Antileishmanial Action of Organometallic Complexes of Pt(II) and Rh(I)

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The three organometallic complexes [(Cis-Pt^{II} (DDH) (2,5-Dihidroxibenzensulfonic)$_2$, Rh^{I} $(CO)_2$ Cl(2-Aminobenzothiazole) and Rh^{I} $(CO)_2$ Cl(5-Cl-2-Methilbenzothiazole)] used in this study had been previously found to have a high in vitro *activity against promastigote and amastigote like forms of* Leishmania donovani. *Here, the cytotoxic effect of these new organometallic complexes on the J-774 macrophages were studied. Only the $Rh^{I}(CO)_2$ Cl (2-Aminobenzothiazole) complex induced substantial toxicity in the cells.*

Also, we assayed the effect of this complex on the parasite's biosynthesis of macromolecules. The $Rh^{I}(CO)_2$Cl (5-Cl-2-Methylbenzothiazole) complex inhibited DNA, RNA, and protein synthesis. On the other hand, the two other compounds tested did not inhibit the incorporation of radioactive precursors. Finally important ultrastructural alterations in the parasites treated with the two non-cytotoxic complexes were observed.

Key words: Organometallic complexes - *Leishmania donovani* - *in vitro* toxicity - mechanism of action

Leishmaniasis is a parasitic disease which affects 12 millon people worldwide (Modabber 1987) and is produced by a species of the genus *Leishmania*, a protozoo belonging to the family Trypanosomatidae. Manifestations of the illness include more or less generalized cutaneous lesions, or, in the case of visceral leishmaniasis (kala-azar), caused by the species *L. donovani* or *L. infantum*, phagocytes of internal organs and tissues (spleen, liver, bone marrow, etc.) are attacked. The drugs for treating these parasitoses have traditionally been pentavalent antimonials, aromatic diamidines and fungicides such as amphotericine B. However, these are extremely toxic and cause a great number of side effects (WHO 1984). Many recent efforts have been made to synthesise and evaluate alternative compounds for treating these parasites (Berman 1988, Croft 1988, Bacchi et al. 1991, Marr 1991).

In the last few years, certain metal complexes have proven anti-tumoral against such protozoan parasites as *Trypanosoma cruzi*, *T. rhodesiense* and *L. donovani*. One property that the tumor cells share with the trypanosomatids is rapid multiplication (Kinnamon et al. 1979, Englund et al. 1982, Wysor et al. 1982, Farrell et al. 1984). The anti-trypanosomal activity of a complex with known anti-cancer activity, cis-platino (neoplatin) has been studied by Balber et al. (1985). Other metal complexes, studied by Farrell et al. (1984), Craciunescu et al. (1988a, b, 1990 a, b), Zinststag et al. (1991), Croft et al. (1992), Sanchez-Delgado et al. (1993), and others, have shown potential anti-cancer activity with metals Pt(II), Pt(IV), Ru(II), Ru(III), Rh(I), Rh(III), Ir(I), Ir(II), Os(II) and Os(III) Ir(IV), Pt(II) and Rh(I).

In the present work, we have selected three organometallic complexes which have previously shown *in vitro* activity against the promastigote forms of *L. donovani* and have also shown a similar activity against the amastigote like forms, these being Pt(II): Cis-Pt(DDH)(Ac. 2,5-Dihydroxibenzensulphonic)$_2$ and those of Rh(I): $Rh(CO)_2$ Cl (5Cl-2-Methylbenzothiazole), $Rh(CO)_2$ Cl (2-Aminobenzothiazole) (Mesa-Valle et al. 1989, 1993). First, we assayed the *in vitro* toxicity of the complexes for the cells of the strain J-774, before evaluating the effect exerted on the parasite's biosynthesis of macromolecules. A study was made of the ultrastructural alterations caused by the two complexes which did not induce toxicity on the cell line assayed.

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MATERIALS AND METHODS

The parasite - The strain of *L. donovani* used was LCR-L 133 (Leishmania Reference Center, Jerusalem), isolated in 1967 from a human case of kala-azar in Begemder (Ethiopia) and maintened in our laboratory until 1980 by culture in Modified NNN Medium (Mesa-Valle et al. 1993).

The organometallic complexes - The complexes assayed are square, flat organometal compounds: cis-Pt(II) (DDH) (Ac. 2,5-Dihidroxybenzensulfonic)$_2$ where DDH is 1,2 diaminocyclohexane; Rh(I)$(CO)_2$ Cl (2-Aminobenzothiazole) and Rh(I)$(CO)_2$ Cl (5-Cl-2-Methylbenzothiazole).

The complexes were synthesised according to the methods described by Craciunescu et al. (1985, 1986).

Parasite cultures - The promastigote forms of *L. donovani* were cultured in TC 199 medium (Gibco) supplemented with 30% foetal bovine serum (Gibco) previously inactivated at 56°C for 30 min (SBFI).

The amastigote like forms were obtained *in vitro* by the methods described previously by Castilla et al. (1995). The viability of the forms were determined by fluorescence microscopy after staining the organism with acridine orange 15µg/ml during 15 min at 38ºC.

For the assays, we used parasites in the exponential phase of growth. The organisms were counted in a Neubauer haemocytometric chamber and the number was adjusted to 1 x 10^6 /ml.

Cytotoxicity assay - The macrophage line used, J-774, was cultivated in RPMI medium. When the macrophages had formed a monolayer, the cells were labelled with Cr^{51} (50 µCi/ml of final concentration), following the technique of Fulfor et al. (1986). Next, the cells were treated with the different complexes at 100, 10 and 1 µg/ml for 6, 12 and 24 hr. The culture medium was withdrawn and transferred to Eppendorf tubes; SDS-NaOH was added to adhering cells, and this was transferred with the cell remains to new Eppendorf tubes. Finally, in a Beckman g scintillation counter, the gamma radiation in the supernatant and in the pellets was measured, and the percentage of specific release of Cr^{51} was calculated using the following values:

$$PSL = \frac{CPMSCont}{CPMSCont + CPMPCont} \times 100$$

$$PLS = \frac{CPMSPro}{CPMSPro + CPMPPro} \times 100$$

PSL= percentage of spontaneous liberation; PSL= percentage of specific liberation; CPM SCont= number of counts per minute of the supernatant control; CPM PCpn= number of counts per minute of pellet control; CPM Spro= number of counts per minute of the supernatant problem; CPM PPro= number of counts per minute of pellet problem.

The effect of the metal complexes on the incorporation of [^{3}H] thymidine, uridine and leucine in the flagellate forms of the parasite - The effect of the organometallic complexes on parasite biosynthesis was measured following the technique described by part of our research group (González et al. 1989). The complexes were assayed at concentrations of 100 and 50 µg/ml and were added to the cultures of the *Leishmania* promastigote forms. The precursors used were thymidine (6-^{3}H, specific activity 20-30 Ci/mmol), uridine (5-^{3}H, specific activity Ci/mmol) and leucine (4.5-^{3}H, specific activity 45-70 Ci/mmol). A concentration of 5 mCi/ml was added to the culture medium.

The recovery of the labelled products, and indirectly the biosynthesis of the macromolecules, were measured after the precipitation of the macromolecules with TCA 10% and filtration in a vacuum through GFC (Watman) filters at 0, 45, 90 and 135 min. The incorporation of the complexes per measure of radioactivity was carried out in a Beckman b Spectrometer using PPO PPOP Cotell.

Electron-microscope transmission - To examine the alterations in parasites caused by the metal-drug complexes we treated the parasites in culture with the complexes at a concentration of 100 µg/ml for 8 hr at 28°C. Afterwards, the samples were centrifuged and the pellet was fixed in glutaraldehyde at 2.5% in cacodylate buffer at pH 7.2 and examined under the electron microscope (TEM) following the technique of Osuna et al. (1983).

RESULTS

Table I shows the effect in the parasite forms after treatment with the three compounds. Table II shows the results obtained on treating the macrophage cell line J-774 with the three organometal complexes. Only the complex Rh(I), with the ligand 2-Aminobenzothiazole, induced substantial toxicity to the cells, reflected in 24.2% specific liberation of Cr^{51} at 24 hr of treatment and at a concentration of 100 µg/ml. It is noteworthy that this complex showed greater activity *in vitro* against the parasites, the percentage of inhibition of growth being 97% at 72 hr and 100 µg/ml (Mesa-Valle et al. 1993).

Fig. 1 indicates the effects produced by the complex cis-Pt(II)(DDH)(2,5-dihydroxybenzen-

TABLE I

Percentage of growth inhibition caused by the metal complexes in the culture of Promastigote and Amastigote like forms of *Leishmania donovani*

	Time of incubation								
	24 hr			48 hr			72 hr		
Concentration (μg/ml)	100	10	1	100	10	1	100	10	1
Promastigote forms									
$Rh(CO)_2Cl$(2-Aminobenzothiazole)	54.9	0	0	60	0	0	97	0	0
$Rh(CO)_2Cl$(5-Cl-2-Methilbenzothiazole)	72.5	0	0	64.4	26.4	0	83.6	14.2	0
Cis-Pt(DDH)(2,5-Dihidroxibenzensulphonic)$_2$	43.3	0	0	47.8	26.3	0	67.2	37	0
Amastigote like forms									
$Rh(CO)_2Cl$(2-Aminobenzothiazole)	67.8	0	0	72.7	0	0	86.5	0	0
$Rh(CO)_2Cl$(5-Cl-2-Methilbenzothiazole)	31.9	0	0	31	0	0	50.8	0	0
Cis-Pt(DDH)(2,5-Dihidroxibenzensulphonic)$_2$	52.7	24.7	0	50.1	29.7	0	57.8	18.6	0

The results are the average of five experiments.

TABLE II

Cytotoxic effect of metal complexes on J-774 Macrophages[a]

	Time of incubation							
	24 hr			48 hr			72 hr	
Concentration (mg/ml)	100	10	1	100	10	1	100	10
$Rh(CO)_2Cl$(2-Aminobenzothiazole)	0	0	0	2.4	3.2	0	24	3.2
$Rh(CO)_2Cl$(5-Cl-2-Methilbenzothiazole)	0	0	0	2.9	0	0	3.2	0
Cis-Pt(DDH)(2,5-Dihidroxibenzensulphonic)$_2$	0	0	0	3.5	0	0	3.0	0

a: the results are the averages of five different experiments, expressed in percentage of specific liberation of Cr^{51}.

sulfonic)$_2$ on the incorporation of [^{3}H] thymidine, [^{3}H] leucine and [^{3}H] uridine, showing that this complex does not inhibit DNA, RNA or protein synthesis. Similar results were obtained for the complex Rh(I): $Rh(CO)_2$ Cl (2-Aminobenzothiazole) (Fig. 2), while the complex $Rh(CO)_2$ Cl (5-Cl-2-Methylbenzothiazole) acts on all three levels, decreasing the incorporation of the three radioactive precursors at the two concentrations used, 50 and 100 μg/ml. Thus, at the greater concentration, the inhibtion in thymidine incorporation was 72% at 135 min, while that of leucine and uridine was 60% (Fig. 3).

The ultrastructural alterations at the nuclear chromatin level in *L. donovani* caused by the Pt(II) complex are shown in Fig. 4. Here, a discontinuity of the double nuclear membrane seems to appear. There is a great quantity of lipid vacuoles, as well as the formation of myeline configurations in tissues both at the cytoplasm and the nuclear levels, all showing a high degree of cellular degeneration. Figs 5 and 6 show the effect at the ultrastructural level of the complex Rh(I)$(CO)_2$ Cl (5-Cl-2-Methylbenzotiazole), with similar effects appreciable for complex Pt(II).

DISCUSSION

Metal complexes with anti-tumor action, such as cis-Pt (neoplatin) have demonstrated their effectiveness in the treatment of trypanosomiasis (Kinnamon et al. 1979, Wysor et al. 1982). Analogues of this complex have been tested against different species of *Trypanosoma* (Farrell et al. 1984, Ruiz-Perez et al. 1986, 1987) and against *Leishmania* (Mesa-Valle et al. 1989, 1993).

Of the complexes assayed in the present work, the Pt(II) complex Pt(DDH)(2,5-Dihydroxybenzensulfonic)$_2$ is a structural analogue of the first (neoplatin). In the same way, this complex is obtained by replacing the NH_3 groups of the neoplatin with (DDH), and the Cl with a ligand of greater liposolubility, (2,5-Dihydroxybenzensulfonic), joined to Pt by a bidentate bond. This structure increases the dynamic stability of the complex, attributable to its greater anti-neoplast effectiveness on the stereochemical disposition of the complex for the interaction with the DNA. Thus, this complex showed activity against *T. brucei* and *T. rhodesiense* (Craciunescu et al. 1986), and some structural analogues showed activity against epimastigote forms of *T. cruzi* (Ruiz-Perez et al.

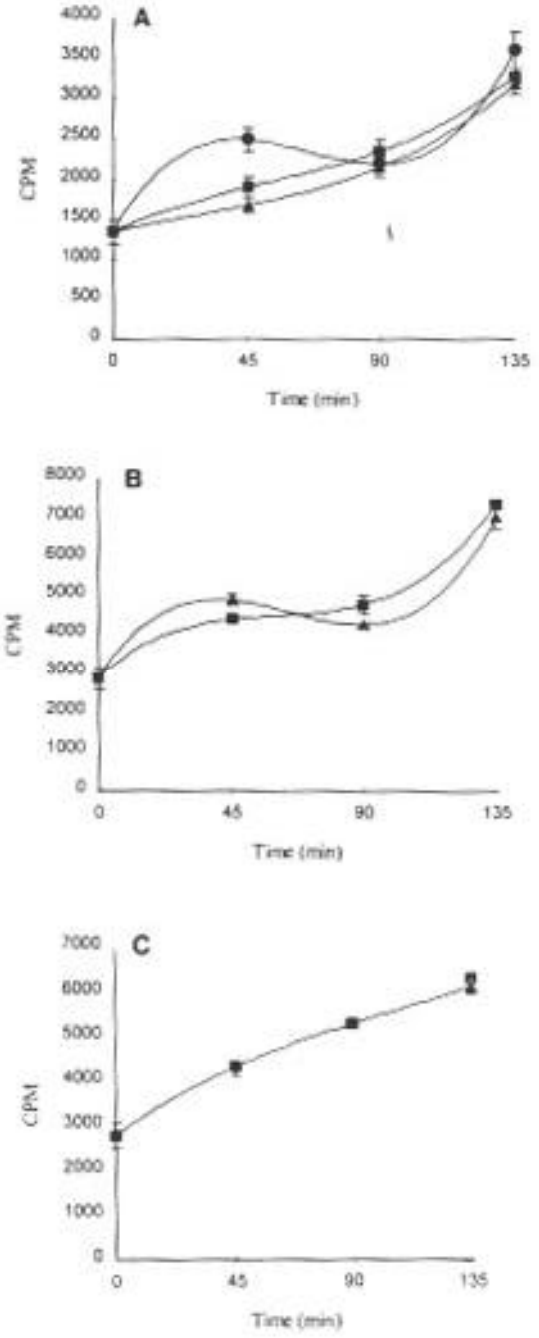

Fig. 1: effect of cis-Pt^{II} (DDH)(2,5-Dihidroxybenzensulphonic)$_2$ on the incorporation by *Leishmania donovani* of: (A).- (^{3}H)leucine, (B).- (^{3}H)thymidine and (C).- (^{3}H)uridine. (●) Control;(■) Drug concentration-100 µg/ml;(s) Drug concentration-50 µg/ml.

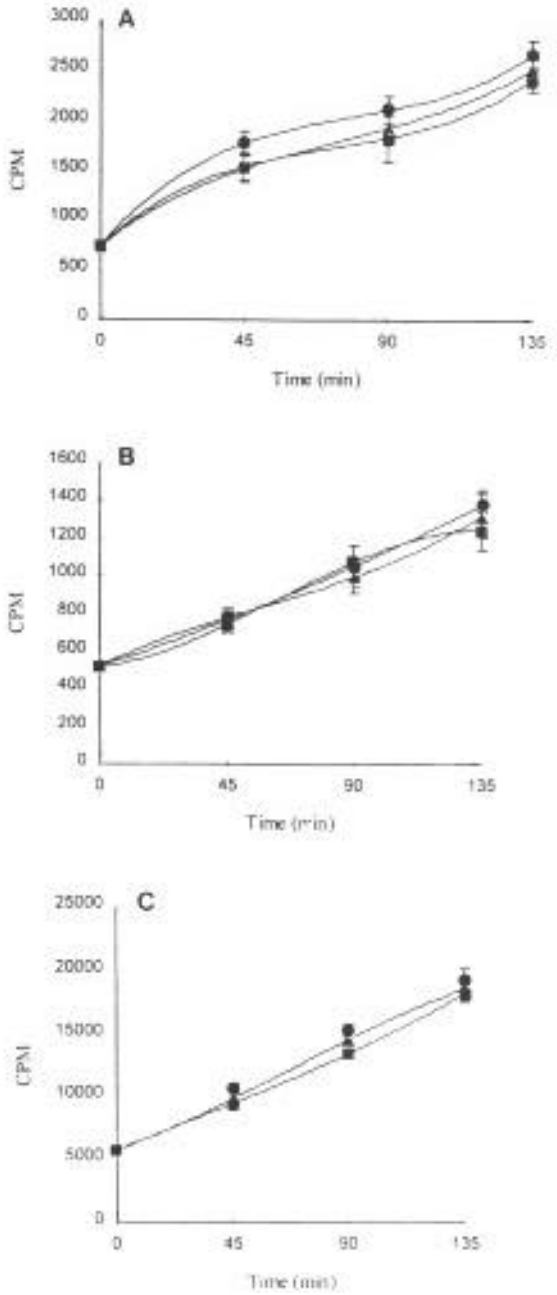

Fig. 2: effect of $Rh^{II}(CO)_2Cl$ (2-Aminobenzothiazole) on the incorporation by *Leishmania donovani* of: (A).- (^{3}H)leucine, (B).- (^{3}H)thymidine, and (C).- (^{3}H)uridine. (●) Control; (■) Drug concentration-100 µg/ml; (s) Drug concentration-50 µg/ml.

1986, 1987) and against promastigote forms of *L. donovani* (Mesa-Valle et al. 1989, 1991).

Willson et al. (1992) showed that derivatives of sulphonic acid inhibit the growth of *T. equiperdum* and the glycolytic enzymes of *T. brucei*. Glycolysis is the essential mechanism in obtaining energy in the protozoa of the family Trypanosomatidae and occurs in the specific cytosolic bodies called glycosomes (Opperdoes et al. 1984).

The three organometallic complexes showed an *in vitro* antiproliferative activity against both

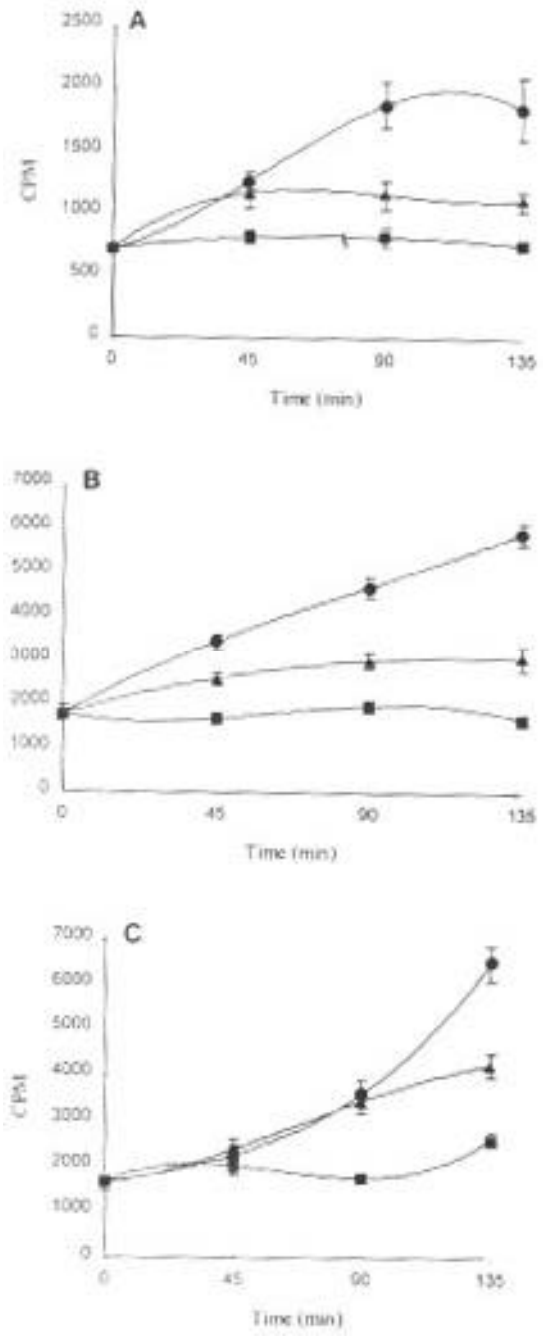

Fig. 3: effect of $Rh^{II}(CO)_2Cl$ (5-Cl-2-Methylbenzothiazole) on the incorporation by *Leishmania donovani* of: (A).-(^{3}H)leucine, (B).- (^{3}H)thymidine, and (C).- (^{3}H)uridine. (●) Control; (■) Drug concentration-100 µg/ml; (s) Drug concentration-50 µg/ml.

the flagellates and amastigote like forms (Table I). The highest efectivity was obtained at the highest concentration and longest time tested.

In an attempt to ascertain whether the leishmanicidal activity *in vitro* (Mesa-Valle et al. 1989) shown by the complex is owed to a blockage in the biosynthesis of macromolecules, we studied the levels of incorporation of [^{3}H] thymidine, [^{3}H] leucine and [^{3}H] uridine. However, the data obtained with this complex do not coincide with those observed previously by us for other complexes with analogous structures used against *T. cruzi* (Ruiz-Perez et al. 1987). In the promastigote forms of *L. donovani*, this complex does not significantly inhibit the incorporation of the labelled analogues by the parasite.

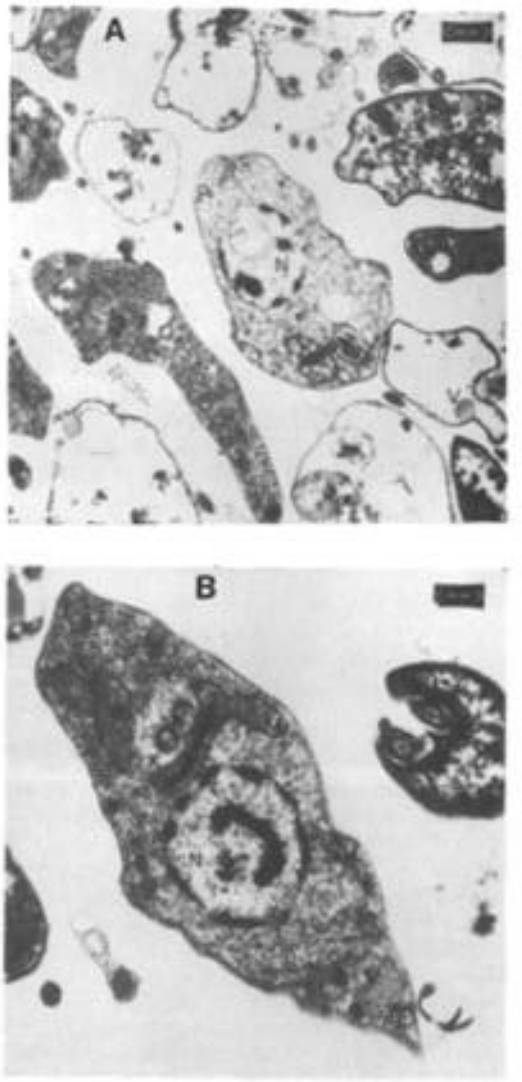

Fig. 4: ultrastructural effects of cis-Pt^{II} (DDH)(2,5-Dihidroxybenzensulphonic)$_2$ of *Leishmania donovani in vitro* Nucleus (N), Kinetoplast DNA (K), Mitochondria (M). (A) (x7.000) and (B) (x12.000)

At the ultrastructural level, treatment of the promastigote forms for 8 hr with the complex produces abnormal condensations of the chromatin and

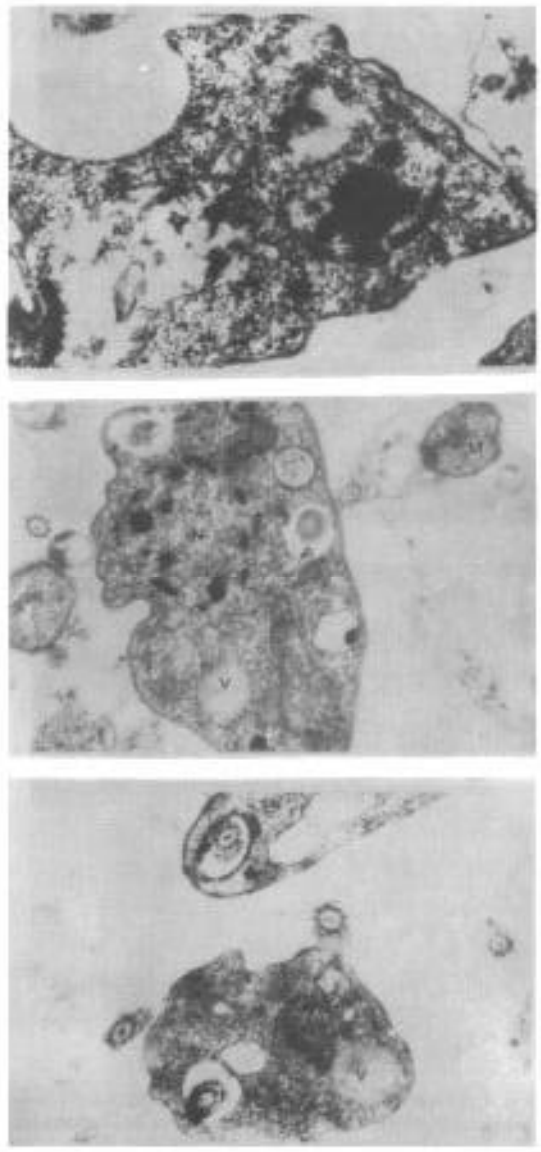

Fig. 5: *Leishmania* exposed for 8 hr *in vitro* to 100 µg/ml $Rh^{II}(CO)_2Cl$ (5-Cl-2-Methylbenzothiazole). Nucleus (N). Mitochondria (M). Kinetoplast DNA (K). (x15.000).

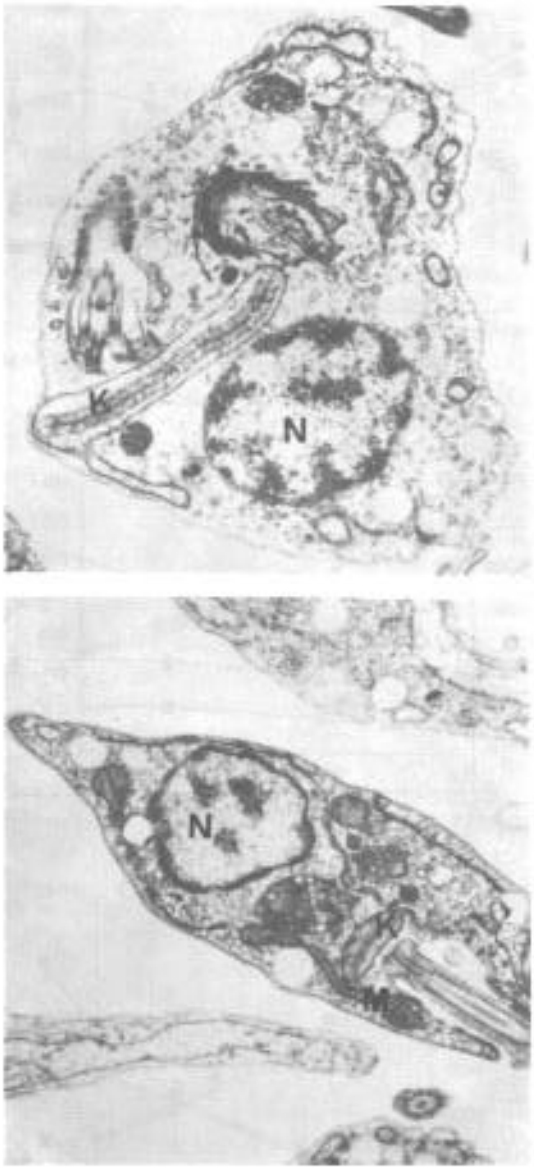

Fig. 6: section showing an normal *Leishmania* (x12.000). Nucleus (N). Mitochondria (M). Kinetoplast DNA (K).

intranuclear vacuoles with membrane alterations, giving the appearance of mitochondrial fingerprint structures. The mitochondria appear swollen and mitochondrial crests are highly evident in the matrix. No alterations appear at the plasma-membrane level.

The complexes Rh, Rh(I) and Rh(III) have shown anti-tumor and anti-bacterial activity similar to that of the derivatives cis-Pt(II) (Dehand & Jordanov 1976, Craciunescu et al. 1986, 1987, 1990a, 1990b). Some of the complexes with greater anti-cancer activity have been found to be strong inhibitors of Ornitine Decarboxylase (Sjoerdsma & Schechter 1984), a target enzyme with a great number of active complexes against various species of *Trypanosoma* and *Leishmania* (Bacchi et al. 1980, Fouce et al. 1991).

In addition, the ligands used in our case were derived from benzothiazole, a structure in which some of its derivatives have shown promising antimicrobial anti-fungus and anti-parasite activity (Hisano et al. 1982, Haugwitz et al. 1982, Zahner et al. 1991a, b, Yalçin & Sene, 1993, Bujdakova et al. 1993), especially as inhibitors of the synthesis of ergosterol (Urbina et al. 1991), the major sterol in the membranes of fungi, yeasts and trypanosomatids. Known inhibitors of ergosterol synthesis, such as

ketoconazol (Goad et al. 1989) show intense anti-leishmania activity (Goad et al. 1985).

The complexes from Rh(I) tested by us have a flat, square configuration similar to the structure of neoplatin. Ruiz-Perez et al. (1987) studied the action of this complex on the levels of incorporation of radioactively in macromolecules. Similar assays carried out by us confirmed that the complex $Rh(I)(CO)_2$ Cl (2-Aminobenzothiazole) does not significantly inhibit the synthesis of macromolecules of the parasite, whereas $Rh(I)(CO)_2$ Cl (5-Cl-2-Methylbenzothiazole) inhibited the incorporation of thymidine by approximately 72% after 135 min at 100 µg/ml, and leucine and uridine incorporation by 60% for the same time and dosage.

The cytotoxicity of the complex Rh(I), with ligand (2-Aminobenzothiazole) to J-774 macrophages at 24 hr and maximum dosage, produced 24.2% specific liberation of Cr^{51},whereas the complex $Rh(CO)_2$ Cl (5-Cl-2-Methylbenzothiazole), which produced the greatest inhibition rates in macromolecule biosynthesis in *Leishmania*, was 3.2% at 24 hr of maximum dosage.

The ultrastructural alterations observed in the promastigote forms of *L. donovani* after treatment with $Rh(CO)_2$ Cl (5-Cl-2-Methylbenzothiazole) show large abnormal chromatin condensations in the nucleus and kinetoplast of the parasite, which is indicative of an intercalation of the metal complex in the DNA. In addition, numerous vacuoles appear, some with multiple membranes and the intravacuolar appearance of fingerprint myeline figures. In some cases a pronounced swelling of the mitochrondria is visible, with conspicuous mitochondrial crests and electrondense deposits in the matrix. *Leishmania* promastigotes lose their typical structure and amoebal cytoplasmic projections appear as a consequence of the loss of tone in the tubules which make up the cytoskeleton. In some cases, discontinuities appear in the nuclear membrane and in the lacunar spaces between the nuclear membrane and the cytosol, as well as between the tubules of the cytoskeleton and the cytoplasm.

These effects on the membranes could be due to a direct influence of the ligand on the biosynthesis of the membrane components, which would lead to irregularities and dysfunctions.

In the present work, we study the manner of action and the cytopathological effects of certain metal complexes which *in vitro* have shown leishmanicidal activity, seeking alternatives to the traditional medicines for leishmaniasis. The best candidate, given its slight toxicity for mammal cells and its high activity against *Leishmania* by inhibiting the synthesis of macromolecules, is the complex Rh(I): $Rh(CO)_2$ Cl (5-Cl-2-Methylbenzothiazole).

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