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# COMMUNICATIONS

## Biodegradable microspheres‖: polyacryl starch microparticles as a delivery system for the antileishmanial drug, sodium stibogluconate

A. J. BAILLIE*, G. H. COOMBS†, T. F. DOLAN, C. A. HUNTER†, T. LAAKSO‡§, I. SJÖHOLM§, P. STJÄRNKVIST‡§, *Department of Pharmacy, University of Strathclyde, Glasgow G1 1XW, UK, †Department of Zoology, University of Glasgow, Glasgow G12 8QQ, UK, ‡Department of Pharmaceutical Biochemistry, Biomedical Centre, University of Uppsala, S-751 23 Uppsala, and §Division of Pharmacy, National Board of Health and Welfare, S-751 Uppsala, Sweden*

Liver parasite burdens of *Leishmania donovani* in the mouse have been determined after treatment with intravenous administration of sodium stibogluconate in the free or carrier form. The carrier form, in which the drug was covalently bound to polyacryl starch microparticles, was up to 100× more effective than the free form in this murine model of visceral leishmaniasis. Empty microparticles had no effect on liver parasite burdens and the enhanced in-vivo antileishmanial activity of the carrier form of the drug was apparently due to passive drug delivery to the infected liver.

Visceral leishmaniasis is probably the archetypal intracellular infection. The cells which act as host to the amastigote forms of the parasite in this disease are mainly the fixed or tissue macrophages of the liver, spleen and bone marrow. As an almost exclusive infection of the reticuloendothelial system (RES), visceral leishmaniasis is ideally suited to carrier-mediated therapy which takes advantage of the often unwanted passive targeting of the carrier to liver and spleen. Several workers have described the efficacy of liposomal drugs against visceral leishmaniasis in animals (for review see Alving 1983) and we (Baillie et al 1986) recently showed that non-ionic surfactant vesicles, niosomes, could also act as vectoring agents in an animal model of the disease.

Microspheres prepared from various macromolecules are also attractive as carriers for delivering drugs to the RES. Polyacryl starch microparticles, prepared by radical polymerization of acryloylated starch in a water-in-oil emulsion (Artursson et al 1984a), have been shown to be capable of delivering entrapped enzymes to the RES (Artursson et al 1984b) and have been studied in-vitro as carriers for low molecular weight drugs (Laakso et al 1987).

We now describe the use of polyacryl starch microparticles as a carrier system for sodium stibogluconate therapy of murine visceral leishmaniasis. This is the first time that such microparticles have been used against experimental leishmaniasis.

* Correspondence.

‖ Part IX of a series.

### *Materials and methods*

*Materials.* Sodium stibogluconate (Pentostam) equivalent to 0.32 mg Sb $mg^{-1}$ was a gift from the Wellcome Foundation, UK. Soluble starch (maltodextrin, mol wt = 5000) was a gift from Dr Lars Svensson, Stadex AB, Malmö, Sweden. Acrylic acid glycicidyl ester and *N*-t-butyloxycarbonyl-1, 6-diaminohexane, were obtained from Fluka AG, Switzerland, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide from Sigma, UK, and *N*-hydroxysuccinimide and *N,N,N′,N′*-tetramethylethylene diamine from Merck, FRG. All other reagents were of analytical grade.

*Synthesis of* N-*acryloyl-1,6-diaminohexane (ADH).* The synthesis was essentially as described by Stahl et al (1978). In a total volume of 60 mL chloroform, 3 g *N*-t-butyloxycarbonyl-1,6-diaminohexane (11·7 mmol) was reacted with 1·2 mL acrylic acid chloride (13·8 mmol) in the presence of 1·65 mL triethylamine (11·9 mmol) at −5 to −10 °C with stirring. The reaction mixture was then allowed to reach room temperature (20 °C) and washed 4 times with 60 mL water. After evaporation of the chloroform the *N*-t-butyloxycarbonyl-*N*-acryloyl-1,6-diaminohexane was recrystallized from toluene (yield, 70%; m.p. 109–110 °C uncorrected).

ADH was obtained with a yield of 94% on removal of the *N*-protecting group by treatment with 40 mL 1 : 1 mixture of 3 M HCl and ethylacetate followed by trituration with ether. The structure was confirmed by NMR.

*Preparation of ADH-polyacryl starch microparticles.* The general procedure followed that of Laakso et al (1986). Purified acryloylated starch (0·12 acrylic groups/glucose residue) and ADH were dissolved in 5 mL 0·2 M pH 8·5 phosphate buffer containing $10^{-3}$ M EDTA.

After addition of 0·08 M ammonium peroxydisulphate, the aqueous phase was emulsified in 300 mL chloroform–toluene (1 : 4) with 32 μM Pluronic F68. Monomer in the aqueous phase of the w/o emulsion was polymerized by addition of 0·1 mL *N,N,N′,N′*-tetramethylethylenediamine. $N_2$ was bubbled through the system throughout the procedure.

Microparticle composition can be characterized by the D-T-C nomenclature (Edman et al 1980) which denotes the quantity of component in the monomer solution used for microparticle formation, where D is acryloylated starch concentration (g/100 mL), T is total acryloyl groups (g/100 mL) and C the proportion (% w/w of acrylic monomer) of cross-linker present. D-T-C of the ADH polyacryl starch microparticles was 10-1·5-0 (with ADH making a 66% contribution to total T) and 10-0·5-0 for empty microparticles.

*Coupling of sodium stibogluconate to ADH-polyacryl starch microparticles.* 50 mg ADH microparticles and 200 mg sodium stibogluconate were mixed in 10 mL 1 M pH 8·0 $NaHCO_3$ buffer. 5 mL of the same buffer containing 100 mg *N*-hydroxysuccinimide and 250 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were added and the whole suspension stirred overnight at room temperature. Unreacted drug and coupling reagents were removed by centrifugal washing in normal saline.

*Antimony determination.* Flame atomic absorptiometry (Pye Unicam SP90 atomic absorptiometer) was used to determine the antimony content of the microparticles. The antimony standard was a solution of $SbCl_3$ in dilute HCl. Method sensitivity was 1 μg Sb $mL^{-1}$.

*Microparticle size determination.* The size of the microparticles was determined by photon correlation spectroscopy using a Malvern Instruments Type 7027 correlator with 60 channels in conjunction with a He/Cd laser (Linconix) operating at 441·6 nm with a power output of 10 mW. The samples were measured in normal saline at 25 °C and at an angle of 90° to the incident beam.

*Parasites. Leishmania donovani* (strain L 82, obtained from London School of Hygiene and Tropical Medicine) was maintained by passage through female 10–14 week old Golden Syrian hamsters (*Mesocricetus auratus*). A suspension of *L. donovani* (0·4 mL, $2 \times 10^8$ amastigotes) was administered intraperitoneally to the hamsters which were killed some 8–14 weeks after inoculation, the infected spleen being removed and homogenized in PSGEMKA buffer (Hart et al 1981). The released amastigotes were washed and resuspended in PSGEMKA buffer and used to infect further hamsters or to infect mice by tail vein inoculation. Mice were given 0·2 mL parasite suspension containing $2 \times 10^7$ *L. donovani* amastigotes.

*Parasite clearance studies.* Female Balb/c mice (Department of Zoology, University of Glasgow) of about 20 g with free access to food and water were used. Mice were infected with *L. donovani* as described above on day 1. With 5 to 6 mice per treatment group, drug was administered in a 0·2 mL volume via the tail vein on days 7 and 8. The mice were then killed on day 14 and multiple liver impression smears prepared and stained using Giemsa's stain. The parasite burden was assessed by counting ≤2 smears and expressed as the number of parasites per 100 host cell nuclei. Two batches of the stibogluconate microparticles were used, one containing 8·4 μg Sb (mg beads)$^{-1}$ and the other 3·3 μg Sb (mg beads)$^{-1}$. Each preparation was diluted with normal saline or concentrated by centrifugation to give the required Sb concentration before injection. Doses of microparticulate stibogluconate used were equivalent to 12, 9, 6, 3 and 1·5 μg Sb per mouse. These represent doses of microparticles in the range 3·64–0·18 mg per mouse.

'Empty' microparticles, containing no drug, were also administered to infected mice. Polyacryl starch microparticles were given to groups of 4 mice at two dose levels, 3·64 and 1·82 mg microparticles per mouse. These quantities are the equivalent of antimony doses of 12 and 6 μg per mouse, respectively, in the form of the low entrapment efficiency microparticles (3·3 μg Sb $mg^{-1}$). ADH polyacryl starch microparticles were given at a dose of 1·82 mg microparticles per mouse.

## Results

The hydrodynamic diameter of the microparticles was found to be 1915 nm ± 95 (s.d.) with a polydispersity factor of 0·43. This diameter is based on a z average diffusion coefficient (Koppel 1972) and is weighted towards larger particles.

Liver parasite burdens after dosing with microparticulate stibogluconate are shown in Fig. 1. Control, undosed, liver burdens were different in the two experiments, 197 parasites/100 host cell nuclei (Fig. 1a) and 352 parasites/100 host cell nuclei (Fig. 1b), but for both levels of infection it is apparent that there was an antimony dose-dependent decrease in parasite burden. The effect of the antimony dose can be expressed in terms of the suppression (%) of parasite burden relative to the controls (Fig. 2). Calculated in this way it can be seen that the two stibogluconate bead preparations tested had equivalent activities, showing the importance of antimony dose rather than efficiency of bead loading with stibogluconate. Extrapolation to 100% suppression (Fig. 2) gives an antimony dose of some 14 μg/mouse. The dose for 50% suppression was found to be about 6 μg microparticulate antimony/mouse.

Free stibogluconate was found to be much less active than the carrier-bound form (Fig. 3). 100% suppression was achieved only when a total dose of some 1500 to 2000 μg antimony/mouse was used with the dose for 50% suppression being about 400 μg/mouse. Thus in

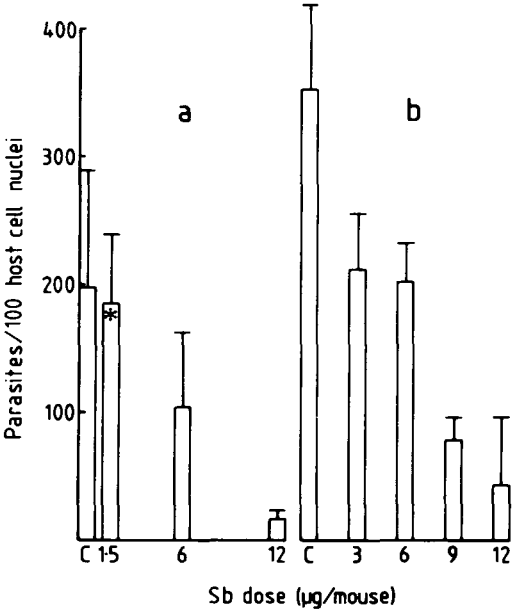


FIG. 1. Histogram of the number of *L. donovani* amastigotes per 100 liver cell nuclei obtained by counting Giemsa stained liver impression smears. Mice were infected on day 1 with $2 \times 10^7$ amastigotes and dosed with the microparticulate form of stibogluconate on days 7 and 8, except for the group indicated * which was dosed only on day 7. The total dose given (antimony equivalents) is indicated in all cases. The microparticles contained (a): 8·4 μg Sb (mg beads)$^{-1}$, and (b): 3·3 μg Sb (mg beads)$^{-1}$, control animals (C) received no treatment on days 7 or 8. Parasite burdens shown are the mean for ⩽5 mice, each liver counted in duplicate, and the bars indicate standard deviations.

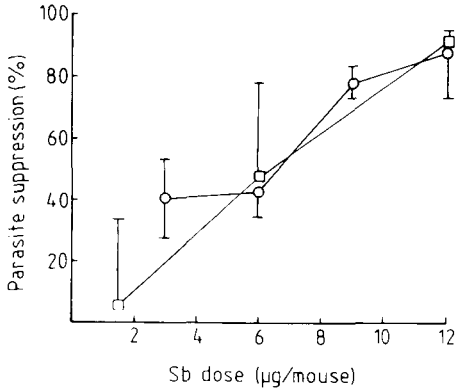


FIG. 2. The data of Fig. 1 plotted as the reduction in liver parasite burden, expressed as % suppression. Mice were dosed with microparticulate stibogluconate, ○—○, 3·3 μg Sb (mg beads)$^{-1}$ and □—□, 8·4 μg Sb (mg beads)$^{-1}$. Percentage suppression was calculated for individual mice relative to the mean parasite burden for the appropriate untreated control group. Mean parasite suppressions with standard deviations are shown.

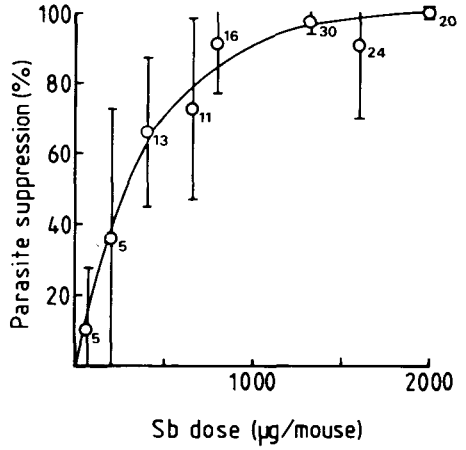


FIG. 3. Dose-response curve for free sodium stibogluconate solution administered i.v. Mice were infected on day 1 with $2 \times 10^7$ amastigotes and dosed with drug on days 7 and 8. The data points indicate the total dose given in terms of antimony equivalent. Liver parasite burdens in dosed animals have been expressed as a percentage of those in untreated control groups. All data points shown are mean values with standard deviations. The number of mice in each group is also indicated.

this model of visceral leishmaniasis, the apparent potency of the carrier form of the drug is up to 100× greater than that of the free form.

Neither empty polyacryl starch nor ADH-polyacryl starch microparticles gave evidence of an effect on liver parasite burden at the dose levels used. It would thus seem that the microparticles had no inherent antiparasitic activity in-vivo and that the parasite suppression shown in Fig. 2 is a function of the drug content of the microparticles. No toxic effects have been observed with stibogluconate-loaded microparticles.

*Discussion*

It would appear that the polyacryl starch microparticles behave as liposomes (review, Alving 1983) and niosomes (Baillie et al 1986) in increasing the efficacy in-vivo of the antileishmanial drug, sodium stibogluconate. Although we have not yet determined antimony tissue levels after dosing with the microparticulate form of stibogluconate, it seems a reasonable assumption that higher levels of drug are attained in the infected RES by using the carrier form rather than free drug. This is supported by the reported high uptake of this type of microparticle by the RES after i.v. injection (Laakso et al 1986). The fundamental function of the carrier is therefore probably one of simple passive vectoring, which achieves effective compartmentalization of the drug.

Differences in in-vivo behaviour between the monolithic microparticulate carrier and vesicular forms such as liposomes and niosomes might however be expected. The stibogluconate is covalently bound to the microparticle rather than simply entrapped within the aqueous

compartments of a vesicle and even after degradation of the microparticle some drug may remain covalently linked to the degradation products. Thus after lysosomal degradation of the microparticle, that proportion of the stibogluconate remaining covalently linked would be less liable than free drug to diffuse from the lysosomal compartment, so helping to maintain effective intracellular antimony levels for longer periods.

The evidence suggests that the microparticulate modified starch is a good carrier system for the therapy of this intracellular infection. The efficacy described here is, as far as can be judged within methodological constraints, as high as that obtained with liposomal delivery in similar animal models of visceral leishmaniasis (Alving 1983).

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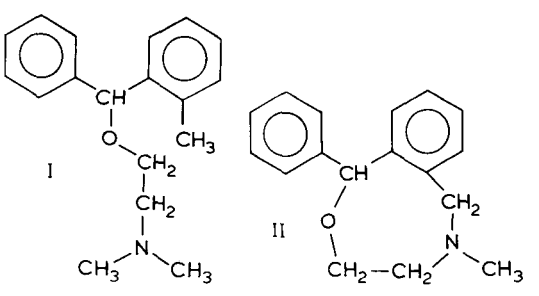
# Cytochrome P450 metabolic intermediate complex of nefopam

R. LEURS, D. DONNELL*, H. TIMMERMAN, A. BAST†, *Department of Pharmacochemistry, Vrije Universiteit, Boelelaan 1083, 1081 HV, Amsterdam, The Netherlands, *Riker Laboratories, 3M Health Care Ltd, Loughborough, UK*

NADPH-catalysed biotransformation of nefopam in liver microsomes obtained from phenobarbitone-pretreated rats leads to the formation of an inactive cytochrome P450 metabolic intermediate (MI) complex. This complex can be detected spectrophotometrically by an absorbance maximum at 459 nm. The extent of the in-vitro MI complexation of 33 μM nefopam, a cyclic analogue of orphenadrine, was almost equal to the extent of the in-vitro MI complexation of 33 μM tofenacine, the mono-*N*-demethylated metabolite of orphenadrine. The time course of the MI complexation of nefopam and studies with two of its major metabolites suggest an initial biotransformation, which has to occur before MI complexation can take place. Maximal MI complexation of nefopam occurred at approximately 25 μM, whereas the MI complexation could not be detected at 100 μM nefopam.

Unexpected accumulation of the antiparkinsonian agent, orphenadrine, in man has been shown under chronic dosing conditions (Labout et al 1982). Product inhibition due to the formation of a cytochrome P450 metabolic intermediate (MI) complex during orphenadrine biotransformation has been suggested to cause this accumulation (Bast et al 1983a). The MI complex is probably a nitroxide radical or a nitroso metabolite, which binds irreversibly to the reduced form of cytochrome P450. The complex can be detected spectrophotometrically by an absorbance maximum near 455 nm (Bast et al 1983a).




A previous study with several orphenadrine analogues revealed some structural features necessary for MI complexation. In particular it was shown that secondary amines gave rise to extensive MI complexation (Bast et al 1984). In this study the in-vitro MI complex formation during biotransformation in phenobarbitone-induced rat liver microsomes of the analgesic, nefopam, is reported. Nefopam (I) is a cyclic analogue of orphenadrine (II) and contains a ring tertiary amine function.

† Correspondence.