthesized via the mevalonate pathway (18). Fosmidomycin, an inhibitor of DOXP reductoisomerase, showed antimalarial activity in vitro and in vivo (21); however, 10% of patients failed to show cure with this treatment (42). To avoid resistance or the rapid emergence of resistance, combinations with antimalarial inhibitors operating on the different points in the same metabolic pathway could be synergistic (39). In P. falciparum all the metabolic pathways and associated enzymes downstream of isopentenyl pyrophosphate (IPP) need to be identified and characterized (39).

In a previous work, we showed the presence of an active isoprenoid pathway for the biosynthesis of dolichol of 11 and 12 isoprenic units (4) and side chain of the 8 and 9 isoprenic units attached to benzoquinone rings of ubiquinones in P. falciparum (7). In mammalian cells, the isoprenic chains of dolichols and ubiquinones comprise 20 to 22 and 10 isoprenic units, respectively (2, 23). In plants, the isoprenoid pathway biosynthesizes terpenes whose antibacterial, antifungal, and antiparasitic activities have been previously reported (5, 34). The chemical structure of some terpenes resembles that of some intermediates of the isoprenoid pathway (10) and may interfere with the biosynthesis of the latter in other organisms. In our laboratory, we demonstrated the inhibition of P. falciparum protein isoprenylation by the monoterpene limonene (29).

One of the oldest antimalarial drugs is a sesquiterpene lactone, artemisinin, whose mechanism of action has been recently described (13).

Therefore, the possibility of developing new antimalarial drugs that could interfere with the biosynthesis of the dolichols, with the isoprenic chain of ubiquinones, and with protein isoprenylation led us to study the effects of different terpenes purified from essential oils (6, 27).

In the present study we investigated the effects of various terpenes (farnesol, nerolidol, limonene, and linalool) and the S-farnesylthiosalicylic acid (FTS) on the biosynthesis of dolichol and the isoprenic side chain of ubiquinones as well as on protein isoprenylation in the intraerythrocytic stages of P. falciparum.

MATERIALS AND METHODS

Parasite culture. The experiments were performed with isolate NF54, clone 3D7 of P. falciparum. Parasites were cultured according to the method of Trager

* Corresponding author. Mailing address: Universidade de São Paulo, Av. Professor Lineu Prestes, 1374, CEP 05508-900 São Paulo, Brazil. Phone: (55) (11) 30917267. Fax: (55) (11) 30917417. E-mail: amkatzin@icb.usp.br.
Inhibition of isoprenoids by terpenes in *P. falciparum*

**Vol. 48, 2004 INHIBITION OF ISOPRENOIDS BY TERPENES IN *P. FALCIPARUM* 2503**

and Jensen (37) as modified by Kimura et al. (25). The gas mixture of the tissue culture flasks (75 cm²) contained 5.05% CO₂, 4.93% O₂, and 90.2% N₂.

Parasite development and multiplication was monitored by microscopic evaluation of Giemsa-stained thin smears. Synchronization was obtained by two treatments with 5% (wt/vol) Plasmasol (PlasmaLab II; Bayer Pharma, Sweden) and the final concentration, 5% hematocrit, 2% parasitemia. Each stage of the parasite (ring, trophozoite, and schizont forms) was then incubated with anti-α-tubulin antibody (29) and then subjected to autoradiography for several days at −70°C. Standards were visualized with iodine vapor.

**Gel electrophoresis.** SDS-PAGE was performed in 12.5% gels as described elsewhere (26). The same number of drug-treated or untreated parasites as mentioned above were solubilized in SDS sample buffer and applied to each well for analysis. All gels were treated with Amplify (Amersham), dried, and exposed to Kodak X-Omat film with intensifying screen sets at −70°C.

**Immunoprecipitation assays.** Samples stored in liquid N₂ were resuspended in 100 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, and a 5-μg/ml concentration of a protease inhibitor cocktail [0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 mM β-mercaptoethanol, chymostatin (5 mg/ml), and a 1-μg/ml concentration each of leupeptin, antipain, and pepstatin A] and then preincubated with protein A-Sepharose beads (Pharmacia) (29). Each stage (ring, trophozoite, and schizont forms) was then incubated with anti-human-Ras or anti-Raf-1 (Novagen, Madison, WI) for 2 h at 4°C. The antigen-antibody complex was precipitated using 100 μl of a 10% protein A-Sepharose slurry. After five washes with PBS, the bound antigen was released in SDS sample buffer and analyzed by SDS-PAGE and autoradiography (24).

**Reverse-phase high-performance liquid chromatography (HPLC).** Each purified parasite (ring, trophozoite, and schizont forms) stage was freeze-dried and successively extracted with hexane (three times; 0.5 ml each) (extract I), chloroform-methanol (1:1; vol/vol; three times; 0.5 ml each) (extract II), and chloroform-methanol-water (10:10:3; vol/vol; three times; 0.5 ml each) (extract III) (4). For the analysis of dolichyl-phosphate (dolichyl-P) and dolichyl-pyrophosphate (dolichyl-PP), either II or extract III was subjected to acid hydrolysis. Samples were hydrolyzed with 100 μl of a solution of 50% HCl in a screw-cap test tube for 3 h at room temperature. After incubation, the sample was neutralized with a saturated solution of LiOH and then extracted with hexane as described above (33).

Aliquots of each extract were monitored for radioactivity. Samples were analyzed on an Ultraphase ODS Beckman column (4.6 mm by 25 cm) with a Gilson HPLC apparatus equipped with UV/VIS 151 and 152 detectors. For dolichol analysis, the eluent was monitored at 210 nm. Fractions of 0.5 min were collected, and aliquots of each fraction were spotted on silica gel plates and visualized by several customizing procedures.

A gradient elution system was used, with methanol-water (9:1, vol/vol) as solvent system at a flow rate of 1.5 ml/min. Standards of dolichols 11 and dodecaprenol and a mixture of dolichols 18 and 19 were injected (4).

Hexane extracts of each parasite stage were analyzed for isoprenoid side chains attached to benzoquinone rings. Whole hexane extracts from each stage were dried under a nitrogen stream, resuspended in methanol and conjected with a mixture of known amounts of authentic Q₁₀,₂₀ standards. Hexane-methanol (75: 25, vol/vol) was used as a solvent system at a flow of 1 ml/min. Standards were detected at 275 nm (7). Each 30-s fraction was collected, and aliquots were monitored for radioactivity. In order to compare the effect of terpenes and FTS on the biosynthesis of dolichol and derivatives or on the isoprenoid side chains of ubiquinones, the same quantities of treated or nontreated parasites were injected. Percentage inhibition was determined as follows: (cpm for control samples − cpm for treated samples)/cpm for control samples × 100.

A comparative statistical analysis of peak areas of HPLC chromatograms of samples treated with terpenes or FTS versus untreated samples was performed for both dolichol and ubiquinones. In order to determine the possible inhibitory effect of these compounds during treatment, an estimate of the degree of inhibition for each phase of the process was made. After evaluating the normality of the distribution, Student’s t test was applied to the data, taking as null hypothesis (H₀) the equality of the means between control and treated populations (95% confidence limits). The average inhibition was then estimated with a significance of 95% for each phase of the experiment.
RESULTS

Inhibition of *P. falciparum* development after treatment with terpenes and FTS. To test the inhibitory effect of terpenes and FTS on *P. falciparum* growth, defined numbers of parasites were cultured in the absence or presence of increasing concentrations of each drug. Three independent determinations of the IC50 for terpenes and FTS demonstrated the precision of the assay. The results of parasitemia and the uptake of [3H]-labeled proteins from terpenes and FTS (48-h)-treated or untreated parasites did not differ significantly in the three parasite stages (Table 2). The results are significantly different at *P* < 0.05. Data are based on at least three independent experiments, which were performed in triplicate. CI, confidence interval.

### Table 1. In vitro IC50s and IC90s of terpenes and FTS for *P. falciparum*

<table>
<thead>
<tr>
<th>Terpene</th>
<th>Mean IC50 ± SE (CI)</th>
<th>Mean IC90 ± SE (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farnesol</td>
<td>64 ± 5.5 μM (47.755–65.262 μM)</td>
<td>108 ± 6.5 μM (78.19–116.31 μM)</td>
</tr>
<tr>
<td>Nerolidol</td>
<td>760 ± 23 nM (618.96–802.95 nM)</td>
<td>2,189 ± 74 nM (1,759–2,307 nM)</td>
</tr>
<tr>
<td>Limonene</td>
<td>1.22 ± 0.13 mM (1.07–1.42 mM)</td>
<td>2.27 ± 0.15 mM (1.99–2.70 mM)</td>
</tr>
<tr>
<td>Linalool</td>
<td>0.28 ± 0.03 mM (0.196–0.324 mM)</td>
<td>0.64 ± 0.01 mM (0.533–1.152 mM)</td>
</tr>
<tr>
<td>FTS</td>
<td>14 ± 1.3 μM (13.61–19.18 μM)</td>
<td>26 ± 3 μM (23.44–50.7 μM)</td>
</tr>
</tbody>
</table>

*The IC50 and IC90 of terpenes and FTS were calculated by probit analysis as described in Material and Methods, item Inhibition tests. Student’s *t* test was applied to the results. The results are significantly different at *P* < 0.05. Data are based on at least three independent experiments, which were performed in triplicate. CI, confidence interval.

Inhibition of dolichol and ubiquinone biosynthesis in *P. falciparum* induced by treatment with terpenes and FTS. The percent inhibition of dolichol and isoprenic chain side of ubiquinone biosynthesis, shown in Table 2, was determined by comparing average peak areas of HPLC chromatograms and a statistical analysis applied as described in Materials and Methods [see “Reverse-phase high-performance liquid chromatography (HPLC)”]. All the terpenes inhibited dolichol biosynthesis in the trophozoite and schizont stages when [3H]GGPP was used as precursor. Linalool and nerolidol showed a similar effect on ring stages. Farnesol inhibited biosynthesis of dolichol in the trophozoite and schizont stages when [3H]GGPP was used as a precursor; this effect was also detected in schizont stages upon treatment with nerolidol. FTS showed a low inhibitory effect on dolichol biosynthesis in the schizont stages (Table 2). The inhibitory effects of terpenes on dolichol biosynthesis were similar when dolichyl-P and dolichyl-PP were analyzed (data not show).

Concerning the biosynthesis of the isoprenic chain attached to the benzoquinone ring of ubiquinones, farnesol, nerolidol, and linalool showed a stronger inhibitory activity on the schizont stage. Farnesol was a more potent inhibitor than limonene in the ring and trophozoite stages when [3H]GGPP was used as precursor. FTS caused low inhibition (21%) of the isoprenic chain side of ubiquinone biosynthesis in schizont stages (Table 2).

To determine whether the inhibitory effect of terpenes and

### Table 2. Percent inhibition of the biosynthesis of dolichol and isoprenic chains of ubiquione in treated *P. falciparum*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Precursor</th>
<th>% Inhibition of <em>P. falciparum</em> treated with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ferneisol</td>
<td>Nerolidol</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>O</td>
</tr>
<tr>
<td>Ring</td>
<td>GGPP</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>FPP</td>
<td>0</td>
</tr>
<tr>
<td>Trophozoite</td>
<td>GGPP</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>FPP</td>
<td>68</td>
</tr>
<tr>
<td>Schizont</td>
<td>GGPP</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>FPP</td>
<td>66</td>
</tr>
</tbody>
</table>

*Abbreviations: FPP: [1(n)-3H]farnesyl triammonium pyrophosphate; GGPP: [1(n)-3H]geranylgeranyl triammonium pyrophosphate; D: dolichol of 55 and 60 carbons; Q: ubiquione of eight isoprenic and nine isoprenic units. B Percent inhibition was determined as described in Materials and Methods [see “Reverse-phase high-performance liquid chromatography (HPLC)”]. Student’s *t* test was applied to the results. The results are significantly different at *P* < 0.05.
The intensity of the bands with \( ^{14}\text{C}\)IPP incorporation between untreated and farnesol- or nerolidol-treated parasites was reduced. No incorporation of \( ^{3}\text{H}\)FPP, \( ^{14}\text{C}\)IPP, or \( ^{3}\text{H}\)GGPP, or \( ^{14}\text{C}\)IPP into uninfected erythrocytes submitted to identical experimental conditions (4).

**DISCUSSION**

In this report we demonstrate that farnesol, nerolidol, limonene, linalool, and FTS have antimalarial activities on intraerythrocytic stages of *P. falciparum* in vitro. In recent reports, we demonstrated that nerolidol inhibits the biosynthesis of the isoprenoid chain attached to the benzoquinone ring in the intraerythrocytic stages of *P. falciparum* (7) and that limonene inhibits protein isoprenylation in the same stages of the parasite (29). In the present study we demonstrate that terpenes such as farnesol and linalool inhibit the biosynthesis of several intermediates and end products of the isoprenoids pathway.

The terpenes tested here may be acting through inhibition of the isoprenyl diphasphate synthases. This family of enzymes catalyzes consecutive 1'-4 condensations of isopentenyl-PP with the allylic substrate to form the linear backbone for all isoprenoid compounds, including prenylated proteins, prenylated quinones, and dolichol (41). In plants the isoprenoid pathway biosynthesizes only terpenes, while in *P. falciparum* this pathway generates other types of isoprenoids, e.g., dolichol, isoprenic side chains of ubiquinones, and isoprenic chain attached to proteins (4, 7, 9). Due to their structural similarity,
FIG. 2. In vitro treatment of *P. falciparum* cultures with terpenes or FTS and metabolic labeling with [3H]GGPP and [3H]FPP. Parasites were untreated or treated for 48 h with terpenes and FTS and labeled for 18 h with a 3.125-μCi/ml concentration of [3H]GGPP (A to C) or [3H]FPP (D to F) in the presence of the drugs. The parasites were harvested and purified by Percoll gradient, lysed, and analyzed by SDS-PAGE and fluorography. (A and D) Ring forms; (B and E) trophozoite forms; (C and F) schizont forms. Lane 1, untreated parasites; lane 2, 5.5 μM FTS; lane 3, 25 μM farnesol; lane 4, 500 nM nerolidol; lane 5, 0.5 mM limonene; lane 6, 0.1 mM linalool. Molecular size standards are indicated on the left.
terpenes might interfere with the parasite’s biosynthesis of polyprenoids by competing with natural substrates in enzyme-substrate reactions or by interfering with the mechanisms of elongation of isoprenic chains.

It is known that farnesyl-PP and geranylgeranyl-PP are substrates of the enzymes prenyltransferases involved in the biosynthesis of dolichol, the isoprenic side chain of ubiquinones and isoprenic chains attached to proteins (41). In this report we used [3H]FPP or [3H]GGPP to determine if the terpenes assayed had specificity for one of these precursor. According to our results, these terpenes appear to have a widespread inhibitory action.

Farnesol had a strong inhibitory effect on the biosynthesis of dolichol and the isoprenic side chain of benzoquinone rings as well as on protein isoprenylation when [3H]FPP was used as precursor. Farnesol has a molecular structure similar to that of the precursors used for labeling these polyprenoids and could be interfering with chain elongation. When schizont stages treated with farnesol were labeled with [14C]IPP in the presence of this terpene, only the spots with Rf coincident with dolichol and ubiquinones were diminished (Fig. 1, lane 3). This fact would suggest that, since farnesyl-PP an intermediate product of the biosynthetic pathway of isoprenoids, the addition of external farnesol might interfere with the elongation of the isoprenic chains that contain more than 10 carbons in their molecule. Similar results were obtained in nerolidol-treated parasites (Fig. 1, lane 4). On the other hand, limonene and linalool might interfere in the synthesis of the first precursor, since all the spots corresponding to geraniol, farnesol, dolichol, and ubiquinones were reduced when parasites were treated with these terpenes (Fig. 1, lanes 5 and 6). Antimalarial activity by essential oils that contain nerolidol has been described previously by us and others (7, 12). The IC50 of nerolidol for isolate NF54, clone 3D7, of P. falciparum was quite different from that described by us for isolate S20 (7); however, both isolates presented identical inhibition of dolichol and ubiquinone biosynthesis without inhibiting of farnesol and geraniol. In other cell systems, farnesol has been found to inhibit the biosynthesis of dolichol and ubiquinones by degradation of 3-hydroxy-3-methylglutaryl-Co A reductase (3). However, this enzyme or the gene sequences related to 3-hydroxy-3-methylglutaryl-Co A reductase are not present in P. falciparum, where isoprenoids are biosynthesized via the DOXP/MEP pathway (17, 21). We thus suggest that farnesol and nerolidol act in P. falciparum by inhibiting the elongation of isoprenic chains.

The antimalarial activity of limonene through inhibition of protein isoprenylation previously described by us was confirmed here (29). This antimalarial effect could be more extensive. Monoterpenes like limonene or linalool probably inhibit the biosynthesis of dolichol and ubiquinones by interfering with the condensation between isopentenyl-PP and dimethylallyl-PP. This hypothesis is suggested by the results of HPTLC, when parasite labeling with [14C]IPP in the presence of limonene or linalool resulted in reduction of spots coinciding with geraniol, farnesol, dolichol, and ubiquinones. These inhibitory effects of limonene on the biosynthesis of dolichol and the isoprenic side chains of benzoquinone rings were first described in hepatoma and Neuro2A cells, respectively (22, 36). It has been proposed that linalool inhibits the biosynthesis of dolichol and ubiquinones. Although this effect has not been described to date, antileishmanial activity via increased nitric oxide production was recently reported in linalool-treated macrophages (11). Antifungal and antibacterial effects of linalool have also been reported but their mechanisms of action are not still known (32).

We believe that the inhibitory effects of nerolidol, farnesol, limonene, and linalool on isoprenoid biosynthesis demonstrated herein are specific because the concentrations of terpenes used did not affect overall protein biosynthesis.

FTS had a low inhibitory effect on the biosynthesis of dolichol and ubiquinones (23 and 21%, respectively). These results agree with data published for other cell systems (15). We used FTS as a control of a modified terpene, and the levels of inhibition of dolichol and ubiquinones biosynthesis obtained were very low. These results might be interpreted as an indication that the inhibitory effect of farnesol and the other terpenes (nerolidol, limonene, and linalool) tested in the present study is specific for dolichol and side chain of ubiquinones biosynthesis.
On the other hand, when parasites were treated with FTS, a reduced signal was detected in anti-Ras immunoprecipitates of the schizont stages, and parasite development was arrested at this stage. FTS inhibits the development of the human pancreatic tumor by dislodging Ras from its membrane-anchoring domains and accelerating its degradation (14, 16, 19).

Farnesol generated the widest range of inhibition of isoprenylation of Ras- and Rap-like proteins in the three stages of *P. falciparum*. The effect produced by farnesol has not been described in eukaryotic cells to date. We suggest that farnesol interferes with the linkage between the protein and a farnesyl-PP group or a geranylgeranyl-PP group. Linalool induced some inhibition of Ras-like protein isoprenylation in ring and schizont stages. A similar effect was described in the human erythroleukemia cell line K562, and the authors suggested that alcohols with an open chain are inactive or have low inhibitory activity on isoprenylation (20).

The present report is the first demonstration that these terpenes have an inhibitory action on isoprenoid biosynthesis at different points of the isoprenoid pathway and inhibited the development of the intraerythrocytic stages of *P. falciparum* at one of the lowest IC₅₀. The other terpenes assessed here also showed antimalarial activity in vitro and inhibited parasite development. This widespread antimalarial activity was more evident in the schizont stages, probably because during the differentiation from trophozoites to schizonts the transport of several molecules is dislodging antagonist. Int J. Cancer. 80:911–918.


