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Research brief

Leishmania amazonensis: Biosynthesis of polyprenols of 9 isoprene units by amastigotes

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Abstract

The isoprenoid metabolic pathway in protozoa of the *Leishmania* genus exhibits distinctive characteristics. These parasites, as well as other members of the Trypanosomatidae family, synthesize ergosterol, instead of cholesterol, as the main membrane sterol lipid. *Leishmania* has been shown to utilize leucine, instead of acetate as the main precursor for sterol biosynthesis. While mammalian dolichols are molecules containing 15–23 isoprene units, *Leishmania amazonensis* promastigotes synthesize dolichol of 11 and 12 units. In this paper, we show that the intracellular stages of *L. amazonensis*, amastigotes, synthesize mainly polyprenols of 9 isoprene units, instead of dolichol.

Index Descriptors and Abbreviations: *Leishmania*; Amastigotes; Polyisoprenoids; Dolichol; Mevalonate; HPTLC, high performance thin layer chromatography; RP-HPLC, reversed-phase high performance liquid chromatography; FBS, foetal bovine serum; Pren-*n*, polyprenol of *n* isoprene units; Dol-*n*, dolichols of *n* isoprene units; CoQ, coenzyme Q; MVA, mevalonic acid; PBS, phosphate-buffered saline

Leishmania parasites alternate between an invertebrate phlebotomine vector, where they multiply as flagellated, extracellular promastigotes, and a mammalian host, where the non-flagellated amastigotes live and reproduce inside parasitophorous vacuoles of macrophages.

Sterol lipid biosynthesis pathways in these parasites raised interest since the demonstration that the main sterol constituent of their plasma membrane was ergosterol and not cholesterol (Goad et al., 1984), a characteristic shared by other trypanosomatids (Halevy and Sarel, 1965) and fungi (reviewed in Borgers, 1980). Sterol biosynthesis inhibitors active against pathogenic fungi were then proposed as potential antileishmanial agents and are still under testing in the chemotherapy of leishmaniasis (Roberts et al., 2003).

Most animals and fungi synthesize sterol and prenol lipids through the classical mevalonic acid pathway (Swiezewska and Danikiewicz, 2005). This was also shown to be the case in *Leishmania*, by the demonstration that mevalonate could be incorporated into ergosterol (Goad et al., 1985) and ubiquinone (Ranganathan and Mukkada, 1995). Interestingly, instead of synthesizing sterols from acetate, as most eukaryotes, promastigotes and axenic amastigotes of *Leishmania mexicana* were shown to utilize mainly leucine as the isoprenoid precursor (Ginger et al., 1999). The presence of other end products or derivatives of isoprenoid biosynthesis as dolichol phosphate-linked saccharides (Previato et al., 1986) and prenylated proteins (Yokoyama et al., 1998) has also been demonstrated in *Leishmania* promastigotes.

We have recently shown that *Leishmania amazonensis* promastigotes synthesize predominantly dolichols containing 11–12 isoprene units (Arruda et al., 2005), therefore, distinct from animal dolichols, composed of 15–23 isoprene residues (Chojnacki and Dallner, 1988).

Most of the work on sterol and prenol synthesis in *Leishmania* was carried out with promastigotes. Since amastigotes live in an environment radically diverse from

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promastigotes, we decided to investigate whether differences in the biosynthesis of isoprenoids could be identified in this form of the cycle.

Amastigotes of *Leishmania amazonensis* (MHOM/BR/1973/M2269) were purified from lesions induced in BALB/c mice, about 8–12 weeks after intradermal inoculation of 1×10^6 parasites in the hind footpads. Lesions were removed and homogenized in PBS; the suspension was cleared of cell debris by centrifugation at 50 g for 8 min; the supernatant was then washed three times in PBS and passed through a 25-gauge needle. Amastigotes recovered from tissue were resuspended in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine and 50 mg/ml gentamicin and kept at 33 °C in a 5% CO_2 atmosphere. This treatment resulted in cultures devoid of intact macrophages. The amastigotes remained viable for at least 48 h, as judged by propidium iodide staining and MTT testing and did not differentiate into promastigotes, as evaluated by morphology. *L. amazonensis* promastigotes were grown in M199 supplemented with 10% FBS.

Biosynthesis of isoprenoids was studied in amastigotes (8.0×10^8) or promastigotes (2.5×10^8) labeled for 20–22 h with 2 μCi/ml of the specific precursor [2-^{14}C]-mevalonic acid (MVA, 72 mCi/mmol, Amersham International, Buckinghamshire, UK). Harvested parasites were washed with PBS and freeze-dried pellets were extracted with hexane as described (Arruda et al., 2005).

Hexane extracts were analyzed by HPTLC in silica gel 60 plates (Merck, NJ, USA) developed as described by Palmer et al. (1986) with hexane:diethylether:acetic acid (80:20:1, v/v/v) at room temperature. Authentic standards of CoQ_{10}, farnesol, geraniol, Pren-9, Pren-12, Dol-11, Dol-12 and ergosterol were run on the same plate and visualized with iodine vapor. Plates were exposed to phosphor screen (Molecular Dynamics) for 10 days and scanned using a STORM 820 (Amersham). Experiments were repeated at least 2 times.

Analyses by RP-HPLC were carried out on an Ultrasphere ODS C18 Beckman column (250 mm × 4.6 mm, 5 μm particle size) as previously described (Arruda et al., 2005). Briefly, samples were resuspended in methanol and a gradient elution system was used, with methanol/water (9:1, v/v) as solvent A and hexane/propan-2-ol/methanol (1:1:2, v/v/v) as solvent B. A linear gradient from 5% to 100% B over a period of 25 min was run; 100% B was then pumped through for additional 5 min. Standards of polyisoprenoids of 6–12 isoprene units, Dol-11, Dol-12, geraniol, farnesol and ergosterol were co-injected. In these conditions, dolichols and polyprenols with the same number of isoprene units are not separated. Fractions of 1 min were collected, and aliquots were subjected to liquid scintillation counting. All experiments were repeated at least twice.

To analyze polyisoprenoids from *L. amazonensis* amastigotes by mass spectrometry, freeze-dried pellets of 5×10^{10} unlabeled parasites were extracted three times with 1 ml of hexane. The pooled extracts were dried under a nitrogen stream and stored at −70 °C. The sample was resuspended in 20 ml of methanol and purified on a Sep-Pak C18 semi-preparative column (Waters, Milford, MA, USA). The column was washed with 15 ml methanol/water (9:1, v/v) and the fraction containing the prenols was eluted with 20 ml hexane/propan-2-ol/methanol (1:1:2, v/v/v). The eluate was dried under a nitrogen stream and analyzed by RP-HPLC as described above, except that the standards were analyzed in a separate run. Fractions with retention times equivalent to the standards of Pren-9, Dol-11 and Dol-12 units were collected and analyzed by mass spectrometry.

ESI(Li^+)-MS and ESI(Li^+)-MS/MS were performed with a Finnigan LCD-Duo ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) as described previously (D'Alexandri et al., 2006a). The samples were resuspended in 50 μl chloroform/methanol (1:1, v/v) containing 2 mM of lithium iodide. All ESI(Li^+)-MS spectra were acquired in the positive-ion-mode and in both full ion-mode and selective ion-monitoring mode (SIM).

ESI(Li^+)-QTOF-MS data with high resolution and high mass accuracy were collected in the positive-ion-mode using an electrospray ionization hybrid quadrupole orthogonal time-of-flight (TOF) mass spectrometer (Q-TOF, Waters, Micromass Ltd., Manchester, UK). An aliquot of each sample in chloroform/methanol (1:1, v/v) containing 2 mM of lithium was analyzed by direct infusion into the ESI source using a Harvard syringe pump model 11 (Harvard Inc., Hollinston, MA) operating at a 5 μl/min flow rate.

In order to investigate the overall pattern of isoprenoid biosynthesis in amastigotes, neutral lipid fractions from parasites labeled in vitro with the specific precursor [2-^{14}C]-MVA were analyzed by HPTLC. Labeled bands with R_f of authentic standards of geraniol and farnesol and a faint band corresponding to the ubiquinone standard were observed (Fig. 1A). The strongest band detected corresponded to the ergosterol standard, confirming that the mammalian stage, besides taking cholesterol from the host cell, is also able to synthesize ergosterol.

The R_f observed for the putative dolichol band was equivalent to the R_f of a Pren-9 standard (Fig. 1A) and did not match the R_f observed for the Dol11-12 standard, suggesting the existence of a stage-specific pattern in the biosynthesis of polyisoprenoids.

Since the separation of polyprenols of 9–12 units by this technique did not allow unequivocal identification, neutral lipids purified from [2-^{14}C]-MVA-labeled amastigotes and promastigotes were then analyzed by RP-HPLC. In extracts from amastigotes, incorporated radioactivity was detected in fractions with retention times identical to a Pren-9 standard (24 min) (Fig. 1B) but not in the fractions with retention times identical to Pren-11/12 and Dol-11/12 standards (26–27 min), confirming what was observed by HPTLC. On the other hand, the peak corresponding to Pren-11/12 and Dol-11/12 was detected in extracts from promastigotes. In amastigotes, the synthesis of labeled