Antileishmanial Activity of Lapachol Analogues

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The antileishmanial activity of lapachol, isolapachol, and dihydrolapachol, along with soluble derivatives (potassium salt) and acetate was obtained. All the compounds were assayed against metacyclic promastigotes of two different species of Leishmania *associated to tegumentar leishmaniasis:* L. amazonensis *and* L. braziliensis. *All compounds presented significant activity, being isolapachol acetate the most active against promastigotes, with* IC_{50}*/24h = 1.6 ± 0.0 µg/ml and 3.4 ± 0.5 µg/ml for, respectively,* L. amazonensis *and* L. braziliensis. *This compound was also assayed in vivo against* L. amazonensis *and showed to be active. Its toxicity in vitro was also established, and at concentration similar to the* IC_{50}*, no toxicity was evidenced. In all experiments, pentamidine isethionate was used as a reference drug. The present results reinforce the potential use of substituted hydroxyquinones and derivatives as promising antileishmanial drugs and suggest a continuing study within this class of compounds.*

Key words: *Leishmania amazonensis* - *Leishmania braziliensis* - lapachol - isolapachol - antileishmanial activity - naphthoquinones

Leishmaniasis is one of the major infectious diseases affecting the poorest regions of the world. Human infections with *Leishmania* protozoan parasites, transmitted by the bite of a sand fly, cause visceral, cutaneous or mucocutaneous leishmaniasis (Davies et al. 2003). *Leishmania*/HIV co-infections, as well as classic forms of leishmaniasis still impose difficulties in terms of diagnosis and treatment. The global burden of leishmaniasis has remained stable for some years, causing 2.4 million disability adjusted life years lost and 59000 deaths in 2001 (WHO 2002). Drug treatment today, besides the pentavalent antimonials, is restricted to a limited number of drugs like pentamidine isethionate, amphotericin B, and miltefosine (Davies et al. 2003, Kayser et al. 2003). All those drug therapies cause serious side effects. Furthermore, general treatment is unaffordable for many afflicted countries revealing an urgent need for new, safer, and cheaper drugs (Kayser et al. 2003).

One strategy to discover new therapeutic leads is to investigate classes of compounds potentially bioactive or old active compounds for alternative uses. Quinones, in general, and, particularly hydroxyquinones, as well as their reduced derivatives, have been tested against several parasite species (Croft et al. 1992, Sauvain et al. 1993, Iwu et al. 1994, Ray et al. 1998, Kayser et al. 2000, Pinto et al. 2000, Ferreira et al. 2002, Hazra et al. 2002). Many naphthoquinones have been identified as possible lead structures against *Leishmania* and other protozoa, but the potential usefulness is limited by their cytotoxicity and low bioavailability (Kayser et al. 2000). Atovaquone, a hydroxynaphtoquinone, showed to have a concentration-dependent in vitro activity against *L. infantum* (Jernigan et al. 1996), while the in vivo activity in a murine model remained low (Murray et al. 1996, Cauchetier et al. 2000). Lapachol was effective in vitro against intracellular amastigotes of *L. braziliensis*, whereas in a hamster model, it was unable to prevent lesions induced by the infection (Teixeira et al. 2001). It was suggested that lapachol could be transformed in vivo into an inactive metabolite (Teixeira et al. 2001). Apart from these studies, lapachol and other quinones are known to possess antitumor, antibiotic, antimalarial, anti-inflammatory, and anti-ulcer activities (Subramanian et al. 1998, reviewed in De Moura et al. 2001). Recent results showed that lapachol, isolapachol and its acetylderivative possess significant activity against *Biomphalaria glabrata*, the intermediate host of *Schistosoma mansoni* (Goulart et al. 1997, dos Santos et al. 2000, 2001, Lima et al. 2002). Their mechanism of biological action is still uncertain, despite some reported studies. Lapachol was shown to be a vitamin K antagonist, and, thus, might target vitamin K-dependent reactions (Dinnen & Ebisuzaki 1997) and also to be bioactivated by P_{450} reductase to reactive species, which promote DNA scission through redox cycling with generation of free radicals (Kumagai et al. 1997). The involvement of reactive oxygen species in the mechanism of action of lapachol and isolapachol was suggested by electrochemical studies in the presence of oxygen (Goulart et al. 2003, 2004). Di-hydrolapachol was active against *Plasmodium lophurae* in ducks (Hooker & Richardson 1948).

The aim of the present study was to conduct an evaluation of the activity of lapachol and analogues 1-6 (Fig. 1) against two species of *Leishmania* from the New World.

MATERIALS AND METHODS

Compounds - Lapachol (1) is from natural origin, extracted from the heartwood of several plants of the

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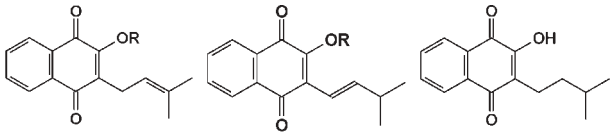

1 R = H **2** R = H **6**

3 R = K^+ **4** R = K^+

5 R = Ac

Fig. 1: compounds submitted to the leishmanicidal assays.

bignoniaceae family, mainly from *Tecoma* and *Tabebuia* species (Thomson 1971) and kindly supplied by Produtos Vegetais do Piauí, Parnaíba, Piauí, Brazil. Isolapachol [2-hydroxy-3-(3-methyl-1-butenyl)-1,4-naphthoquinone] (2) was easily synthesized, following established procedures, by reaction of 2-hydroxy-1,4-naphthoquinone (Sigma-Aldrich, Steinheim, Germany) and isovaleraldehyde (Sigma-Aldrich), in acidic medium (Hooker 1936b). Lapachol and isolapachol salts (3 and 4, respectively) were prepared by the use of an equivalent amount of KOH in ethanol, distillation of the solvent and several washings with cold ether to eliminate residues of the initial compounds. Acetylisolapachol (2-acetoxy-3-(3-methyl-1-butenyl)-1,4-naphthoquinone) (5) was prepared from 2 g of isolapachol (2) (8.2 mmol) by reaction with anhydrous sodium acetate (2 g, 3.95 mmol) and acetic anhydride (13 ml, 10,58 mmol), with stirring, under reflux during 30 min. Work up consisted in water addition and filtration of the precipitate (Hooker 1896). The dihydrolapachol (2-hydroxy-3-(3-methylbutyl)-1,4-naphthoquinone) (6) preparation involved catalytic hydrogenation of lapachol (1) (0.295 g, 1.22 mmol), using PtO_2 on charcoal (10%) in ethanol, separation of the catalyst by filtration and distillation of the solvent (Hooker 1936a). All the compounds presented analytical and spectral (IR, NMR) data in full agreement with the structures shown in Fig. 1.

Culture media, parasites, assays - *L. amazonensis* (MHOM/BR/77/LTB0016 strain) promastigotes were grown at 26°C in Schneider's *Drosophila* medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), pH 7.2. *L. braziliensis* (MCAN/BR/98/R619) promastigotes were grown in the same medium, same temperature and pH, but supplemented with 20% FCS and 2% human urine (Howard et al. 1991, Shamsuzzaman et al. 1999). Parasites were harvested from the medium on day 4, when a high percentage of infective forms (metacyclic promastigotes) was found. After being harvested, the parasites were counted in Neubauer's chamber and adjusted to a concentration of $4x10^6$ promastigotes/ml using the supernatant of each culture as diluent.

Drugs were dissolved in DMSO (the highest concentration was 1.4%, which was not hazardous to the parasites) (Canto-Cavalheiro et al. 1997) and added to parasites suspensions in a final concentration between 0.156 µg/ml and 320 µg/ml. After 24 h of incubation, the parasites were counted and compared to the controls, containing DMSO and parasites alone. Pentamidine isethionate was used as a reference drug. The drug concentration corresponding to 50% of parasite growth inhibition was expressed as the IC_{50}.

Toxicity of compound 5 to mammalian cells - Peritoneal macrophages were collected from outbread mice, counted in a Neubauer chamber, adjusted to a concentration of 1-2 $x10^6$ cells/ml and plated in the Lad-Tek chamber (no. 154534 Nalge Nunc International, Naperville, IL 60563, US). Macrophages were incubated for 2-3 h at 37°C under CO_2 atmosphere with further washing with preheated RPMI, to remove the non-adherent cells. After washing, RPMI containing 100 U penicillin, 50 mg/ml streptomycin and 10% FCS, was added and the cells were incubated for 24 h at 37°C. Compound 5 was added to the cultures in two concentrations, 1.6 µg/ml and 3.2 µg/ml, which correspond to 1X and 2X the IC_{50}/24h, obtained from the assay with *L. amazonensis* promastigotes. The incubation was performed for 24 and 48 h in the same conditions described above. The slides were washed with PBS, fixed with methanol, dried, stained with Trypan blue and/or Giemsa and analyzed using an optical microscope (Pinho et al. 1981).

In vivo activity of compound 5 - Swiss mice were divided in four groups: a) non-infected mice (NInf); infected mice (Inf) injected subcutaneously (s.c.) in the footpad with $1x10^7$ of infective promastigotes in 50 µl culture medium; infected and treated mice (T1) injected s.c. with 5 at the dose of $1XIC_{50}$ (1.6 µg/ml/animal) at 1 day post-infection (dpi) and 7 dpi; infected and treated mice (T2) injected s.c. with 5 at the dose of $2XIC_{50}$ (3.2 µg/ml/animal) at 1 dpi and 8 dpi (culture medium, saline, whatever was used to resuspend the parasites). All groups were analyzed during seven weeks according to the footpad size.

RESULTS AND DISCUSSION

In this report, we evaluated the effect of 1 to 6 against two different species of *Leishmania* from the New World: *L. amazonensis* which has been associated with all clinical forms of leishmaniasis (Barral et al. 1991, Leon et al. 1992), and *L. braziliensis* that usually presents high invasive capacity resulting in deforming mucocutaneous manifestations, and is endemic in the state of Rio de Janeiro, Brazil.

In general, the compounds showed activity against promastigote forms in the range of IC_{50}/24h 1.6 to 7.8 µg/ml for *L. amazonensis* and 3.4 to 54.0 µg/ml for *L. braziliensis* (Table). The differences in their activities against both parasites could be associated to the culture conditions, since *L. braziliensis* promastigotes were cultivated with a high amount of fetal calf serum. This fact would result in a lower availability of the compound, since some serum protein or protein from urine could aggregate with the assayed drug, which has already been described (Da Silva et al. 2002). Besides, considering that both parasites have their own metabolic characteristics, it would be expected a difference in the activity of different compounds, which probably would be metabolized in different ways. Furthermore, it was previously observed that a particular parasite (*Trypanosoma evansi*) isolated from several sources, presented a significant difference concerning the resistance to the same drug (Gomes-Cardoso et al. 1999). Our data showed that among the lapachol analogues as-

TABLE

Antileishmanial activity (IC_{50}), in µg/ml, of the assayed hydroxyquinones and derivatives

Compounds	IC_{50} µg/ml	
	Leishmania amazonensis	*L. braziliensis*
Lapachol (1)	5.2 ± 0.7	11.9 ± 6.9
Isolapachol (2)	4.4 ± 2.9	9.3 ± 2.7
Lapachol, K^+ salt (3)	7.7 ± 4.1	21.4 ± 2.9
Isolapachol, K^+ salt (4)	7.8 ± 0.2	15.8 ± 0.0
Acetylisolapachol (5)	1.6 ± 0.0	3.4 ± 0.5
Dihydrolapachol (6)	7.3 ± 0.3	54.0 ± 9.0
Pentamidine Isethionate	0.28 ± 0.05	11.6 ± 1.6

IC_{50}: values indicate the effective concentration of a compound in µg/ml necessary to achieve 50 % growth inhibition.

sayed against *L. amazonensis* and *L. braziliensis*, the most effective was the acetylisolapachol (5), being the highest activity observed against *L. amazonensis* (IC_{50}/24h = 1.6 ± 0.0 µg/ml) (Table). As expected, pentamidine isethionate, the reference drug, was the most active against *L. amazonensis* (IC_{50}/24h = 0.28 ± 0.05 µg/ml). However, previous data from our laboratory showed that pentamidine isethionate is highly toxic, in vitro, against peritoneal macrophage of mice as well as in vivo (Temporal et al. 2002).

The most active derivative in vitro, compound 5, was chosen for additional assays. When macrophages were incubated with the compound, during 24 and 48 h, in a concentration equivalent to IC_{50}/24 h (1.6 µg/ml), the morphology observed was similar to those of the control group. However, when the concentration was 3.2 µg/ml, only 20% of the cells were preserved during 24 h incubation. With 48 h incubation, compound 5 was injected into outbread mice in two different concentrations, such as, the equivalent to IC_{50} from the in vitro experiment and twice this value. No difference was observed comparing treated/non-treated groups of mice in both concentrations, up to 4th week, after parasite injection. However, from the 5th week, an increase in the effect of the compound 5 in both concentrations used can be seen, with a decrease in the footpad size (Fig. 2).

Concerning the toxicity of hydroxyquinones, important for further in vivo studies, no systematic study had been undertaken, even though such information would be valuable in view of the potential and therapeutic use of such substrates (Munday et al. 1995). Concerning lapachol, its toxicology was studied extensively (Duke 2002). In an early study, it showed to be a hemolytic agent in dogs and monkeys although renal histology was reported to be normal in both species (Morrison et al. 1970). Hemolytic anemia was found to be the limiting toxicity in animal studies (Duke 2002). It has also shown strong interceptive effect in rats with 100% fetal/embryo mortality, without affecting the mothers (Guerra et al. 1999). Human toxicity was verified at dose greater than 1.5 g per day (Duke 2002). For isolapachol, no data, except those from our group (Lima et al. 2002), had been hitherto reported.

Our results are promising since this hydroxyquinone derivative 5 showed to be active both in vitro and in vivo against L. *amazonensis* and in vitro against *L. braziliensis.* Furthermore, the in vitro experiment with macrophage demonstrated that in a concentration equivalent to IC_{50}, this compound is not hazardous to the host cell, fact that suggests a continuing study within this class of compounds toward in vivo assays. The present results reinforce the potential use of substituted hydroxyquinones and derivatives as very promising antileishmanial drugs.

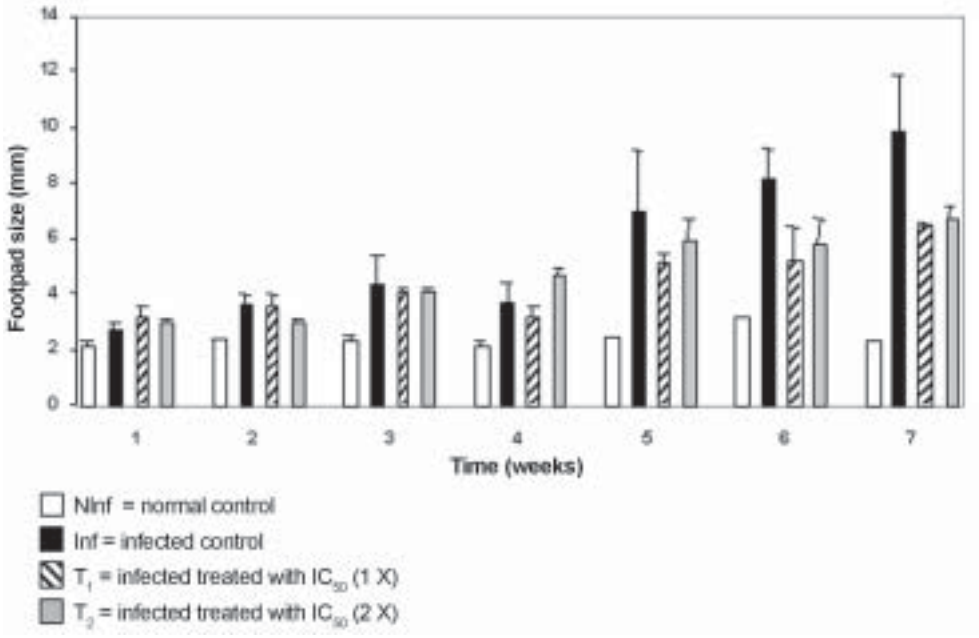

Fig. 2: in vivo activity of acetylisolapachol 5 in Ninf, Inf, T1, and T2 groups, during seven days post-infection. From the 5th week, a decrease in the footpad size in T1 and T2 can be observed.

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