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Characterization of the isoprenoid chain of coenzyme Q in *Plasmodium falciparum*

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Abstract

Little is known about isoprenoid biosynthesis in parasitic protozoa. The presence of dolichol and isoprenylated proteins has been detected in *Plasmodium falciparum*, but no studies are available about the biosynthesis of the isoprenic side chain attached to the benzoquinone ring of coenzyme Q. In the present study, using metabolic labelling with different intermediates, we demonstrated the presence of an active isoprenoid pathway for the biosynthesis of the isoprenic chain of coenzyme Q. Our results show that *P. falciparum* is able to synthesize different homologs (coenzyme Q_8 and coenzyme Q_9), depending on the given intermediate. Parasites treated with nerolidol at doses 2.2 times below the IC_{50} showed a decreased ability to synthesize the isoprenic chain attached to coenzyme Q at all intraerythrocytic stages. Treatment with nerolidol arrested development of the intraerythrocytic stages of the parasites, indicating that the drug may have an antimalarial potential.

Keywords: Malaria; Apicomplexan; Coenzyme Q; Isoprenoid; *Plasmodium falciparum*

1. Introduction

Today malaria represents one of the most important health problems in the world. It is estimated that almost 300 million clinical cases occur every year, and over one million people die [1]. *Plasmodium falciparum* is the most dangerous among the four species of the genus *Plasmodium* that affect humans because its infection is often lethal, especially among children, pregnant women and non-immune individuals. This parasite is becoming resistant to most of the drugs currently used for malaria treatment and therefore it is essential to find new drug targets for the development of new treatments.

Isoprenoids play important roles in all living organisms, e.g. as components of structural cholesterol, steroid hormones in mammals, carotenoids in plants and ubiquinones in all living organisms [2].

Recently, the identification of two genes encoding the enzymes 1-deoxy-D-xylulose-5-phosphate (DOXP) synthase and DOXP reductoisomerase has suggested that isoprenoid biosynthesis in *P. falciparum* depends on the DOXP pathway, and it was shown that DOXP reductoisomerase activity can be inhibited by fosmidomycin [3]. However, further intermediates of this pathway have not been detected so far. Vial [4] has suggested two important points related to the isoprenoid pathway in *P. falciparum*: (a) the presence of the DOXP pathway described by Jomaa et al. [3] and the absence of the mevalonate pathway in *P. falciparum*, and (b) all the metabolic pathways and associated enzymes downstream of isopentenyl pyrophosphate (IPP) need to be identified and characterized. Protein prenyl transferase activities of *P. falciparum* have been

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Abbreviations: IPP, isopentenyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography

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reported [5]. Our group showed the presence of an active isoprenoid pathway for the biosynthesis of dolichols of 11 and 12 isoprenic units in *P. falciparum* [6] using advanced precursors of this pathway. In *P. falciparum* the biosynthesis of the isoprenic chain attached to the benzoquinone ring has not been studied previously.

Coenzyme Q is a molecule composed of a benzoquinone ring with a side chain made of several isoprenic units, whose number confers its identity to coenzyme Q. A polyprenyl diphosphate synthase is involved in the elongation of the side chain [7]. This isoprenic chain is then attached to *p*-hydroxybenzoic acid (PHBA), which was synthesized via the shikimic acid pathway in apicomplexan parasites [8]. The isoprenic chain allows the molecule to attach to the inner membrane of mitochondria, where it participates in many metabolic processes, like the electron transport chain and pyrimidine biosynthesis; it also plays a role as an antioxidant, preventing lipid peroxidation in cell membranes [9].

Little is known about the biosynthesis and regulation of coenzyme Q in protozoa. *Trypanosoma brucei* [10] and *Leishmania major* [11] synthesize the polyprenyl side chain of ubiquinone via the mevalonate pathway [12] and *Plasmodium* spp. incorporates PHBA into ubiquinone [4,13]. Incorporation studies with [^{14}C]shikimic acid were carried out on *Plasmodium knowlesi, Plasmodium cynomolgi* and *Plasmodium berghei*. However, these investigations focused on the biosynthesis of vitamin K, which was not detected, and no attempt was made to measure incorporation into ubiquinone [14].

Terpenes are molecules consisting of isoprenic units and their antibacterial, antifungal and antiparasitic activity has been determined [15]. Cultures of *P. falciparum* treated with limonene arrest development and inhibit isoprenylation of protein [16]. The antimalarial activity of nerolidol was determined in cultures of *P. falciparum*, but it is unknown which metabolic pathway is inhibited [17].

The fact that coenzyme Q has an essential role in mitochondrial metabolism, together with the lack of information about the biosynthetic pathway used by the parasite to synthesize isoprenoid chain attached to benzoquinone ring, prompted us to study the biosynthesis of this compound in *P. falciparum* by metabolic incorporation of many related radioactive precursors.

We also show that the biosynthesis of the isoprenic chain attached to the benzoquinone ring of coenzyme Q can be partially inhibited by nerolidol. This sesquiterpene arrests the development of the intraerythrocytic stages of *P. falciparum* [17].

2. Materials and methods

2.1. Materials

Nerolidol, coenzyme Q_7, coenzyme Q_{10} and dolichol 11 were purchased from Sigma (St. Louis, MO, USA). Coenzyme Q_9 was purchased from Fluka (Buchs, Switzerland). Coenzyme Q_8 was isolated from *Escherichia coli* by extraction with hexane and further purification by high-performance liquid chromatography (HPLC) as described [18]. Geraniol and farnesol were a kind gift from Dr. N. Franca Roque (Department of Fundamental Chemistry, Chemistry Institute, USP, Brazil). Percoll® was purchased from Pharmacia (Uppsala, Sweden). [G-^{3}H]hypoxanthine (270 GBq. mmol^{-1}), [1-^{14}C]acetic acid sodium salt (59.0 mCi mmol^{-1}), [1-^{14}C]IPP ammonium salt (55.0 Ci mmol^{-1}), [1-(*n*)-^{3}H]farnesyl pyrophosphate (FPP) triammonium salt (17.0 Ci mmol^{-1}), and [1-(*n*)-^{3}H]geranylgeranyl pyrophosphate (GGPP) triammonium salt (16.5 Ci mmol^{-1}) were obtained from Amersham International (Buckinghamshire, UK). *R*-[5-^{3}H]mevalonic acid ammonium salt (40 Ci mmol^{-1}) was purchased from DuPont NEN (Wilmington, DE, USA). Albumax I was obtained from Gibco BRL Life Technologies (Rockville, MD, USA). High-performance thin-layer chromatography (HPTLC) silica gel aluminum plates (Kieselgel 60, 20×20 cm, Merck, Darmstadt, Germany) were used. All solvents were of analytical or HPLC grade. Kodak X-Omat AR films were used for autoradiography. Radioactivity was measured with a Beckman LS 5000 TD β-counter.

2.2. Parasite Cultures

A *P. falciparum* isolate (S20) was obtained from a patient living in Porto Velho (Rondônia, Brazil) [19]. Cultures were grown by the method of Trager and Jensen [20], modified by Kimura et al. [21]. Development and multiplication of the culture were monitored by microscopic evaluation of Giemsa-stained thin smears. Ring-infected erythrocytes (0–20-h cultures), young trophozoite-infected erythrocytes (20–30-h cultures), old trophozoite-infected erythrocytes (30–40 h culture), and schizont-infected erythrocytes (40–48-h cultures) were purified on a 40/70/80% discontinuous Percoll® gradient (10 000×*g*, 30 min, 25°C). This yielded an upper band for old trophozoite or schizont stage (40%), a band for young trophozoite stage (70–80% interface), and a pellet for the ring stage and uninfected red blood cells [22].

2.3. Metabolic labelling of P. falciparum cultures

P. falciparum cultures with at least 12% parasitemia were labelled for 16 h with [1-^{14}C]acetic acid sodium salt (6.25 μCi ml^{-1}), *R*-[5-^{3}H]mevalonic acid (25 μCi ml^{-1}), [1-^{14}C]IPP ammonium salt (3.125 μCi ml^{-1}), [1-(*n*)-^{3}H]FPP triammonium salt (3.125 μCi ml^{-1}), or [1-(n)-^{3}H]GGPP triammonium salt (3.125 μCi ml^{-1}). After the labelling period, each stage (ring, young trophozoite and schizont forms) was purified on a Percoll® gradient as described above and freeze-dried prior to lipid extraction.

2.4. Lipid extraction

Each purified and freeze-dried stage was extracted with hexane (4×0.3 ml). The pooled extracts were dried under a nitrogen stream and resuspended in 500 μl of hexane. Aliquots of each extract were monitored for radioactivity.

2.5. Thin-layer chromatography

Hexane extracts of similar amounts of parasites or uninfected erythrocytes were analyzed by HPTLC. Plates were developed with hexane:diethylether:acetic acid (80:20:1, by volume) at 4°C [23]. Authentic standards of coenzyme Q_{7-10}, farnesol, geraniol, and dolichol 11 were run on the same plate.

Plates were sprayed with EN^{3}HANCE (DuPont NEN) and exposed to autoradiography for several days at −70°C. Standards were visualized with iodine vapor.

2.6. HPLC

Hexane extracts were analyzed on a reverse-phase column (Ultrasphere ODS, 4.6 mm×25 cm; Beckman, Fullerton, CA, USA) using a Shimadzu HPLC apparatus, equipped with an SPD-M10 AV Diode Array detector (Shimadzu Corp., Tokyo, Japan). Whole hexane extracts from each stage were dried under a nitrogen stream, resuspended in ethanol and co-injected with a mixture of known amounts of authentic standards of Q_{7-10}. Hexane:methanol (75:25, v/v) was used as a solvent system at a flow of 1 ml min^{-1}. Standards were detected at 275 nm [18]. One-minute fractions were collected and aliquots were monitored for radioactivity.

2.7. Nerolidol inhibition test

Nerolidol diluted in methanol was used at concentrations of 1–500 nM in different experiments. A control with methanol was performed in parallel.

The method of Desjardins et al. [24] was used to determine the IC_{50} and IC_{90} values of nerolidol. Briefly, ring-stage parasite cultures (5% hematocrit, 2% parasitemia) were exposed to increasing drug concentrations (5, 10, 25, 50, 100, 250 and 500 nM). After 24 h in culture, [G-^{3}H]hypoxanthine was added (5 μCi ml^{-1}), and after an additional 24-h incubation period, cells were harvested. All tests were done in triplicate. Suspensions of similarly treated uninfected erythrocytes were used for background subtraction. Parasitemia and parasite morphology were determined using Giemsa-stained smears immediately before the beginning of the assay and at the end of it. The IC_{50} value of growth inhibition was calculated by probit analysis (Minitab Statistical Software 13.30™, Minitab Inc.) [16].

Inhibition tests with 50 nM of nerolidol were carried out on flat-bottom microtiter plates (Falcon). Freshly synchronized cultures of 5% hematocrit and 1% parasitemia (ring-stage parasites) were exposed to several dilutions of the compound to be tested in normal culture medium. After 24, 48, and 72 h (if not otherwise stated), the percentage of each form was determined. After counting, values for each form were expressed as percent of total number of parasites (counting multinuclear schizont-infected red blood cells as one cell). The results of three independent tests were evaluated for significant discrepancies between forms per time point in treated versus untreated parasites by Student's *t*-test.

2.8. Nerolidol treatment and labelling of *P. falciparum* cultures

P. falciparum asynchronous cultures with an initial parasitemia of 7% were treated with nerolidol (50 nM) for 25 h [17]. Further labelling with [1-^{14}C]IPP ammonium salt (3.125 μCi ml^{-1}) for 15 h in the presence of this terpene was performed. Untreated cultures of *P. falciparum* were carried out in parallel. Each stage (ring, young trophozoite and old trophozoite forms) was purified as described above.

3. Results

3.1. Labelling of coenzyme Q with different intermediates of the isoprenoid pathway in *P. falciparum*

In order to investigate the biosynthetic pathway of coenzyme Q in *P. falciparum*, the following labelled intermediates were used for incorporation studies: [1-^{14}C]acetic acid, *R*-[5-^{3}H]mevalonic acid, [1-^{14}C]IPP, [1-(*n*)-^{3}H]FPP and [1-(*n*)-^{3}H]GGPP. HPTLC analysis of hexane extracts from schizont stages is shown in Fig. 1A. Under identical experimental conditions, uninfected erythrocytes did not incorporate or metabolize significantly these precursors (Fig. 1B). Spots coincident with the mixture of authentic standards of coenzyme Q_{7-10} were detected in *P. falciparum* when [1-^{14}C]acetic acid, [1-^{14}C]IPP, [1-(*n*)-^{3}H]FPP and [1-(*n*)-^{3}H]GGPP were used as precursors. These standards of coenzyme Q_{7-10} are not separated in this solvent [23]. Other radiolabelled isoprenoids of this pathway such as farnesol/geraniol and dolichol of 11 isoprenic units were detected, as previously described [6]. The fastest moving components (R_F: 0.68, Fig. 1A, lane 1 and R_F: 0.92, Fig. 1A, lanes 2–4) probably correspond to degradation products since the lability of this kind of compounds is well known [18] and were not further investigated. Labelling with *R*-[5-^{3}H]mevalonic acid was completely inefficient, even when using a large amount of this precursor (25 μCi ml^{-1}, data not shown).

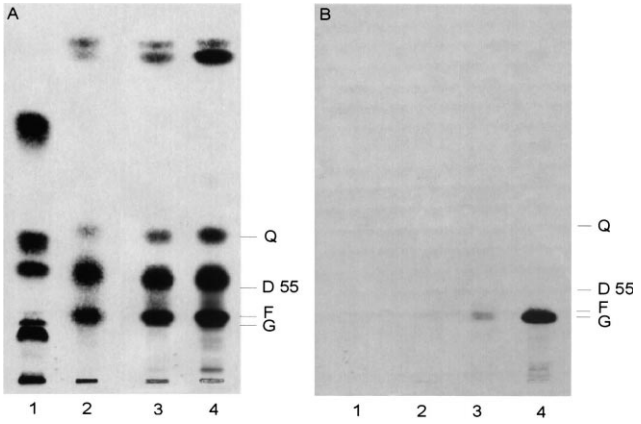

Fig. 1. A: HPTLC analysis of hexane extracts obtained from schizont stages metabolically labelled with the different intermediates of the isoprenoid pathway. B (control): HPTLC analysis of hexane extracts obtained from uninfected erythrocytes metabolically labelled with the different intermediates of the isoprenoid pathway. A,B: Lane 1, [^{14}C]acetic acid, lane 2, [^{14}C]IPP, lane 3, [^{3}H]FPP, lane 4, [^{3}H]GGPP. Solvent system: hexane:diethylether:acetic acid (80:20:1, by volume). G: Geraniol; F: farnesol; D 55: dolichol of 11 isoprenic units; Q: mixture of standards of coenzyme Q_{7-10}, respectively.

3.2. Identification of coenzyme Q by HPLC in all intraerythrocytic stages of P. falciparum

Parasites were labelled with [1-^{14}C]IPP, [1-(*n*)-^{3}H]FPP and [1-(*n*)-^{3}H]GGPP. Each stage was purified on a Percoll® gradient, and the hexane extracts were analyzed by HPLC in order to determine the length of the isoprenic chain present in coenzyme Q. Fig. 2 shows the radioactive profiles obtained by labelling with [1-^{14}C]IPP. One peak eluting at 7 min coincident with an authentic sample of Q_9 was observed, mainly in the schizont stage.

When parasites were labelled with [1-(*n*)-^{3}H]FPP, one major peak coincident with Q_8 was obtained (Fig. 3). Similar results were obtained for all stages of *P. falciparum*, but were more evident for the schizont stage (Fig. 3c). When [1-(*n*)-^{3}H]GGPP was incorporated, Q_9 was identified using the same procedure (Fig. 4). It should be noted that the most efficient labelling of coenzyme Q was obtained with this precursor.

Standards of farnesol and geraniol showed retention times of 2–3 min, whereas dolichol 11 was eluted at 13 min.

3.3. Nerolidol inhibits P. falciparum growth in vitro by decreasing the progression of ring to trophozoite stage

To determine the inhibitory effect of nerolidol on the growth of *P. falciparum* parasites, defined numbers of parasites were cultivated in the absence or presence of increasing concentrations of nerolidol. Three independent determinations of the IC_{50} for nerolidol demonstrated the precision of the system. The results concerning parasitemia and [G-^{3}H]hypoxanthine uptake by parasitized erythrocytes on microtiter plates were similar. The IC_{50} for ner-

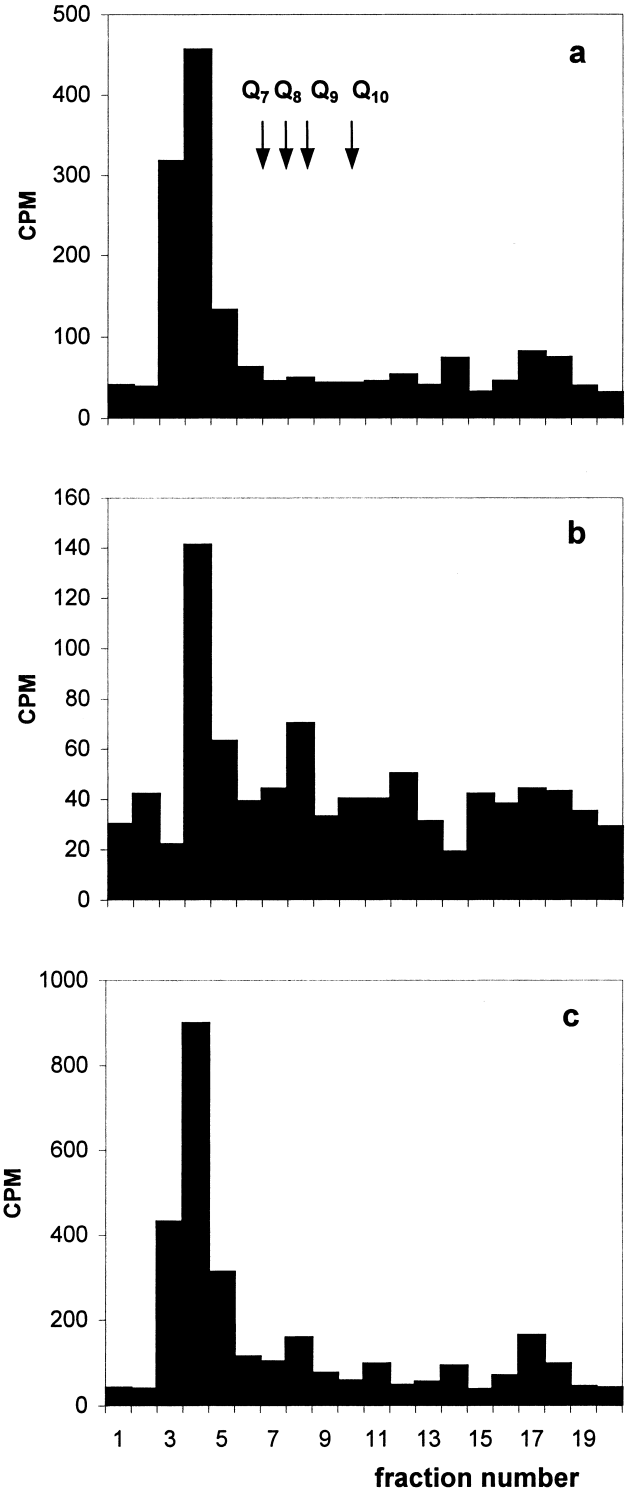

Fig. 2. Hexane extracts from parasites metabolically labelled with [^{14}C]IPP were analyzed by reverse-phase HPLC using hexane:methanol (75:25, v/v) as a solvent system. A mixture of Q_{7-10} standards was co-injected in each case. Fractions of 1 ml (1 min) were collected and monitored for radioactivity. a: Ring stage; b: trophozoite stage; c: schizont stage fractions.

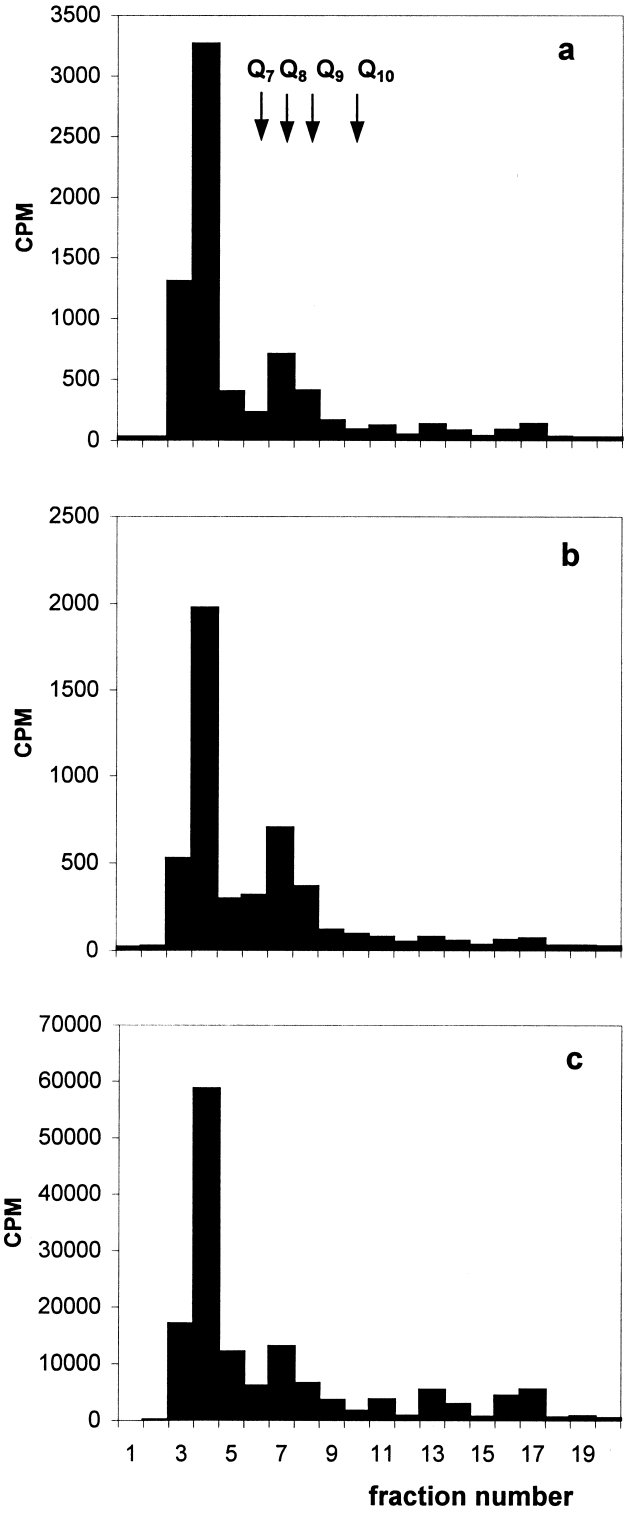

Fig. 3. Hexane extracts from parasites metabolically labelled with [^{3}H]FPP, were analyzed by reverse-phase HPLC using hexane:methanol (75:25, v/v) as a solvent system. A mixture of Q_{7-10} standards was co-injected in each case. Fractions of 1 ml (1 min) were collected and monitored for radioactivity. a: Ring stage; b: trophozoite stage; c: schizont stage fractions.

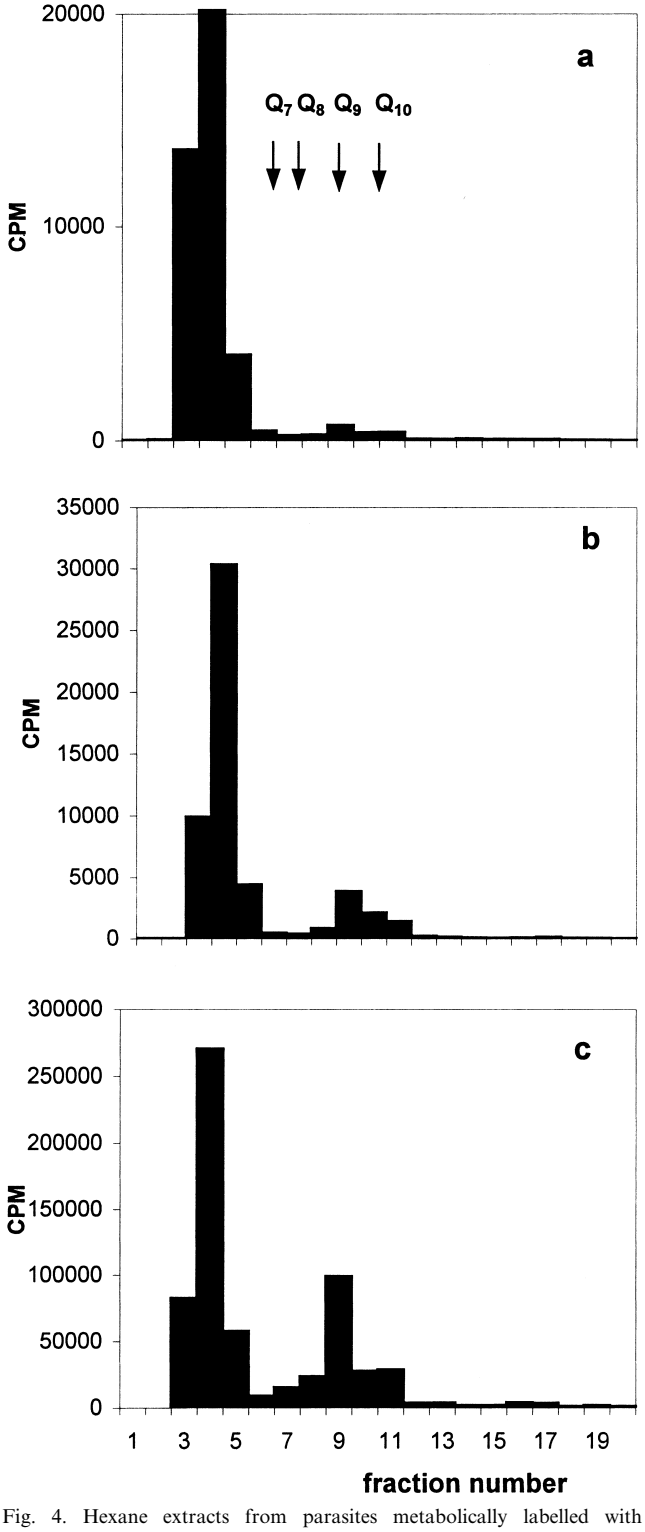

Fig. 4. Hexane extracts from parasites metabolically labelled with [^{3}H]GGPP were analyzed by reverse-phase HPLC using hexane:methanol (75:25, v/v) as a solvent system. A mixture of Q_{7-10} standards was co-injected in each case. Fractions of 1 ml (1 min) were collected and monitored for radioactivity. a: Ring stage; b: trophozoite stage; c: schizont stage fractions.

olidol was 120 nM with a 95% confidence interval of 107–142 nM. Similarly, the IC_{90} was determined to be 230 nM (95% confidence interval, 199–280 nM). At the IC_{90} concentration, parasites died after 3 h of treatment.

To determine the concentration of nerolidol that interfered with the incorporation of isoprenoid precursors in the isoprenic chain, 50 nM was chosen because under these conditions only a small percentage of parasites died and the overall protein biosynthesis was not affected.

3.4. Inhibition of coenzyme Q biosynthesis by treatment with nerolidol

Metabolic incorporation of [1-^{14}C]IPP was determined in parasites treated or not with 50 nM nerolidol. The different stages containing the same amount of treated and untreated parasites were purified, freeze-dried and extracted as described. Further analysis of these extracts was performed by HPTLC.

Nerolidol-treated parasites showed inhibition of the biosynthesis of coenzyme Q and other intermediates of the isoprenoid pathway (dolichol) in ring, young trophozoite and schizont stages (Fig. 5). However, other isoprenoid compounds, such as geraniol and farnesol, showed no differences when treated and untreated parasites were compared.

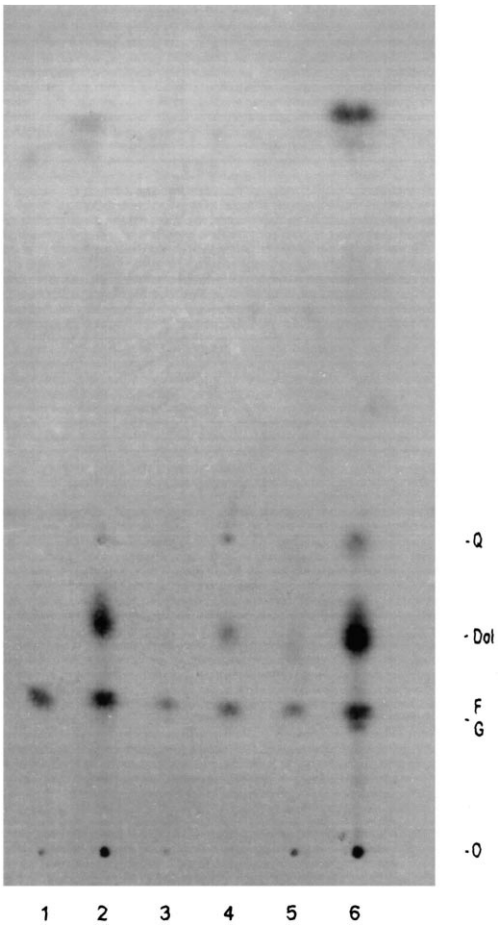

Fig. 5. HPTLC analysis of hexane extracts from parasites treated or not with 50 nM nerolidol and labelled with [^{14}C]IPP. Lane 1, treated ring stage; lane 2, untreated ring stage; lane 3, treated trophozoite stage; lane 4, untreated trophozoite stage; lane 5, treated schizont stage; lane 6, untreated schizont stage. O: Origin, G: geraniol; F: farnesol; Dol: dolichol of 11 isoprenic units; Q: mixture of standards of coenzyme Q_{7-10}.

4. Discussion

The results presented here demonstrate the presence of an active isoprenoid pathway for biosynthesis of isoprenic chains of coenzyme Q in *P. falciparum* intraerythrocytic stages. We have identified coenzyme Q_8 and coenzyme Q_9 by metabolic labelling of parasites with [1-^{14}C]acetic acid, [1-^{14}C]IPP, [^{3}H]FPP, and [^{3}H]GGPP in all intraerythrocytic stages. As far as we know, this is the first report on the incorporation of [1-^{14}C]IPP into *P. falciparum*. It is well known that the considerable increase in total lipid content associated with *P. falciparum* invasion is due to the existence of an intense lipid transport system in infected erythrocytes [25]. The efficient uptake of [^{3}H]FPP and [^{3}H]GGPP and, to a lesser extent, [1-^{14}C]IPP, may be ascribed to this transport mechanism for lipid-like components. The only inefficient precursor was mevalonic acid, a classical precursor used to study the mevalonate pathway, but this pathway is probably absent in *P. falciparum* [3,4].

The parasite is capable of synthesizing two different homologs of coenzyme Q, depending on the given radioactive intermediate. When labelling is performed with [^{3}H]FPP, coenzyme Q with an isoprenic chain of 40 carbons (Q_8) is detected; when it is performed with [^{3}H]GGPP, Q_9 (45 carbons) is found. These findings can be explained by the fact that both FPP and GGPP are substrates of the prenyltransferase involved in the biosynthetic pathway of the isoprenic chain of ubiquinone as shown in other systems [26]. This difference in the length of the isoprenic chain according to the given precursor was also observed in *P. falciparum* dolichols [6].

In order to check whether this difference could be induced by the length of the given isoprenoid intermediate, the basic isoprenic unit [1-^{14}C]IPP was used as a metabolic marker. It would be expected that by labelling with [1-^{14}C]IPP, both coenzymes Q would be detected. Surprisingly, HPLC analysis showed a single radioactive peak, which co-eluted with a coenzyme Q_9 standard. This result obtained with the incorporation of IPP is difficult to explain. It may be possible that *trans*-prenyltransferase utilizes preferentially GGPP over FPP as a substrate, as reported for rat liver microsomes [27]. In fact, from the results shown in Fig. 2 we cannot rule out the possibility that a smaller amount of Q_8 is present in trophozoite and schizont stages. The polyprenyl diphosphate synthase essentially defines the length of the side chain of ubiquinone in different species [28].

Schnell et al. [29] found the presence of coenzyme Q_8

and coenzyme Q_9 in *P. falciparum* by using [^{14}C]PHBA to label the benzoquinone ring in infected blood from the monkey species *Aotus trivirgatus*. We suggest that, as coenzyme Q acts as an antioxidant, the host's organism could be forcing the parasite to produce different homologs in order to resist different kinds of oxidative damage imposed by the immune system [30]. As the culture lacks this kind of phenomenon, one can speculate that the parasite retains the capacity of biosynthesizing only one species of coenzyme Q.

The branching point in the biosynthesis of sterols and the ubiquinone side chain occurs at the level of FPP. FPP undergoes polymerization that eventually forms the completed polyprenyl pyrophosphate side chain [13]. Some terpenes such as nerolidol, with a chemical structure similar to that of FPP, could be inhibiting the biosynthesis of the polyprenyl pyrophosphate side chain attached to ubiquinone. We then assessed the effects of nerolidol treatment on parasite cultures, at concentration 2.2 times below the IC_{50}. This concentration allowed us to analyze the influence of nerolidol treatment on the isoprenic chain of coenzyme Q, since there was no inhibition of the overall protein biosynthesis. Treatment of parasites with 50 nM nerolidol arrests the development of intraerythrocytic stages of *P. falciparum*. Moreover, the biosynthesis of both dolichol and the isoprenic chain of coenzyme Q are inhibited. But, the initial products of the isoprenoid pathway (geraniol and farnesol) are detected in untreated and nerolidol-treated parasites labelled with [1-^{14}C]IPP (Fig. 5). One explanation for this observation could be that nerolidol interferes with the mechanism of elongation involved in the biosynthesis of dolichol and the isoprenic chain attached to the benzoquinone ring of coenzyme Q.

On the other hand, in *P. falciparum* cultures treated with 0.5 mM of limonene (a prenyl transferase inhibitor) and labelled with the same precursor, it was observed that the biosynthesis of geraniol, farnesol, dolichol and the isoprenic chain attached to the benzoquinone ring of coenzyme Q was not inhibited. These products were detected by HPTLC (data not shown). In our laboratory, we have characterized the protein prenylation in *P. falciparum* and have demonstrated that limonene arrests the development of the parasites by inhibiting this process [16].

The mechanisms of action of nerolidol and limonene on the parasite are different, probably due to their different chemical structures: nerolidol is an alcohol with an unsaturated linear structure; limonene lacks the HO-group and presents a six-member ring backbone.

Our results provide evidence that *P. falciparum* has an active isoprenoid pathway for the biosynthesis of the isoprenic chain of coenzyme Q, and that *P. falciparum* is able to synthesize different homologs of this molecule depending on the given intermediate. Parasites treated with nerolidol showed a decreased ability to synthesize coenzyme Q in all intraerythrocytic stages, indicating that the drug could be considered to have potential antimalarial activity.

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