Research Article

Cytotoxicity and In Vitro Antileishmanial Activity of Antimony (V), Bismuth (V), and Tin (IV) Complexes of Lapachol

Marcele Neves Rocha, 1 Paula Monalisa Nogueira, 1 Cynthia Demicheli, 2 Ludmila Gonçalvez de Oliveira, 2 Meiriane Mariano da Silva, 2 Frédéric Frézard, 3 Maria Norma Melo, 4 and Rodrigo Pedro Soares 1

1 Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz/FIOCRUZ, 30190-002 Belo Horizonte, MG, Brazil
2 Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil
3 Departamento de Fisiologia e Biofísica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil
4 Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil

Correspondence should be addressed to Rodrigo Pedro Soares; rsoares@cpqrr.fiocruz.br

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Leishmania amazonensis is the etiologic agent of the cutaneous and diffuse leishmaniasis often associated with drug resistance. Lapachol [2-hydroxy-3-(3′-methyl-2-butenyl)-1,4-naphthoquinone] displays a wide range of antimicrobial properties against many pathogens. In this study, using the classic microscopic in vitro model, we have analyzed the effects of a series of lapachol and chlorides complexes with antimony (V), bismuth (V), and tin (IV) against L. amazonensis. All seven compounds exhibited antileishmanial activity, but most of the antimony (V) and bismuth (V) complexes were toxic against human HepG2 cells and murine macrophages. The best IC50 values (0.17 ± 0.03 and 0.10 ± 0.11 µg/mL) were observed for Tin (IV) complexes (3) [(Lp)(PhSn)] and (6) (Ph3SnCl2), respectively. Their selective indexes (SIs) were 70.65 and 120.35 for HepG2 cells, respectively. However, while analyzing murine macrophages, the SI decreased. Those compounds were moderately toxic for HepG2 cells and toxic for murine macrophages, still underlying the need of chemical modification in this class of compounds.

1. Introduction

Leishmania amazonensis, a New World species, has been identified as a dermatotropic species often associated with drug resistance [1]. Current antileishmanial therapies are toxic to human and some simply fail [2, 3]. In the Americas, for over six decades, parenteral administrations of pentavalent antimonials (Sb-V), sodium stibogluconate (Pentostam), and meglumine antimoniate (Glucantime) have been used for treating leishmaniasis. In places where resistance to antimonials is common, such as India, other chemotherapeutic treatments include amphotericin B and pentamidine [2, 4]. Therefore, the absence of a low toxic and safe oral drug still underlines the need for new antileishmanial compounds.

Lapachol, [2-hydroxy-3-(3′-methyl-2-butenyl)-1,4-naphthoquinone] (Figure 1) is a natural compound extracted from the core of Bignoniaceae trees. In Leishmania, lapachol analogues, derivatives, and complexes have been tested by several groups. Lapachol, isolapachol, and some of their derivatives were active in vitro and in vivo against Leishmania braziliensis and L. amazonensis, respectively [5]. Bismuth (III), antimony (V), and tin (IV) complexes were active against Helicobacter pylori, Leishmania major, and Leishmania donovani, respectively [6–8].

The design of bifunctional metal complex, where both the ligand and the metal exert pharmacological activity, represents a promising strategy for achieving more effective and selective drugs. In the present study, lapachol was coupled
with three different metals: triphenyltin (IV), triphenylbismuth (V), and triphenylantimony (V). We have tested the \textit{in vitro} activity and cytotoxicity of synthesized antimony (V), bismuth (V), and tin (IV) lapachol and chloride complexes against intracellular \textit{L. amazonensis}, HepG2 cells, and murine macrophages.

2. Materials and Methods

2.1. Synthesis of the Lapachol Metal Complexes and Tested Metal Chlorides. The \((\text{LP})(\text{Ph}_3\text{Bi})\text{O}_9\text{.5} (1)\) and \((\text{LP})(\text{Ph}_3\text{Sb})\text{OH} (2)\) complexes were synthesized by following the procedure described by [9]. To prepare \((\text{LP})(\text{Ph}_3\text{Sn})\) the same procedure was used. Triethylamine (70 \(\mu\text{L}\)) was added to a mixture of lapachol (0.121 g, 0.5 mmol) and triphenyltin (IV) chloride (193 mg, 0.5 mmol) in chloroform (20 mL). The resulting mixture was stirred for 4 h at room temperature. Removal of the solvent under vacuum yielded a solid material. The material was subsequently dissolved in acetone and precipitated in water. The triethylammonium hydrochloride formed during the reaction was dissolved and removed by water. Elemental analyses were carried out using a Perkin-Elmer 240 Elemental Analyzer. Atomic absorption analyses of bismuth, antimony and tin contents were carried out on a Hitachi Atomic Absorption Spectrophotometer (Model 8200).

The following equations can be proposed to illustrate the formation of \((\text{LP})(\text{Ph}_3\text{Sb})\text{OH}\) complex as follows:

\[
\begin{align*}
\text{LpH} + \text{Et}_3\text{NH} & \underset{\text{THF}}{\longrightarrow} \text{Lp}^- + [\text{Et}_3\text{NH}]^+ \\
\text{Lp}^- + \text{Ph}_3\text{SbCl}_3 + [\text{Et}_3\text{NH}]^+ & \underset{\text{THF}}{\longrightarrow} \text{LpSbPh}_3\text{Cl} + [\text{Et}_3\text{NH}]\text{Cl} \\
\text{LpSbPh}_3\text{Cl} + [\text{Et}_3\text{NH}]\text{Cl} + \text{H}_2\text{O} & \rightarrow \text{LpSbPh}_3\text{OH} + [\text{Et}_3\text{NH}] + \text{HCl}
\end{align*}
\]

(1)

The same process can be proposed for all complexes. The yields, melting points, and elemental analyses of the compounds prepared are given in Table 1. Triphenylbismuth dichloride (4), triphenylantimony dichloride (5), triphenyltin chloride (6), and lapachol (7) were obtained from Aldrich. Triethylamine was obtained from Sigma. The predicted structures of all tested compounds are shown in Figure 1.

2.2. Parasites. The World Health Organization (WHO) reference strain \textit{L. amazonensis} (IFLA/BR/1967/PH8) was used and typed as previously described [10]. Promastigotes forms

![Diagram](image-url)
were grown at 25°C in M199 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Culti-
lab), 40 mmol/L HEPES (Amersham), 0.1 mmol/L adenine (Sigma), 0.0005% hemin (Sigma), 0.0002% biotin (Sigma), 50 units/mL penicillin, and 50 mg/mL streptomycin (Invitro-
gen) [11].

2.3. In Vitro Classic Microscopic Tests. Animals were kept in the Animal Facility of the Centro de Pesquisas René
Rachou/FIOCRUZ in strict accordance to the Guide for the
Care and Use of Experimental Animals [12]. The procedures
were approved by the Internal Ethics Committee in Ani-
mal Experimentation (CEUA) of Fundação Oswaldo Cruz
(FIOCRUZ), Brazil (Protocol L-042/08). Mice were eutha-
nized with CO2 in an induction chamber prior to macrophage
removal. Balb/c mice were injected intraperitoneally with
2 mL of 3% sodium thioglycollate medium. After 72 h, per-
itoneal macrophages were removed by washing with cold
RPMI 1640 medium and enriched by adherence to round
glass coverslips (13 mm) placed in a 4-well culture plate. Cells
(2 × 105 cells/well) were cultured (37°C, 5% CO2, 18 h) in
RPMI supplemented with 10% heat-inactivated FBS (fetal
bovine serum) prior to infection with parasites. Macrophages
were exposed to stationary phase promastigotes (2 × 106/well)
at a final ratio of 1:10. The plates were incubated at 37°C, 5% CO2,
for 5 h in BOD to allow internalization of parasites [13].
Then, the medium was removed for the remaining noninter-
 nalized parasites. Negative control included only infected
macrophages and medium. Incubations were tested in dupli-
cate in two independent experiments [14, 15]. The subst-
cences were serial diluted with RPMI 1640 medium sup-
plemented with 10% FBS at five different concentrations
(50 → 3.1 μg/mL). For compounds (3) and (6), the dilution
was 10 → 0.016 μg/mL. Amphotericin B was used as refer-
ce drug. Infected macrophages were exposed daily to the
compounds for 3 consecutive days. After this period, cover-
slips were collected, stained with Panoptic (Laboclin), and
subsequently mounted with Entellan (Merck) on glass slides.

2.4. Cytotoxicity Tests. The cell lineage HepG2 A16 was
derived from a human hepatocellular carcinoma cell line
HepG2 (ATCC HB-8065) and obtained from America
Type Culture Collection line (ATCC) [16]. Balb/c murine
peritoneal macrophages were obtained as described above.
Cytotoxicity was determined using the MTT method (3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
(Sigma)). HepG2 cells were kept in RPMI medium supple-
mented with 10% FBS, and confluent monolayers were trypsi-
nized, washed in RPMI, and transferred to 96-well microtiter
plates (4 × 104 cells/well) for 16–18 h. Murine macrophages
were used in the concentration 2 × 105 cells/well in 96-
well microtiter plates. The compounds were serial diluted
in different concentrations (10 → 0.16 mg/mL). In both
tests, the medium was removed, and the compounds were
incubated for 24 h (37°C, 5% CO2). Colorimetric reaction
was developed following the incubation with MTT (37°C,
5% CO2, 4 h) and addition of acidified isopropanol [17].
The reaction was read spectrophotometrically (Spectramax M5,
Molecular Devices, San Francisco, CA) with a 570 nm filter
and a background of 670 nm. Incubations were tested in
triple in two independent experiments. The minimum
dose that killed 50% of the cells (MLD50) was determined [18],
and the values were plotted to generate dose-response curves
using Microcal Origin Software (Northampton, MA, USA)
[15, 19]. The selective indexes (SIs) of compounds were
calculated using the MLD50/IC50 ratios to HepG2 and
peritoneal macrophages [20, 21].

3. Results
The in vitro classic microscopic test enables direct counting
to determine the percentage of infected cells and/or the number
of amastigotes [22]. Here, the IC50 values were calculated
based on the percentage of infected macrophages [15]. The
in vitro antileishmanial activities, cytotoxicity and selective
indexes (SIs) of lapachol metal complexes and chlorides (1–
6), lapachol (7) and amphotericin B are shown in Table 2.
Lapachol and compounds (1), (2), and (5) were considered
inactive (IC50 > 10 μg/mL) and toxic (SI < 20) for HepG2 cells
and macrophages [20, 21]. The tin (IV) lapachol complex (3)
and chloride (6) were active against intracellular amastigote
forms of L. amazonensis (Figures 2(a) and 2(b)) and less
toxic for HepG2 cells (SIs ranging from 70.65 to 120.35)
(Figures 2(d) and 2(e)) (Table 2). One triphenyl bismuth
chloride (4) (Figure 2(c)) was also active and a little more
toxic for HepG2 cells (Figure 2(f)) than (3) (Figure 2(d))
and (6) (Figure 2(e)) (SI = 34.03). All compounds were
toxic for murine macrophages (SI < 20). Amphotericin B,
an antileishmanial reference drug, exhibited an IC50 value
approximately fourfold higher than (3) and (6) (0.73 ± 0.60
μg/mL) (Table 2).

4. Discussion
Leishmaniases are considered by the WHO as one of the
major six important infectious diseases worldwide. Over
the past years, the absence of research and development
for new medicines targeting diseases affecting people in
developing countries has become a global concern [23].

| Compound | Yield (%) | M.p. (°C) | C found (calc.) (%) | H found (calc.) (%) | Metal found (calc.) (%) | Formula for calc. |
|----------|-----------|-----------|-------------------|-------------------|----------------------|------------------|
| (1)      | 79        | 126–129   | 57.31 (57.40)     | 4.09 (4.23)       | 29.03 (29.98)        | (Lp)(Ph3Bi)O3     |
| (2)      | 76        | 154–156   | 65.30 (64.82)     | 4.52 (4.78)       | 20.64 (19.19)        | (Lp)(Ph3Sb)OH     |
| (3)      | 79        | 107–109   | 66.41 (67.03)     | 4.40 (4.77)       | 21.74 (20.07)        | (Lp)(Ph3Sn)       |

*a M.p.: melting point.
Currently, the development of new drugs, combinations, or protocols against tropical and neglected diseases is of great importance in public health [24–27]. However, side effects, treatment failure due to parasite resistance, HIV coinfection, and intravenous administration are the major concerns hindering leishmaniasis chemotherapy [2, 3].

Lapachol derivatives and complexes have exhibited anti-tumor, anti-inflammatory, antiangiogenic, analgesic, and antimicrobial properties [6, 28–32]. Lapachol and some of its analogues demonstrated activity in vitro against *L. braziliensis* and *L. amazonensis* [5]. The use of metal complexes against *Leishmania* may represent a potential alternative against...

Figure 2: *In vitro* antileishmanial activity of compounds (3), (4), and (6) against intracellular *L. amazonensis* ((a), (b), and (c)) and cytotoxicity against hepatoma HepG2 cell ((d), (e), and (f)). Curves were obtained using Microcal Origin Software. IC₅₀ = half-maximal inhibitory response; MLD₅₀ = the minimum lethal dose. Figures are a representation of one experiment.
Table 2: Antileishmanial activity, cytotoxicity, and selective indexes of tested compounds for HepG2 cells and murine macrophages.

| Compound | Formula | IC_{50} | HepG2 | Macrophages |
|----------|---------|---------|--------|-------------|
|          |         |         | MLD_{50} | SI | MLD_{50} | SI |
| 1        | (Lp)(Ph,Bi)O\_3.5 | 29.05 ± 18.45 | 58.38 ± 8.47 | 2.01 | 32.4 ± 8.20 | 1.11 |
| 2        | (Lp)(Ph,Sb)OH | 18.27 ± 5.58 | 325.22 ± 89.40 | 17.81 | 130.65 ± 40.52 | 7.15 |
| 3        | (Lp)(Ph,Sn) | 0.17 ± 0.03 | 12.01 ± 0.17 | 70.65 | 1.6 ± 0.57 | 9.41 |
| 4        | Ph\_3BiCl\_3 | 5.40 ± 0.16 | 183.75 ± 4.77 | 34.03 | 25.15 ± 0.49 | 4.67 |
| 5        | Ph\_3SbCl\_3 | 11.61 ± 7.85 | 157.46 ± 37.13 | 13.56 | 30.75 ± 6.01 | 2.65 |
| 6        | Ph\_3SnCl\_3 | 0.10 ± 0.11 | 12.04 ± 8.42 | 120.35 | 0.73 ± 0.13 | 7.30 |
| 7        | Lp\(^d\) | 15.48 ± 5.23 | 201.77 ± 5.32 | 13.03 | 184.65 ± 6.58 | 11.92 |
| Amphotericin B |         | 0.73 ± 0.60 | 644.59 ± 126.57 | 883.00 | 179.95 ± 8.84 | 246.51 |

\(^a\)IC_{50}: the inhibitory concentration that killed 50% of the L. amazonensis in \(\mu g/mL\).
\(^b\)MLD_{50}: the minimum lethal dose that killed 50% of the cells in \(\mu g/mL\).
\(^c\)SI: selective index, calculated based on the MLD_{50}/IC_{50} ratios.
\(^d\)Lp: Lapachol.

5. Conclusions

Lapachol and a series of six lapachol and chloride metal complexes have been evaluated for their in vitro activity against intracellular amastigote forms of L. amazonensis. The tin (IV) lapachol and chloride complexes (3 and 6) exhibited higher antileishmanial activity compared to amphotericin B. The triphenyl bismuth (V) compound (4) also exhibited antileishmanial activity with moderate cytotoxicity. Lapachol compounds with bismuth (V) and tin (IV) were less toxic when compared with lapachol alone for HepG2 cells. In conclusion, tin, and in a less extent, bismuth complexes were moderately toxic for HepG2 cells and toxic for murine macrophages.

Conflict of Interests

The authors have declared that no conflict of interests exists.

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