Action of new organometallic complexes against *Leishmania donovani*

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The action of 16 newly synthesized metal complexes having the general structure \textit{cis}-Pt(II)-X$_n$-L$_n$ have been tested \textit{in vitro} against the promastigote forms of *Leishmania donovani*. The metal complexes at 24 h and maximum dosages inhibited growth from 0%, e.g. in \textit{cis}-Pt-nifurtimox, to 100%, e.g. in \textit{cis}-Pt-(2,3,4,5,6-pentafluoroaniline)$_2$Br$_2$ or \textit{cis}-Pt-pentamidine-I$_2$. A study of the cytotoxicity of these latter complexes on the phagocytic cell line J-774 showed neither high cytotoxicity nor cytolysis. At the maximum dosage after 24 h of permanent contact with the cells (extreme, non-physiological conditions), cytolysis did not exceed 30%. For most of the compounds, cytosis ranged from 0%, for \textit{cis}-Pt-oxamniquine-Cl$_2$ to 27.7%, for \textit{cis}-Pt-pentamidine-I$_2$. The compound \textit{cis}-Pt-(2,3,4,5,6-pentafluoroaniline)$_2$-Br$_2$ caused up to 1.4% cytolysis under the above conditions. Parasites exposed to \textit{cis}-Pt-pentamidine-I$_2$ showed notably reduced DNA, RNA and protein synthesis, unlike those exposed to other compounds. Parasites examined by electron microscopy showed effects mainly on the nucleus, though in some cases the mitochondria were affected, altering the internal membranes of the cytoplasmic organelles. The \textit{in-vivo} activity of the complex \textit{cis}-Pt-guanethidine-Cl$_2$ was evaluated in parasitized Wistar rats, in which the number of amastigotes per gram of spleen was reduced by 75% compared with controls.

**Introduction**

The World Health Organization considers leishmaniasis to be one of the most important parasitic diseases. This disease affects at least 12,000,000 people in the warm areas of the world, producing a grave illness associated with up to 90% mortality in untreated cases.

One of the principal subjects of current research in the field of leishmaniasis is the development of new agents for use in chemotherapy. The present treatment is based on the administration of pentavalent antimony salts and/or pentamidine. Recognized antifungal agents, such as amphotericin B, prove very effective in vitro against the parasite, but due to toxicity and difficulty in administration, these are used only for the treatment of cutaneous leishmaniasis resistant to antimony (Sb) salts.

Other drugs have been used with varying degrees of efficacy: antimalarials such as chloroquine and mepramine, certain antibiotics such as rifampicin, monomycin, trimethoprim and even nifurtimox, an agent known for its effectiveness against Chagas’ disease.

More recently, new agents have been sought for the treatment of visceral leishmaniasis. Bauman et al. and Fouce et al. studied the role of a polyamine analogue (α-difluoromethylornithine) against *L. donovani* and *L. infantum*. Marr and Morman et al. demonstrated the therapeutic efficacy of purine analogues against Chagas’ disease and visceral leishmaniasis. Ram et al. have synthesized and assayed the leishmanicidal activity of carbazolylpyrimidines, agents which are less likely to show toxicity than those used at present.

We have therefore assayed \textit{in vitro} the action of 16 new metal-drug complexes against *L. donovani* and determined their effect in cultures of J-774 macrophage cell lines. In addition, we evaluated the \textit{in-vivo} action of \textit{cis}-Pt-(guanethidine)-Cl$_2$ against parasitized Wistar rats.

**Materials and methods**

Culture and maintenance of the parasite

The strain of *L. donovani* used was LCR-L 133 (L. donovani Reference Centre, Jerusalem), isolated in 1967 from...
a human case of kala-azar in Begemder (Ethiopia) and maintained in our laboratory since 1982 by successive 15 day subcultures using a biphasic medium consisting of a liquid phase of minimal essential medium (MEM) with 10% inactivated fetal bovine serum incubated in air at 28°C. To maintain infectivity of the parasite, golden hamsters were inoculated at least once every 6 months and 30–45 days later the parasites were isolated from the spleen. TC-199 supplemented with 20% inactivated fetal ovine serum at 56°C for 30 min in Roux flasks (Falcon) of 75 cm² surface area were used for assays. At the exponential-growth phase the liquid medium was centrifuged, the number of flagellates counted and the culture distributed in aliquots of 1 x 10⁶ parasites/mL. The assays were carried out in 24-well microtitre plates.

Metal complexes

The metal complexes were synthesized by Dr G. Craciunescu, in the Department of Inorganic Chemistry of the Pharmacy Faculty at Complutense University in Madrid. The compounds assayed (Table I) had the general structure cis-Pt(II)-Xₙ-Lₙ where X is a halogen, the group NH₃ or dianimopimelic acid (DAP) and L is a ligand.

Treatment of parasites with metal complexes

The metal complexes were dissolved in dimethylsulphoxide (DMSO) at a concentration of 0.1% after being determined to be non-toxic and capable of permitting parasite growth, as previously demonstrated. The concentrations tested were 100, 10 and 1 µg/L, dissolved in TC-199 medium (Gibco, UK) plus 20% inactivated fetal bovine serum (IFCS). The effect of each complex, and its concentration, were determined at 24, 48 and 72 h, estimating the number of parasites using a Neubauer haemocytometer and calculating the percentage of growth inhibition using the following formula:

\[
\% GI = \frac{T_c - T_p}{T_c} \times 100
\]

where % GI is the percentage of growth inhibition for each time period and each dosage, T_c is the number of parasites per mL in the control wells and T_p is the average number of parasites per mL corresponding to the different agents tested and the respective dosages.

After determining the effectiveness of the complexes, cis-Pt-oxamniquine-Cl₂, cis-Pt-(2,3,4,5,6-pentafluoroaniline)₂-Br₂, cis-Pt-guanethidine-Cl₂ and cis-Pt-pentamidine-I₂ were selected as the most active. The free ligand molecules guanethidine, oxamnique, pentamidine and 2,3,4,5,6-pentafluoroaniline were assayed as controls.

In all cases, a positive control, glucantime (antimoniate of meglumine) and a negative control, the medium containing 0.1% DMSO, were tested.

Cytotoxicity assay

The cell strain used was J-774 (ATCC TIB 67), isolated by Dr P. Ralph (Salk Institute, San Diego, CA, USA). The cell line was cultivated on RPMI medium (Gibco) with 20% IFBS at 37°C in 5% CO₂. The cytotoxicity assays were carried out in 24-well microtitre plates using a cell suspension adjusted to 1 x 10⁶ cells/mL. After allowing the cells to adhere the medium

<table>
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<th>Complex number</th>
<th>Xₙ</th>
<th>Lₙ</th>
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<tbody>
<tr>
<td>1</td>
<td>(NH₃)₂</td>
<td>(Benzylorange)₂</td>
</tr>
<tr>
<td>2</td>
<td>(NH₃)₂</td>
<td>(2-benzylamine-5-hydroxynaphthalenesulphonic acid)</td>
</tr>
<tr>
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<td>Cl₂</td>
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</tr>
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<td>5</td>
<td>Cl₂</td>
<td>(octodryl)₂</td>
</tr>
<tr>
<td>6</td>
<td>Cl₂</td>
<td>(4-nitromidazole)₂</td>
</tr>
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<td>(20-amine-4-phenylthiazole)₂</td>
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<tr>
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<td>2-piperazinyl-(1)-ethanol</td>
</tr>
<tr>
<td>9</td>
<td>Cl₂</td>
<td>Lampit</td>
</tr>
<tr>
<td>10</td>
<td>Cl₂</td>
<td>oxamnique</td>
</tr>
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</tr>
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<td>12</td>
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</tr>
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<td>13</td>
<td>Br₂</td>
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</tr>
<tr>
<td>14</td>
<td>Br₂</td>
<td>(trancyclipromine)₂</td>
</tr>
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<td>15</td>
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<td>pentamidine</td>
</tr>
<tr>
<td>16</td>
<td>DAP</td>
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was replaced with RPMI with 10% FBS plus 50 mCi/mL of Cr$^{51}$ (specific activity 200–900 Ci/mmol) (Dupont, USA).

The assay technique for cytotoxicity was that of Fulford & Marcino-Cabral. After allowing 1 h for labelling, the medium was replaced by fresh cold medium containing the dissolved metal complexes selected as described above. The complexes were kept in contact with the cells for 6, 12 and 24 h, after which the wells were shaken and the medium transferred to Eppendorf tubes and centrifuged at 16,000 g for 5 min. The supernatant was transferred to tubes containing fresh medium. Each pellet containing the cells was treated with 1 mL of 0.1% SDS–1 M NaOH and transferred to new tubes.

Radioactivity was determined with a gamma-spectrometer (LKB Wallac 1275 M inigamma Gamma Counter), with the aim of calculating the specific release of Cr$^{51}$, using the following formulae:

\[
\% \text{ spontaneous liberation} = \frac{S_c}{S_c + P_e} \times 100
\]
\[
\% \text{ specific liberation} = \frac{S_e}{S_e + P_e} \times 100
\]

where S and P are the radioactivity in the supernatant and pellet, respectively (in cpm), and subscripts c and e indicate control and experimental samples, respectively.

Study of the effect of the metal complexes on macromolecule synthesis by the parasite

To measure the effect of the metal complexes on the biosynthesis of nucleic acids and proteins, we studied the incorporation of three radioactively labelled precursors: [6-\text{H}]thymidine (specific activity 26–30 Ci/mmol), [5-\text{H}]uridine (specific activity 25–30 Ci/mmol) and [4,5-\text{H}]leucine (specific activity 35–70 Ci/mmol).

The biosynthesis of DNA was measured using thymidine incorporation in the precipitable material, RNA biosynthesis by uridine incorporation and protein synthesis by leucine incorporation. For this, cultures with 5 × 10^6 parasites/mL were centrifuged and, after removal of the supernatant, the pellet was resuspended in 1 mL of culture medium to which 100 μg/mL and 50 μg/mL of the complexes selected were added. After incubation at 28°C for 30 min, the culture medium was removed and replaced in each case with a culture medium without the metal complex but with the addition of 5 μCi/mL of radioactive analogue. After 45, 75 and 135 min, the cultures were centrifuged and the pellet, after three washings with cold medium, was precipitated with 10% TCA for 2 h at 4°C. A 5% trichloracetic acid solution was then centrifuged at 1500 g for 10 min before dissolving the precipitate in 3 volumes of TC-199 with 20% FCS, to inactivate the remaining trypsin.

The amastigotes were purified by centrifugation in discontinuous Percoll gradient previously made isotonic, leading to amastigotes lying in the interphase between regions of density 1.07 and 1.06 g/cm$^3$. After the amastigotes had been collected, the Percoll was removed by centrifugation at 150g for 10 min before dissolving the interphase in PBS (0.1 M). The pellet containing the...
amastigotes was resuspended after determining their number and viability, the number of amastigotes obtained being referred to the weight of the spleen. The amastigotes were transferred to TC-199 medium with 10% FCS supplemented with 20 μCi/mL of [3H]leucine. The amastigotes were incubated for 1 h at 37°C in 5% CO2. Afterwards, the radioactive medium was removed by centrifugation, and the pellet containing the amastigotes was treated with a solution of 0.1 M NaOH and 0.1% (w/v) SDS. The radioactivity of the sample was determined by liquid scintillation counting in a Beta 7500 Beckman scintillation counter.

**Results and discussion**

Table II shows the percentages of growth inhibition obtained by treating the flagellate forms of the parasite with the various metal complexes at different concentrations and times. In addition, the growth-inhibition percentages were calculated for the free ligands of the four most active complexes. Most notable were the high percentages of inhibition obtained with complexes 4, 10, 11, 12 and 15. Also noteworthy, with complex 12, the inhibition percentages declined over the 72 h of culture; this was possibly due, as with the other complexes described in the literature, to an alteration of the complex on being kept in solution, or perhaps was due to the enzymatic activity of the parasite.

In addition, it is worth noting that nifurtimox was completely inactivated by the formation of the metal complex. This agent has recognized antiparasite activity, being used specifically against *Trypanosoma cruzi*. It acts through a reduction in its nitro groups and the formation of products capable of producing toxic free radicals specifically attacking parasite membranes. Nifurtimox, as a free ligand, produces a 60% growth inhibition at 24 h and after exposure to 100 μg/mL for 48 h, shows 100% inhibition against *L. donovani*. A similar phenomenon, concerning loss of biological activity of nifurtimox upon formation of a Pt(IV) complex salt, was described by Mesa-Valle et al. Oxamniquine (6-hydroxymethyl-2-isopropylaminomethyl-7-nitro-1,2,3,4-tetrahydroquinoline), the free ligand of complex number 10, is a metabolite of the well-known schistosomicide UK-3883, used currently as an antiparasite drug against *Schistosoma mansoni*. This ligand is obtained by biotransformation, by the fungus *Aspergillus sclerotiorum*, of the parent drug mentioned above. The exact mechanism of activity of this ligand is not known.

<table>
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<tr>
<th>Compound</th>
<th>100 μg/L</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
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<td>44.6</td>
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<td>48</td>
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<td>Complex 7</td>
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<td>100</td>
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<td>Oxamniquine</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>94</td>
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<tr>
<td>Pentfluoroaniline</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>Glucantime</td>
<td>14.8</td>
<td>0</td>
<td>0</td>
<td>16.2</td>
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</table>

*See Table I for formulae for complexes 1–16.*
New metallic complexes against L. donovani

As in the case of the previous complex, the growth-inhibition rates are lower for the complex than for the free ligand. However, in the case of guanethidine and 2,3,4,5,6-pentafluoroaniline, the metal complex is more effective than the free ligand, far more so in the case of the latter in which the free ligand does not inhibit growth, whereas at maximum dosage the metal complex kills all the parasites.

The cytotoxicity of the seven complexes which were selected as being the most active at maximum dosage and at the greatest incubation time, is shown in Table III. As an example, pharmacokinetic data for oxamniquine indicate that the maximum dosage in serum is attained less than 2 h after oral administration, lasts for approximately 1 h, and reaches a maximum of 18.4 g/L of drug after administration of 50 mg/kg body weight. The complex that induced the maximum cytotoxicity, 27.7%, was cis-Pt-pentamidine-I₂, while cis-Pt-oxamniquine-Cl₂ induced no cytotoxicity.

The data concerning the biosynthesis of macromolecules by parasites treated with the different metal complexes, indicate that complexes, 4, 10 and 11 do not significantly reduce the rates of incorporation of the tritiated analogues (Figures 1, 2 and 3). Complex 15, however, drastically reduced the incorporation of radiolabelled thymidine, uridine and leucine, indicating a significant blockage in the biosynthesis of macromolecules (Figure 4). This complex has pentamidine as a ligand. Bonding specifically to the nucleic acids, this molecule interferes with the incorporation of nucleotides in the RNA and DNA. Deraadt & Seed found that pentamidine interfered with DNA synthesis, inhibiting the incorporation of thymidine. In addition, pentamidine can inhibit thymidylate synthetase and oxidative phosphorylation, thereby inhibiting the biosynthesis of DNA, RNA, proteins and phospholipids.

Perhaps in this last situation, the complex cis-Pt-penta- midine-I₂ can induce greater cytotoxicity of J-774 cells by interfering with the biosynthesis of phospholipids of the cell membranes. The growth inhibition caused by free ligand is practically the same as that induced by the complex.

This last complex (Figure 5) shows ultrastructural alterations in the cytoplasm, with vacuolizations possibly result-
Figure 2. Effect of cis-Pt(II)-oxamniquine-Cl₂ on the incorporation by *L. donovani* of (a) [³H]leucine, (b) [³H]thymidine and (c) [³H]uridine. ●, Control; ■, drug concentration 100 μg/L; ▲, drug concentration 50 μg/L.

Figure 3. Effect of cis-Pt(II)-guanethidine-Cl₂ on the incorporation by *L. donovani* of (a) [³H]leucine, (b) [³H]thymidine and (c) [³H]uridine. ●, Control; ■, drug concentration 100 μg/L; ▲, drug concentration 50 μg/L.

Figure 4. Effect of cis-Pt(II)-(pentamidine)-I₂ on the incorporation by *L. donovani* of (a) [³H]leucine, (b) [³H]thymidine and (c) [³H]uridine. ●, Control; ■, drug concentration 100 μg/L; ▲, drug concentration 50 μg/L.
New metallic complexes against L. donovani

Figures 5 and 6 show the effects of metallic complexes on L. donovani. From a widening of the mitochondria, as well as the appearance of membrane configurations and intramitochondrial vacuoles in the kinetoplast sac (Figures 5 and 6), perhaps due to the alteration in phospholipid biosynthesis described above.

In addition, transverse cuts in some cases reveal spaces corresponding to the mitochondria with minor electron-dense content, possibly owing to a loss of functionality of the mitochondria and of the mitochondrial matrix.

The DNA of the kinetoplast appears electron-dense and in the nucleus there are electron-dense condensations of chromatin, possibly resulting from the combination of the metal with the DNA (Figure 6). The units of the nuclear membrane appear widened, and in some cases have spaces and alterations, probably as a consequence of the action on phospholipid biosynthesis.

In the rest of the complexes, and cis-Pt-(2,3,4,5,6-pentafluoroaniline)$_2$Br$_2$ in particular (Figure 7), the ultrastructural manifestations are similar to those found in the previous complex, with notable hyperchromicity shown by the mitochondrial membranes (Figure 7b).

Figure 8 reflects the treatment of the parasites with cis-Pt-oxamniquine-Cl$_2$. The most outstanding feature is the appearance of electron-dense condensations in the chromatin and in the kinetoplast, together with fingerprint-like membrane configurations both in the cytoplasm and in the nucleus, probably due to the intrusion of vacuoles in the interior of the nucleus (Figure 8a), giving rise to the intranuclear configurations mentioned above.

Also striking is the appearance of vacuoles with extraordinarily electron-dense content, as in Figure 9, which shows alterations induced in the forms of the parasite treated with cis-Pt-guanethidine-Cl$_2$, where multimembrane ‘fingerprint’ vacuoles are again visible, as well as

Figure 5. Section showing a normal Leishmania (×12,000), width = 0.583 μm. N, nucleus; M, mitochondria; K, kinetoplast DNA.

Figure 6. Ultrastructural effects of cis-Pt(II)-pentamidine-I$_2$ of L. donovani in vitro (×20,000), width = 0.350 μm. Abbreviations as in Figure 5. The nuclear membrane widens (b and c). The chromatin appears disorganized. Disorganization of the parasite’s mitochondria can be seen in all panels.
membranes with electron-dense content and condensations, and hyperchromicity of the kinetoplast and chromatin. The peripheral chromatin appears extraordinarily electron-dense, and has intranuclear vacuoles.

The results obtained when cis-Pt-guanethidine-Cl₂ was assayed in vivo are presented in Figure 10. The reduction in parasitism in treated rats compared with control rats was nearly 65%, while the reduction in the number of amastigotes per gram of spleen reached 75%.

These results imply that, although in many metal complexes the antitumour activity can be related to antitrypanosomal results, from the complexes assayed, those with an NH₃ group in their general structure do not show a
New metallic complexes against *L. donovani* clear leishmanicidal effect. Those which carry a halogen in their structure are the most active, especially those possessing pentamidine as a ligand, possibly attributable to the combined action on the biosynthesis of the nucleic acids on the part of the free ligand and the metal, without disregard for other actions on the metabolism of the parasite. Similar results were previously obtained with *Trypanosoma* and *Leishmania*.

The fact that complexes with a halogen in their structure are more active confirms our results obtained with *T. cruzi*, in which it was demonstrated that the metal complexes of cis-Pt containing chlorine were much more effective than those possessing another halogen. Nevertheless, cis-Pt-pentamidine-Cl$_2$ has been shown to be less effective against *T. cruzi*, while the present study indicates that cis-Pt-pentamidine-Cl$_2$ is active against *L. donovani*.

**References**


New metallic complexes against *L. donovani*

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