ACTION OF PENTAMIDINE-BOUND NANOPARTICLES AGAINST LEISHMANIA ON AN IN VIVO MODEL

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Summary :

The efficiency of antileishmanial agents may be enhanced by improving their bioavailability with a colloidal drug carrier. We have investigated the action of free pentamidine, compared with pentamidine bound to polymethacrylate nanoparticles, in a rodent model.

BALB/c mice were infected, via the tail vein, with 4×10^7 L. major (MON 74) promastigotes. Twelve days after infection, seven groups of mice were treated respectively with methylglucamine antimoniate (Glucantime®) 5.56 mg/kg i.p. x 5 d., pentamidine bound nanoparticles (100 µM), unloaded polymethacrylate nanoparticles, unloaded nanoparticles associated with free pentamidine (100 µM) 0.1 ml i.v. x 3 d and free pentamidine isethionate (2.28 mg/kg and 0.17 mg/kg i.v. x 3 d.). Twenty-one days post infection, the mice were sacrificed and the *Leishmania* load in the liver calculated from the number of amastigotes/500 liver cells and total liver weight in treated and untreated mice. Results demonstrated a 77 % amastigote reduction in the group treated with targeted pentamidine relative to the control group. The ratio free pentamidine/bound-pentamidine was approx. 12.

KEY WORDS : nanoparticles. *Leishmania*. mouse. pentamidine.

Résumé : ACTIVITÉ LEISHMANICIDE IN VIVO DE NANOPARTICULES CHARGÉES DE PENTAMIDINE.

L'efficacité des molécules antileishmaniennes peut être améliorée en augmentant leur biodisponibilité à l'aide de vecteurs colloïdaux. Des nanoparticules de polyméthacrylate chargées de pentamidine ont été évaluées comparativement à la pentamidine libre sur un modèle de leishmaniose murine où les souris sont infectées par voie i.v. avec 4×10^7 promastigotes de *L. major* (MON 74). Douze jours après infection, sept lots de souris ont été traités avec de l'antimoniate de méthyl glucamine (Glucantime®, Sb^{V+} 56,6 mg/kg, i.p. x 5 j.), des nanoparticules chargées de pentamidine (100 µM), des nanoparticules non chargées ou ces dernières associées à la pentamidine (100 µM), [0.1 ml i.v. x 3 j.] de la pentamidine libre (2,28 mg/kg et 0.17 mg/kg, i.v. x 3 j.) et du sérum physiologique. Vingt et un jours après l'infection, les souris sont tuées et la charge parasitaire calculée à partir du nombre d'amastigotes observés pour 500 noyaux d'hépatocytes. Les résultats montrent une diminution de 77 % du nombre d'amastigotes dans le lot de souris traitées par la pentamidine vectorisée. Le rapport des D.E. pentamidine libre/pentamidine vectorisée est d'environ 12.

MOTS CLÉS : nanoparticules. *Leishmania*. souris. pentamidine.

INTRODUCTION

Leishmania are obligatory intracellular parasites of mononuclear phagocytic cells of man and other vertebrates. Because the mononuclear phagocytic system of liver, spleen and bone marrow are able to clear particles from the circulation visceral leishmaniasis has been considered the ideal disease for passive drug targeting.

The number of cases of leishmaniasis refractory to the usual treatment is now increasing (Croft *et al.*, 1991). This can be linked not only to the genetic nature of some strains, but also to the immunodepressed state of some patients (Croft *et al.*, 1991; Thakur *et al.*, 1991). The treatment with pentamidine is not fully effective and has toxic side effects, nevertheless it is used (Soto *et al.*, 1994). Colloidal drug carriers bound to pentamidine could enhance effi-

ciency and reduce toxicity. Recently, polyresistant visceral leishmaniasis was put in complete remission with the injection of liposomal amphotericin B (Croft *et al.*, 1991). Davidson *et al.* (1994) have reported on 31 cases treated with Ambisome®, in a multi-centre trial, without significant adverse events and without relapse during 12-14 months of follow-up. Colloidal (e.g. liposomes, nanoparticles) drug carrier that can entrap antimony derivatives is not available, but targeted pentamidine can be used after linkage to methacrylate polymer nanoparticles (Paul, 1990). The targeted drug principle using nanoparticles has been applied successfully with antineoplastic drugs (Astier *et al.*, 1988; Chiannikulchai *et al.*, 1989) or antibiotics (Fattal *et al.*, 1989). Furthermore, another kind of nanoparticles prepared from biodegradable polyisobutylcyanoacrylate polymers, has shown *in vitro* activity against African trypanosomes (Lherm *et al.*, 1987) and the extracellular *Leishmania* amastigotes (Bories *et al.*, 1991). This property was tested in our laboratory within an *in vitro* model using the monohistiocytic line U 937 and *Leishmania* major amastigotes. In this model, pentamidine-loaded polymethacrylate nanoparticles were twenty-time more active (Deniau *et*

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al., 1993) than free pentamidine. The aim of this study is to extend these observations to an *in vivo* model.

MATERIAL AND METHOD

The strain of *Leishmania* was isolated in Portugal and identified by Pr Rioux (Montpellier, France) as *Leishmania major*, MON 74 (WHO reference : MHOM/PT/92/CRE26). The strain is maintained in hamsters or NNN-medium but was cultivated, for this study, in RPMI-1640 medium (Eurobio, France), buffered with bicarbonate, containing 20 % heat-inactivated fetal calf serum (D.A.P., France), 20 % Schneider-medium (Schneider's drosophila medium, Gibco Ltd, U.K.), 1 % L-glutamine (Biomérieux, France) and antibiotics. These promastigotes reached their metacyclic phase after an 8-day period, at 27°C. This cutaneous strain develops a visceral infection regularly in the hamster when infected by the intraperitoneal route.

ANIMALS

Female BALB/c mice (18 ± 2g) were purchased from IFFA CREDO (L'Arbresle, France). Infection with *Leishmania*, via the tail vein, was done on day 0. The mice were then randomly divided into seven different groups.

DRUGS

Methylglucamine antimoniate (Injectable meglumine antimoniate, Glucantime®, Rhône-Poulenc Rorer, France) was used as the reference antileishmanial drug. Mice were dosed intraperitoneally at 200 mg/kg (Sb^{v+} 56.6mg/kg) a day over a five-day period.

Pentamidine isethionate (Pentacarinat®, Roger Bellon, France) diluted in physiological saline was dosed intravenously at 2.28 mg/kg (expressed in pentamidine base) or 0.17 mg/kg. This last dose corresponded to the quantity loaded on the nanoparticles. The methacrylate nanoparticles were prepared using an emulsion polymerization technique (Hansen *et al.*, 1982). They were loaded with pentamidine solution (1mM) in buffer saline solution at pH 7.2, at the Henri Mondor Hospital Pharmacy. Unloaded, they were 270 nm in diameter, but in phosphate buffered solution their size increased slightly to 330 nm (measured with a Nanosizer, Coultronic, France). A 1 ml nanoparticle suspension contained 17 µg of pentamidine base bound on 2 × 10¹¹ nanospheres, by an ionic process involving the free carboxylic groups of polymer. At this concentration, under stirring and at room temperature, a 100 % binding was immediately obtained.

The loaded and unloaded nanoparticles were injected via the tail vein.

The method for treating experimental visceral leishmaniasis in mice was an adaptation of the model of Neal *et al.* (1985). The different groups are presented in Table I.

INOCULATION OF BALB/C MICE

The infective promastigotes were centrifuged at 700 g for 10 min. The pellet was resuspended in normal saline. It was homogenized and adjusted to 4×10⁷ promastigotes in a volume of 0.1 ml.

The injection was strictly intravenous, via the tail vein.

In this model, 12 days after initial infection, visceralization was obtained. Drug dosing was performed on days 13, 15, and 17 (days 13, 14, 15, 16, and 17 for the Glucantime®).

At day 21, after initial infection, the animals were killed by cervical dislocation. Mice were weighed, livers and spleens were removed and also weighed. Smears were prepared from livers and spleens. A liver sample was prepared for an electronic microscope study.

After Giemsa staining of the smears, the liver parasite burden (I) was calculated from the number of amastigotes/500 hepatocytes and related to the liver weight (P in mg), following the Stauber formula (Stauber L.A. *et al.*, 1958).

$$I = \frac{A \text{ (number of amastigotes)}}{N \text{ (number of hepatocytes)}} \times 2 \times 10^5 \times P$$

The parasite suppression (Y) was calculated from the ratio of the mean amastigote counts in drug treated groups to the mean amastigote counts in untreated control group (100 % of the parasite burden).

$$Y = 100 \left(1 - \frac{\text{Parasite burden of the treated mice}}{\text{Parasite burden of the control mice}} \right)$$

STATISTICAL ANALYSIS

Since data did not show equal variances (F-test) and since a gaussian distribution was not demonstrated for all experimental groups, the non-parametric Mann-Whitney U-test was routinely used to test the null hypothesis. Alternatively, equal variances and normal distribution were obtained after log transformation of the data, thus permitting the use of t-test and variance analysis (N.T. BAILEY, 1992). Similar significance levels were obtained using both procedures. A two-tailed P value < 0.05 was considered as significant.

Group [#]	Treatment	Drug and dosage [†]
1	Infected control	Normal saline 0.1 ml × 3d., i.v.
2	Glucantime	Methylglucamine antimoniate 200 mg/kg × 5 d, i.p.
3	Pentamidine-loaded nanoparticles [*]	Pentamidine isethionate 0.17 mg/kg × 3d., i.v.
4	Drug-free nanoparticles [*]	–
5	Drug-free nanoparticles [*] + pentamidine [§]	0.17 mg/kg × 3 d., i.v.
6	Pentamidine	Pentamidine isethionate 2.28 mg/kg × 3 d., i.v.
7	Pentamidine	Pentamidine isethionate 0.17 mg/kg × 3 d., i.v.

Table I – Experimental treatment groups

[#] Each group contained 10 mice except 12 mice for group 3.

[†] Pentamidine dosages were expressed in base.

^{*} Administered polymer was 12.5 mg/kg × 3 d. throughout.

[§] Prepared extemporaneously into syringe by mixing unloaded nanoparticles suspension and pentamidine isethionate solution.

RESULTS

Following infection with 4×10^7 promastigotes, *L. major* MON 74 kills BALB/c mice within two months. The parasite burden reached 13×10^8 per liver after 12 days and remains at this level until death. This development allowed time to test for drug suppression of parasites. Tail necrosis appeared after a 7-day period if the injection is out of the vein. This cutaneous leishmaniasis delays and diminishes the visceralization. The mice with a point of necrosis on their tail were discarded from this study.

After necropsy, the liver parasite burden in infected control group was, on average, 12.94×10^8 amastigotes (Table II). As expected, the parasite burden was 86 % reduced in mice treated with methylglucamine antimoniate (200 mg/kg × 5 d.; $P = 0.0002$ vs untreated mice). Free pentamidine was also efficient since the mean suppression was 35 % at 0.17 mg/kg and 52 % at 2.28 mg/kg × 3d. ($P = 0.003$ and 0.023 respectively vs untreated group). However, the difference between the two regimens ($P = 0.975$) was not significant. The parasite suppression was higher when mice were treated with pentamidine-bound nanoparticles than with pentamidine alone (77 % vs 35 %, $P = 0.0003$). However, pentamidine-bound nanoparticles were less efficient than Glucantime® (86 % versus 77 %, $P = 0.0026$). Unloaded nanoparticles had no effect since the amastigote count was similar to the control (group 1 vs group 4 $P = 0.14$). When free-pentamidine and nanoparticles were mixed just before administration, amastigote counts were close to those obtained with pentamidine-bound nanoparticles but a large variation was observed between animals (Table II).

Preliminary toxicological evaluation of nanoparticles in mice suggested that the carrier had no action on the cells at an acute intravenous 50 % lethal dose of 720 mg/kg of polymeric compound.

In mice treated with loaded pentamidine, parasite suppression was observed in bone marrow and spleen, but not quantified.

DISCUSSION

The aim of this study was to compare the action of free pentamidine with the action of pentamidine targeted on polymethacrylate nanoparticles and to confirm *in vivo*, the result previously obtained *in vitro* (Deniau *et al.*, 1993).

It followed two preliminary studies. First, the interactions between intracellular amastigote *Leishmania* and unloaded or pentamidine-loaded nanoparticles were studied on an *in vitro* model using a strain of *L. major* MON 25 (IPAD/MA/86/LEM898) and the monocyte line U 937. In this model, a twenty-time enhancement of pentamidine efficiency was observed after targeting (Deniau *et al.*, 1993). The same results were obtained by using a different strain, *L. major* Mon 74 (MHOM/PT/92/CRE26). Second, an *in vivo* model using BALB/c inbred mice and the strain of *L. major* MON 74 was established which produced a rapid visceralization of infection, instead of cutaneous leishmaniasis (Fusai *et al.*, 1993). This strain was chosen in our study because of its fast and regular visceralization when injected into a hamster by the i.p. route.

The model was based on the models created by Neal *et al.*, (1985) for their chemotherapeutic studies on visceral leishmaniasis. The main difference was the use of stationary promastigotes instead of amastigotes obtained from the spleen of hamster to infect the mice.

The mice treated with a 2.28 mg/kg pentamidine regimen had immediate side effects after the injection of free pentamidine, but did not die. The group receiving nanoparticles loaded with 0.17 mg/kg of pentamidine (group 3), showed a homogeneous result, the reduction of the parasite burden was 77 % and was comparable to that of the observed reference drug (the ratio ED free-pentamidine/ ED bound-pentamidine is approx. 12). Moreover, at this dose of bound-pentamidine, there was no evidence of shock after the injection.

The nanoparticles were loaded with 100 µM of pentamidine suspension. Using a higher pentamidine

Group	Weigth (grams)				Amastigotes count per 500 hepatocytes	Stauber count (10 ⁸)	Percent of suppression (mean)
	Body		Organ (wet)				
	Initial	Final	Liver	Spleen			
1	17.7 ± 1.7	18.5 ± 2.1	1.37 ± 0.24	0.28 ± 0.06	2556 ± 840	12.94 ± 3.50	0
2	17.3 ± 0.9	19.4 ± 1.3	1.25 ± 0.19	0.27 ± 0.06	366 ± 207	1.85 ± 1.21	86**
3	17.0 ± 0.1	19.1 ± 0.3	1.21 ± 0.15	0.28 ± 0.07	655 ± 187	3.09 ± 0.99	77**
4	18.0 ± 1.8	19.6 ± 1.6	1.35 ± 0.26	0.39 ± 0.19	2078 ± 456	11.28 ± 3.30	14§
5	17.4 ± 1.5	19.0 ± 1.7	1.20 ± 0.19	0.25 ± 0.11	976 ± 1036	5.20 ± 5.66	60*
6	17.6 ± 2.2	18.7 ± 2.5	1.14 ± 0.20	0.29 ± 0.11	1338 ± 797	6.26 ± 4.27	52*
7	17.0 ± 0.1	19.1 ± 0.6	1.34 ± 0.08	0.27 ± 0.02	1581 ± 693	8.40 ± 4.50	35*§

Table II – Suppression of experimental leishmaniasis in mice treated with free pentamidine and pentamidine bound-nanoparticles. The suppression percentage was calculated using the Stauber count vs. untreated mice (group 1) as described in the experimental section. Data are mean ± standard deviation for 10 mice per group, except group 3 (n = 12).

* P < 0.01 vs. untreated mice (group 1); ** P < 0.001 vs. untreated mice; § P < 0.001 vs. pentamidine-nanoparticles treated mice (group 3)

concentration, the nanoparticles would not be loaded homogeneously. *In vitro*, the efficiency of 50 µM pentamidine loaded nanoparticles was reduced despite an increase of the number of loaded nanoparticles.

CHOICE OF THE PARTICULATE DRUG CARRIER

Intracellular sustained-release form is an interesting concept for treatment of mononuclear phagocytic system parasites.

Various drug carriers have already been tested, all of which have advantages and disadvantages.

Croft *et al.* (1991) using liposomes loaded with amphotericin B (Ambisome®) obtained good results both *in vitro* and *in vivo* and in man.

Various colloidal drug carriers have also been tested. Cyanoacrylate-based nanoparticles are biodegradable, which is an important consideration for a product injected intravenously. However, *in-vitro*, they are often toxic to mammal cells (Deniau *et al.*, 1993). Unloaded polyisooalkylcyanoacrylate nanoparticles have a specific action on Kinetoplastidae e.g. *Trypanosoma brucei* (Lherm *et al.*, 1987), *Leishmania donovani* (Gaspar *et al.*, 1992; Bories *et al.*, 1991). They have been linked with primaquine, doxorubicine and dehydroemetine, in order to destroy intracellular *Leishmania* (Rodrigues *et al.*, 1994; Fouarge *et al.*, 1989).

Biodegradable polymer (polylactide) and copolymer (glycolactide) have a number of free carboxylic groups lower than polymethacrylate ones and the pentamidine-binding yield is lower. A previously binding study of pentamidine with these biodegradable polymers was realized, only 20 % of the dose (100 µM) was bound (unpublished data).

Polymethacrylate nanoparticles are slowly biodegradable (Astier *et al.*, 1988; Paul, 1990), but they are harmless. When unloaded, they have no leishmanicidal activity. They are entrapped by the parasited cells, but their presence in the cells did not seem to have any effect on parasite load, as was confirmed in this experiment.

Previous studies have shown that these nanospheres exhibited methacrylic acid residues on their surface which could form ionic bonds with pentamidine. This stoichiometric binding is stable above pH 6.5. In the phagolysosome the pH is about 5.5. Consequently, after phagocytosis pentamidine could be released from its carrier inside the lysosome. This quality is particularly interesting in the case of leishmaniasis and electron microscopy has demonstrated the uptake of nanospheres by parasitised cells (unpublished data).

HYPOTHESIS ON THE MECHANISM OF ACTION

Antileishmanial activity was obtained in our model with a dose of targeted pentamidine twelve-time less than the usually prescribed dose. However, the nanoparticles mode of action remains unclear.

The nanoparticles loaded with leishmanicidal molecules can be efficient through the releasing of drugs on the target, which is the aim of targeting.

The pentamidine, after release inside the cells, could act on the mitochondrion and the kinetoplast of the parasite. The hypothesis of penetration of loaded carriers through the flagellar pocket is not possible in case of methacrylate polymer because of the big size of nanospheres.

A study in electronic microscopy has shown that *in vitro*, methacrylate polymers enter the U 937 cells and that they enter the parasitised cells too. But no particles have been seen inside *Leishmania* (Durand R., 1992). First electron microscopy results performed *in vivo* seem to assess this (unpublished data).

Efficiency of methylglucamine antimoniate was not surprising. This result was very close to those obtained by other authors and confirmed the validity of this model. Meglumine was slightly more efficient when tested on this model, using *L. donovani* LV9 (the parasite burden decrease reached 99 %).

Results obtained with free pentamidine were better than in previous published experiments. Our strain was perhaps more susceptible than others. Trotter in 1980 had observed that pentamidine was not really

efficient against experimental murine visceral or cutaneous leishmaniasis, because she suspected that her model was biased, and so she could not draw any conclusions. Moreover, the mode of administration was subcutaneous.

Our results confirmed that particular unloaded nanoparticles are neither active nor toxic.

The most interesting results were the efficacy of pentamidine-bound nanoparticles which approached the efficacy of Glucantime®.

In the group receiving both free pentamidine and unloaded nanoparticles, a relative efficacy was observed. Considering the immediate and easy binding of an ionic linkage, it was probable that an incomplete linkage occurred during the mixing as suggested by the strong variability of the amastigote count.

CONCLUSION

This study emphasized the potential of colloidal drug carriers. They have already proved to be useful in antineoplastic therapy and after possible improvement, in the therapeutic field of leishmaniasis resistant to pentavalent antimonial drugs.

It will be useful to test this rodent model with other strains of *Leishmania*, to study the mode of action of these drug carriers, to improve the technique in order to validate the drug carriers therapeutic interest and to determine *in vivo* the effective dose ED 50 and ED 90.

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