

IN VIVO ANTILEISHMANIAL ACTION OF Ir-(COD)-PENTAMIDINE TETRAPHENYLBORATE ON *LEISHMANIA DONOVANI* AND *LEISHMANIA MAJOR* MOUSE MODELS

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Summary :

Ir-(COD)-pentamidine tetraphenylborate which has previously been studied on promastigote forms of *Leishmania*, was investigated for its antileishmanial properties compared with pentamidine used as reference compound. *In vitro*, the iridium complex had the same IC₅₀ value on intracellular forms of *Leishmania* as pentamidine (15 µM). *In vivo*, the compound could not be injected intravenously due to the DMSO excipient so that the treatments were performed intraperitoneally or subcutaneously. On the *L. donovani* LV9 /Balb/C mouse model, the iridium complex was not toxic after intraperitoneal treatment at 232 mg/kg/day × 5 or 147 µmoles/kg/day × 5, whereas all the mice died within five days when treated at the same dose with pentamidine isethionate. However, only 23 % of parasite suppression was observed with the iridium complex. On a *L. major* MON 74/Balb/C mouse model, susceptible to intravenously administered pentamidine at 6.7 µmoles/kg/day × 5 (54 % of parasite suppression), the iridium complex exhibited 32 % of parasite suppression after a treatment at 76 µmoles/kg/day × 5 administered subcutaneously. This slight activity is of interest since pentamidine isethionate is not active under these conditions. Transmission electron microscopy of amastigotes from infected and treated mice show aggregation of ribosomal material, distension of the nuclear membrane and kDNA depolymerization. The mechanism of action therefore involves several targets: membranes, ribosomes and kDNA. According to our results, the Iridium complex is a suitable candidate to be encapsulated in drug carriers such as liposomes or nanoparticles.

KEY WORDS : Iridium pentamidine complex, pentamidine, *Leishmania*, electron microscopy, mechanism of action.

MOTS CLÉS : complexe iridié de pentamidine, pentamidine, *Leishmania*, microscopie électronique, mécanisme d'action.

Résumé : ACTION ANTILEISHMANIENNE *IN VIVO* DU TÉTRAPHÉNYLBORATE D'IR-(COD)-PENTAMIDINE SUR DES MODÈLES MURINS À *LEISHMANIA DONOVANI* ET *LEISHMANIA MAJOR*

L'action antileishmanienne du tétraphénylborate d'Ir-(COD)-pentamidine qui a précédemment été étudiée sur les formes promastigotes de *Leishmania* est ici évaluée *in vivo* en comparaison de l'iséthionate de pentamidine, produit de référence. *In vitro*, le complexe iridié présente une valeur d'IC₅₀ sur la forme intracellulaire de *Leishmania* équivalente à celle de la pentamidine (15 µM). *In vivo*, puisque le composé ne pouvait pas être injecté par voie intraveineuse du fait de l'utilisation du DMSO comme excipient, les traitements ont été réalisés par voie intrapéritonéale ou sous-cutanée. Sur le modèle à *L. donovani* LV9 chez la souris Balb/C, le complexe iridié ne s'est pas montré toxique après un traitement par voie intrapéritonéale à la dose de 232 mg/kg/jour × 5 soit 147 µmoles/kg/jour × 5, tandis que toutes les souris traitées avec l'iséthionate de pentamidine aux mêmes doses sont mortes dans les cinq jours. Cependant, on observe une suppression de la charge parasitaire de 23 % seulement avec le composé iridié. Sur le modèle à *L. major* MON 74 chez la souris Balb/C, qui est sensible à l'iséthionate de pentamidine administré par voie intraveineuse à la dose de 6,7 µmoles/kg/jour × 5 (54 % de suppression de la charge parasitaire), le complexe iridié a montré 32 % de suppression de la charge parasitaire après un traitement par voie sous-cutanée à la dose de 76 µmoles/kg/jour × 5. Cette activité, bien que légère est intéressante puisque l'iséthionate de pentamidine n'est pas actif dans ces conditions. La microscopie électronique d'amastigotes prélevés chez les souris traitées montre une aggrégation du matériel ribosomal, une distension de l'enveloppe nucléaire et une dépolymérisation de l'ADN kinétoplastique. Par conséquent, le mécanisme d'action de ce composé met en jeu plusieurs cibles : les membranes, les ribosomes et l'ADN kinétoplastique. A la suite de cette étude, le complexe iridié mériterait d'être encapsulé dans des vecteurs de médicaments tels que les liposomes ou les nanoparticules.

INTRODUCTION

Visceral and cutaneous leishmaniasis are caused by hemoflagellate protozoa which are obligate parasites of the mononuclear phagocyte system

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(MPS). Leishmaniasis cause considerable morbidity and mortality worldwide. The first line treatments are pentavalent antimonials and amphotericin B, both of which show high potential toxicity. Moreover, failure and relapse under antimonials and amphotericin B have also been reported (Modabber, 1992). The treatment with pentamidine is not fully effective and has also toxic side effects (Soto *et al.*, 1994). Thus, the search for new antileishmanial drugs remains a priority since no currently available drug is both safe and active. Several antitumor agents directed towards DNA as their molecular target have been screened for their

trypanocidal activity, since the metabolic pathways of Kinetoplastidae and tumor cells present some similarities. By this means, organometallic complexes were found to have antitrypanosomal activity (Kinnamon *et al.*, 1979). The association of a metallic structure with an organic compound decreases the toxicity and usually increases the biological activity (Farrell *et al.*, 1984). Some metallic drug complexes have been found to be active against *Leishmania donovani* amastigotes in mouse peritoneal macrophages *in vitro* (Croft *et al.*, 1992). Moreover, some organometallic compounds derived from pentamidine are active against African trypanosomes (Loiseau *et al.*, 1992). In addition, compounds combining platinum and pentamidine appeared promising in the *T. b. brucei*/sheep model (Dreyfuss *et al.*, 1988; 1993). An antitrypanosomal screening using *Trypanosoma b. brucei* CMP strain allowed a very active metallic pentamidine derivative, called pentamidine di-(iridium cyclo-octadiene) tetraphenylborate or Ir-COD-pentamidine tetraphenylborate, to be selected (Loiseau *et al.*, 1992). This compound was found to be 16-fold more active than pentamidine isethionate and was well tolerated. Organometallic complexes have interesting antimicrobial effects which could be exploited, particularly against drug resistant Kinetoplastida strains. Previous pharmacokinetic studies in sheep plasma have shown that the metal part increases the half-life of the drug compared to pentamidine isethionate, explaining the higher activity at low doses (Dreyfuss *et al.*, 1993; 1995; Loiseau *et al.*, 1997). Such compounds could be of potential interest as antileishmanial drugs, to replace pentamidine which is sometimes used in cases of antimonial-resistance.

MATERIALS AND METHODS

PARASITES

Two strains of *Leishmania* were used throughout this study:

- *L. donovani* (MHOM/ET/67/L82) or LV9 was routinely maintained in male golden hamsters (Wright's strain). After six-eight weeks amastigotes were isolated from the spleen of infected animals for *in vitro* and *in vivo* studies. This strain was provided by Dr S.L. Croft (London School of Hygiene and Tropical Medicine, UK).
- *L. major* (MHOM/PT/92/CRE26), zymodeme MON 74, was a cutaneous strain which regularly developed a visceral infection in the hamster when infected by the intraperitoneal route. This strain, kept in liquid nitrogen, was provided by Prof. M. Deniau (CHU Henri Mondor, Créteil, France).

ANIMALS

Female BALB/c mice (18 ± 2 g) were purchased from Iffa Credo (L'Arbresle, France).

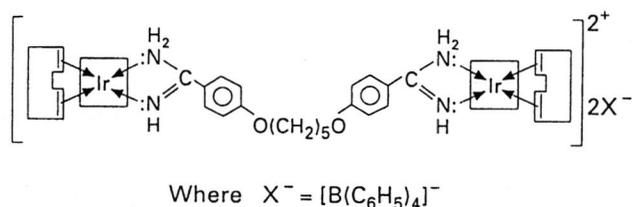


Fig. 1. – Chemical structure of Ir-(COD)-pentamidine tetraphenylborate. COD = cyclo-octadiene.

DRUGS

The structure of Ir-(COD)-pentamidine tetraphenylborate is shown in Figure 1. Pentacarinat® (Pentamidine isethionate) was kindly supplied by Roger Bellon Laboratory (France). Glucantime®, a pentavalent antimonial used as the reference antileishmanial drug, was supplied by Rhône-Poulenc Rorer, France.

ACTIVITY ON INFECTED MACROPHAGES

In vitro evaluation

Leishmanicidal activity against intramacrophagic amastigotes was performed on infected mouse peritoneal macrophages according to the method of Neal & Croft (1984).

In vivo evaluation

Female BALB/c mice were infected with 10^7 amastigotes obtained from the spleen of an infected hamster and injected into mice by the retro-orbital sinus. One week after infection, the mice were randomly divided into groups of ten. Ir-(COD)-pentamidine tetraphenylborate in DMSO and pentamidine isethionate in sterile water were given by the subcutaneous or intraperitoneal routes at doses up to 232 mg/kg (50 mg pentamidine equivalent/kg) and 87 mg/kg respectively. Mice were treated once a day for five consecutive days and sacrificed three days after the end of treatment. Drug activity was estimated by counting the number of amastigotes/500 liver cells in Giemsa stained impression smears prepared from the weighed livers of treated and untreated mice (Neal & Croft, 1984).

TRANSMISSION ELECTRON MICROSCOPY

Liver samples were prepared for electron microscopy after intraperitoneal administration of Ir-(COD)-pentamidine tetraphenylborate to infected BALB/c mice at 232 mg/kg (50 mg pentamidine equivalent/kg). Samples were also prepared from mice treated with excipient alone.

Small portions of liver were cut into 1 mm³ pieces and fixed at room temperature for 60 min. in a 2.8 % glutaraldehyde solution in a 0.1 M Sørensen phosphate buffer (pH 7.4), washed twice in this buffer and post-

fixed for one hour in a 2 % osmium tetroxide solution in the same buffer. Potassium ferricyanide was added to both fixatives. The samples were then stained for 12 hours in 0.5 % uranyl acetate in distilled water. Following dehydration, the material was embedded in a 1:1 mixture of Araldite-Epon®. 50 nm sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. The preparations were examined with a Phillips EM 201 electron microscope (100 Kv) at the Centre Interuniversitaire de Microscopie Électronique, (Université de Jussieu Paris VI).

RESULTS

IN VITRO ACTIVITY ON AMASTIGOTES FORMS IN MACROPHAGES

The iridium complex had the same IC50 value on intracellular forms of *Leishmania* as pentamidine (15 µM).

IN VIVO EFFECT

In vivo, the compound could not be injected intravenously due to the DMSO excipient so that the treatments were performed intraperitoneally or subcutaneously. The iridium complex was not toxic towards the *L. donovani* LV9 /Balb/C mouse model after a treatment at 232 mg/kg/day × 5 or 147 µmoles/kg/day × 5 administered intraperitoneally whereas all the mice died within five days when treated with the same dose with pentamidine isethionate. However, only 23 % of parasite inhibition was observed with the iridium complex (Table I).

In the *L. major* CRE 26/Balb/C mouse model, susceptible to intravenously administered pentamidine at 6.7 µmoles/kg/day × 5 (54 % of parasite inhibition), the iridium complex exhibited 32 % of parasite inhibition after a treatment at 76 µmoles/kg/day × 5 subcutaneously administered (Table II). This slight activity is of interest since pentamidine isethionate is not active under these conditions.

Drug	Treatment ^a Dose (i.p.)		Stauber count × 10 ⁸ (± SEM)	Percentage of parasites inhibition (mean)
	mg/kg/day	µmol/kg/day		
Ir-(COD)-pentamidine tetraphenylborate	232 × 5	147 × 5	5.13 ± 2.31	23
^b Pentacarinat®	87 × 1	147 × 1	/	/
Glucantime®	200 × 5	546 × 5	0.02 ± 0.02	97
	54	147 × 5	6.01 ± 1.04	10
*Control	/	/	6.67 ± 1.33	0

^a : n = 10 mice per group.

^b : toxic dose: death of 4 mice at day 2
3 mice at day 3
2 mice at day 4
1 mouse at day 5.

^c : Treatment with 0.2 ml excipient per day for five days.

Table I. – Effect of Ir-(COD)-pentamidine tetraphenylborate on the *L. donovani* LV9/BALB/c mouse model compared with reference drugs. 232 mg/kg of Ir-(COD)-pentamidine tetraphenylborate corresponds to 50 mg equivalent pentamidine/kg. 200 mg/kg of Glucantime® corresponds to 56 mg Sb^v/kg.

Drug	Treatment ^a Dose		Stauber count × 10 ⁸ (± SEM)	Percentage of parasites inhibition (mean)
	mg/kg/day	µmol/kg/day		
Ir-(COD)-pentamidine tetraphenylborate	120 × 5 (s.c.)	76 × 5 (s.c.)	1.50 ± 0.71	32
	60 × 5 (s.c.)	38 × 5 (s.c.)	1.73 ± 0.94	22
Pentacarinat®	4 × 5 (i.v.)	6.7 × 5 (i.v.)	1.02 ± 0.72	54
^bControl	/	/	2.21 ± 1.33	0

^a : n = 10 mice per group.

^b : Treatment with 0.2 ml excipient per day for five days by the subcutaneous route.

Table II. – Effect of Ir-(COD)-pentamidine tetraphenylborate on the *L. major* CRE 26/BALB/c mouse model compared with reference drugs.

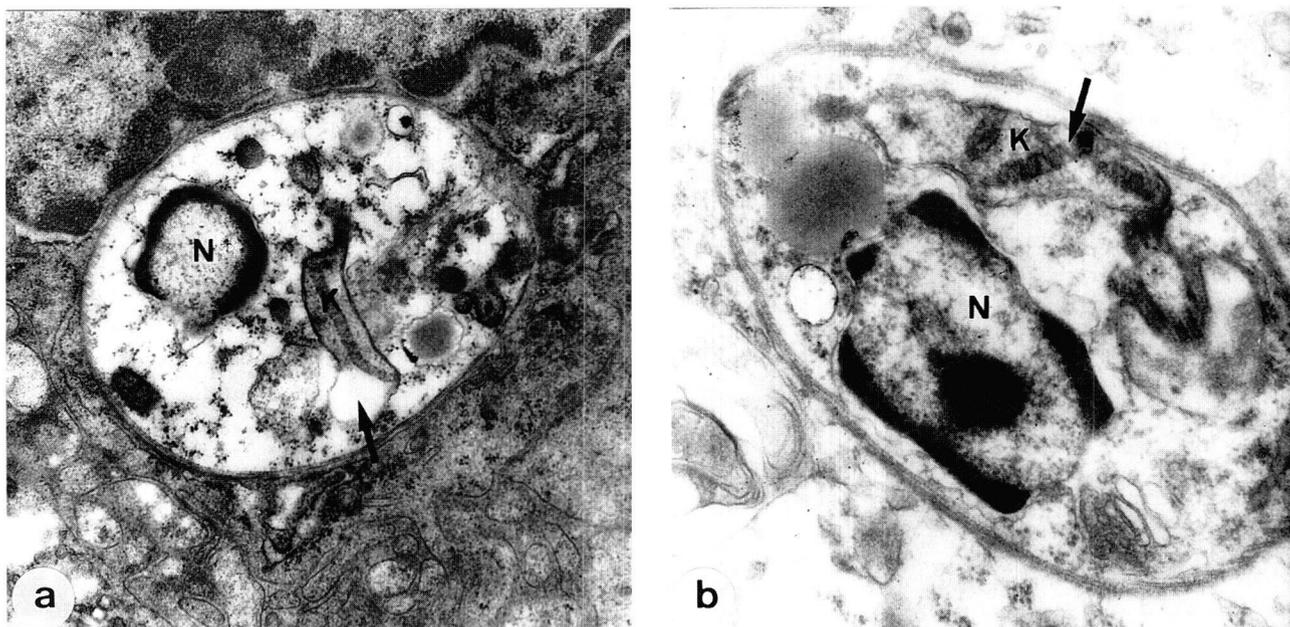


Fig. 2. – Electron micrograph of sections of *L. donovani* LV9-infected liver of BALB/c mice three days after treatment with Ir-(COD)-pentamidine tetraphenylborate administered intraperitoneally at 50 mg pentamidine equivalent /kg/day × 5. Within these treated parasites, the most striking features are:
 a) a clear cytoplasm due to a depleted ribosomal material, a nuclear membrane convoluted and a kinetoplast presenting major alterations: swollen mitochondrion, low electron density of probably depolymerized kDNA (× 15,000).
 b) fragmentation of the kDNA in a deflated mitochondrion. In addition, the alterations already observed in Figure 2a can also be noted here (× 20,000).

ELECTRON MICROSCOPY

The *in vivo* treatment with the Iridium complex at 50 mg equivalent pentamidine/kg/day × 5 induced significant damage in amastigotes compared with controls treated with DMSO only. In the cytoplasm of the parasite, many electron-lucent areas surrounded by aggregated ribosomes are observed. The peri-nuclear space (between the two nuclear membranes) is swollen. The mitochondrion, deflated or swollen, appears amorphous and no longer organized. The kinetoplast has become less electron-dense and is sometimes disrupted into several fragments (Fig. 2a and 2b). Croft & Brazil (1982) have observed similar structural damage in *L. m. amazonensis* promastigotes and amastigotes treated with 10 μM of pentamidine *in vitro*.

DISCUSSION

A previous study has shown that Iridium-(COD)-pentamidine tetraphenylborate enters and accumulates within promastigotes (Mbongo *et al.*, 1998). The apparent K_m value was lower than that of pentamidine for the same parasite strain (Basselin *et al.*, 1996). Thus, the apparent K_m values of Iridium-COD-pentamidine tetraphenylborate and pentamidine

isethionate were 17.4 μM and 73 μM, respectively and the apparent V_m were 1.3 and 2 nmoles/mg protein per two hours, respectively (Mbongo *et al.*, 1998). Drug-metal complexes are sensitive to pH and their structure can be modified in the presence of water (Van der Veer & Reedijk, 1988). Several hypotheses could explain the behavior of Iridium-COD-pentamidine tetraphenylborate under acidic conditions of extraction:
 a) partial dissociation of iridium complex cation and tetraphenylborate anion;
 b) release of ligands (i.e pentamidine);
 c) formation of a new complex with perchlorate anion (Fig. 3). Considering the results obtained from HPLC experiments, we concluded that the iridium

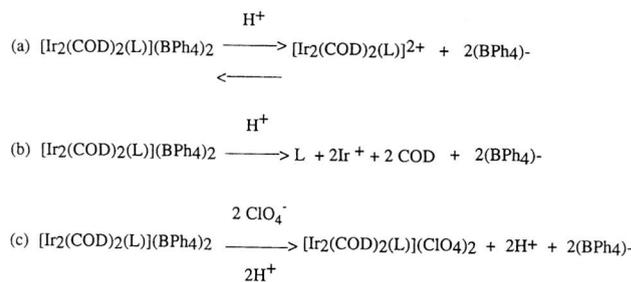


Fig. 3. – Hypothetical dissociation of Ir-COD-pentamidine tetraphenylborate in acidic medium (HClO₄). COD = 1,5 cyclooctadiene; L = pentamidine; Ph = C₆H₅.

complex is taken up by the cells in its undissociated neutral form. This is consistent with the lipophilic properties of tetraphenylborate which enables the iridium complex to cross the parasite membranes.

These previously obtained results (Mbongo *et al.*, 1998) suggest that the iridium complex is relatively stable within the cells, probably because of strong binding to intracellular components such as macromolecules. Therefore, it could act as a carrier for pentamidine. The electron microscopy of amastigotes reported in the present study indicates an action on several targets within the parasite: membranes, ribosomes and kDNA.

The undissociated neutral form of the complex could interact easily with membranes and the alterations observed by electron microscopy confirm such an interaction.

We have previously compared the interaction of pentamidine and the iridium-complex with isolated ribosomes. The organometallic complex stabilised ribosomes, whereas pentamidine had no effect under the same conditions. This difference between the behaviour of pentamidine and of the iridium complex is due to the distribution of positive charges around the cation structure and their accessibility to anions groups of nucleoproteins. The iridium complex is positively charged, thus it may interact more strongly with opposite charges of ribosomes than does pentamidine. Changes in the cytoplasmic ribosome content of drug treated- amastigotes *in vivo* and the effect of iridium complex on stability *in vitro* were not associated with any inhibition of macromolecular biosynthesis, indicating that ribosomes are not the main target of either the iridium complex or pentamidine.

As far as interactions with kDNA are concerned, they were similar to those observed with pentamidine, which interacts with the AT bases of kDNA in Kinetoplastida (Bell *et al.*, 1990).

Despite its toxicity, pentamidine remains a molecule of great interest for curing early stages of African trypanosomiasis, antimonial-refractory leishmaniasis and pneumocystosis (Sands *et al.*, 1985). Thus, new, less toxic, pentamidine derivatives would be valuable in antiparasitic chemotherapy. Since organometallic complexes have previously shown activity against *L. donovani* promastigotes (Mesa-Valle *et al.*, 1989) and amastigotes (Croft *et al.*, 1992), the combination of a metallic structure with the pentamidine molecule appeared promising. *In vitro*, on the both *Leishmania* strains evaluated, the advantage of the iridium complex compared with pentamidine could not be established as clearly as in the case of the trypanosomes (Mbongo *et al.*, 1997, 1998; Loiseau *et al.*, 1992).

Although its activity *in vivo* in mice is not strong enough, the iridium complex is less toxic than pentamidine isethionate. The relative selectivity of these

compounds towards parasite cells remains to be investigated. Pharmacokinetic data carried out in sheep have shown that after a single dose, the compound was eliminated slowly from the blood (Loiseau *et al.*, 1997). Further studies will be undertaken, for example encapsulation in nanoparticles able to concentrate the active principle in the reticulo-endothelial system since previous encapsulation strategies were successful for pentamidine (Durand *et al.*, 1997; Fusai *et al.*, 1997).

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