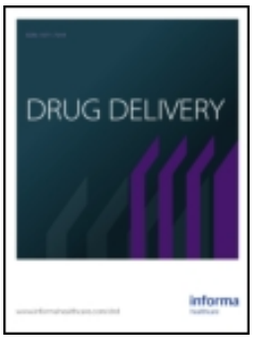

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Targeting of Liposomal Andrographolide to *L. donovani*-Infected Macrophages in Vivo

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Despite the rapid development in medicinal and pharmaceutical technology, the targeting of drugs to phagocytic cells in macrophage-related diseases still remains a major unsolved problem. By using the mannosyl-fucosyl receptors on macrophages, attempts were made to target antileishmanial drugs encapsulated in mannosylated or fucosylated liposomes to treat experimental leishmaniasis in the hamster model. Mannosylated liposomes were found to be more potent in delivering antileishmanial drugs to phagocytic cells. Liposomes loaded with an indigenous drug, andrographolide, a labdane diterpenoid isolated from Indian medicinal plant *Andrographis paniculata*, were prepared and tested against experimental leishmaniasis in a hamster model. Mannosylated liposomes loaded with the drug were found to be most potent in reducing the parasitic burden in the spleen as well as in reducing the hepatic and renal toxicity. In addition, mannosylated drug-loaded liposome-treated animals showed a normal blood picture and splenic tissue histoarchitecture when compared with those treated with free drug or regular liposomal drug. Such a drug-vehicle formulation may be considered for clinical trials.

Keywords Andrographolide, Drug Delivery, *Leishmania donovani*, Macrophages, Mannose-Grafted Liposomes

Despite the rapid development of medicinal and pharmaceutical chemistry in recent decades, chemotherapy is still a major challenge for in vivo drug targeting to macrophages to combat leishmaniasis (UNDP/World Bank/WHO 1987), a intracellular protozoan parasitic disease common in humans in rural areas in developing countries.

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Toxicity is the major obstacle in the therapy of leishmaniasis with the most potent drug, pentavalent antimonial (TDR News 1980). On several occasions it was ineffective to several unresponsive strains of *Leishmania* spp. Substitution of pentavalent antimonials by other drugs, such as pentamidine and amphotericin B, do not show effective results without any toxic syndrome (Utz et al. 1964). Hence, it is necessary to develop a delivery system effective for vectoring drugs to the reticuloendothelial system (RES) without drug toxicity (Medda et al. 1993).

By using the presence of mannosyl-fucosyl receptors on the surface of reticuloendothelial cells, mannose-coated liposomes (Banerjee et al. 1996) and liposomes with several self-targeting molecules (Medda et al. 1999) were found to be effective in the site-specific delivery of antileishmanial drugs to cells with an appreciable reduction of parasitic load in a experimental animal model. Very recently, liposomal oligonucleotides (Chakraborty et al. 1999; Chaudhuri 1997) and liposomal parasite antigen as vaccines (Afrin and Ali 1997) were found to be highly effective against the leishmania parasite.

Our study's purpose was to design an effective drug delivery system and to check the efficacy of an indigenous drug, andrographolide, a labdane diterpenoid (Fujita et al. 1984; Cava et al. 1965) extracted from the leaves of kalmegh plant, *Andrographis paniculata*, against an in vivo experimental model of leishmaniasis. The compound was found to be effective in curing various diseases and has a long history as an Ayurvedic drug or as an Ethnomedicine in India.

Andrographolide, being a natural compound, had less inherent toxicity. But even to minimize the residual toxicity, we investigated the efficacy of the drug in a liposomal system against an in vivo hamster model undergoing experimental leishmaniasis.

MATERIALS AND METHODS

Phosphatidylethanolamine (PE), cholesterol, dicetyl phosphate (DCP), *p*-aminophenyl-α-D-mannoside, glutaraldehyde, and Trypan blue dye were purchased from Sigma Chemicals

(St. Louis, MO). Andrographolide was extracted and purified by Dr. S. Mukhopadhyay of the Medicinal Chemistry Department of our Institute. All other reagents were of analytical grade.

Preparation of Liposomal Andrographolide

The multilamellar liposomes were prepared with PE, cholesterol, DCP, and andrographolide in molar ratio 7:1:1:1 as described by Gregoriadis and Ryman (1972). In short, PE, cholesterol, DCP, and andrographolide are taken in a molar ratio 7:1:1:1 in $CHCl_3$ and methanol solvent (2:1) in a round bottom flask. The lipid film was made by drying the organic solvents and was desiccated overnight. The thin dry film was swollen in PBS (pH 7.2) for 1 hr and sonicated for 30 sec. The suspension was centrifuged ($10{,}000 \times g$) for 30 min in Beckman Ultracentrifuge and washed twice in PBS. To estimate intercalated andrographolide, the pellet was dissolved in Triton X and an aliquot of 100 μl was taken, diluted in PBS to make 1 ml. The O.D. (optical density) was measured at 226.5 nm ($\varepsilon_m = 14 \times 10^3\ M^{-1}\,cm^{-1}$). Total andrographolide intercalation (%) was assessed from the concentration of the drug in the dissolved pellet divided by total amount of drug added during liposome preparation.

Coupling of *p*-Aminophenyl-α-D-Mannopyranoside with Liposomes Intercalated with Andrographolide

Andrographolide intercalated multilamellar liposomes were prepared using PE:Chol:DCP:andrographolide in a molar ratio 7:1:1:1 by method described earlier (Das et al.). After centrifugation, the washed pellet was dissolved in PBS (pH 7.2). The NH_2-group of PE liposomes was coupled with *p*-aminophenyl-α-D-mannopyranoside using glutaraldehyde as the coupling agent (Ghosh and Bacchawat 1980).

Trypan Blue Exclusion Test/Toxicity of Andrographolide on Macrophages

Peritoneal macrophages were isolated from ThG-injected Balb/c mice in RPMI 1640 medium containing 10% FCS. In each Eppendorf tube, containing 500 μl of macrophage suspension (5×10^6 cells), andrographolide was added in increasing amounts to prepare three concentrations: 50, 250, and 500 μg/ml. A separate set of untreated controls was also prepared. The macrophages were incubated with the drug for 1 hr at 37°C. A negative control was used with heat-killed macrophages; 500 μl of 0.4% Trypan blue solution was added to untreated, heat-killed, and drug-treated macrophages and incubated for 10 min. Percent viability was calculated on each set according to the following relationship:

$$\text{Cell viability (\%)} = \{\text{Total viable cells (unstained)}/\text{Total cells (stained + unstained)}\} \times 100$$

Animal Experiment

Our colony of golden hamsters (*Mesocristatus auratus*), originally from Haffkine Research Institute (Bombay, India), was used to maintain *L. donovani* strain AG83. Amastigotes were isolated from hamster spleens according to the Looker et al. method (1983) with some modification (Das et al. 1990). Each animal was injected intracardially with 2×10^6 amastigotes. A total of 12 animals were injected each time, and treatment was started 30 days postinfection. The animals were divided into four groups with three animals in each group for testing of drug efficacy with (i) drug-intercalated mannose-grafted liposomes, (ii) drug-intercalated regular liposomes, (iii) free drug and, (iv) no drug (infected untreated control). The dose given to each animal was 2.5 mg/kg body weight. For LD_{50} determination, the dose treatment used varying amounts—1.10, 2.20, 5.50, and 11.10 mg/kg body weight—given for 4 doses to each animal.

For chemotherapy, a multiple dose treatment was followed using hamsters of average body weight of 90–100 gm. In practice, 250 μg of andrographolide intercalated in 0.5 ml of liposomal suspension containing 3.3 mg of PE was injected subcutaneously into each hamster every 3 days for a total of 6 doses in 15 days. For free drug treatment, the same amount of andrographolide (250 μg/0.5 ml PBS) was injected each time. The animals were killed by cervical dislocation after 7 days from the last injection. The parasitic load in the spleen was calculated using the Stauber formula (Stauber et al. 1958).

$$\text{Total No. of amastigotes} = (\text{No. of amastigotes/host cell nucleus}) \times (\text{weight of spleen in mg}) \times 2 \times 10^5$$

Estimation of Drug Toxicity

Specific serum enzyme levels and splenic tissue histology were assessed to find out the drug toxicity. Serum alkaline phosphatase and serum glutamate pyruvate transaminase were measured (Bergmeyer 1963; Giubault 1976). Serum urea and creatinine levels were measured using a standard kit. The splenic histology was studied by microscopic examination of hematoxylin- eosin-stained sections (Gurr 1962).

RESULTS AND DISCUSSION

Toxicity of the drugs has proved to be a major problem in the chemotherapy of leishmaniasis. Toxicity in general could be due to two main reasons: the diffused delivery to noninfected tissues and the inherent toxicity of the drug molecules. The first line of drugs, the antimonials, are reported to be highly toxic (Baveridge 1963) with increased levels of various marker enzymes and heptomegaly (Marsden 1985), and other typical skin reactions of heavy metals (Sampio et al. 1980). The second line of drugs, pentamidine and amphotericin B, were effective against antimonial unresponsive cases and relapses (Bryceson 1970) but still proved to be highly toxic with cumulative effects of nephrotoxicity, hepatotoxicity, anemia, and many

other complex disorders (Walzer et al. 1974). In our study, we attempted to establish the efficacy of a drug-vehicle combination for chemotherapy of experimental leishmaniasis. The use of mannose-grafted liposomes as a drug delivery system was supported by the fact that intercalated drugs in the liposomes can be directed specifically to the reticuloendothelial systems through recognition of mannosyl-fucosyl receptors on the macrophage surface (Banerjee et al. 1996).

We used andrographolide, a labdane diterpenoid isolated from the indigenous plant *Andrographis paniculata.* The compound has a long history of various therapeutic properties such as antimicrobial, stimulant, liver protectant, and many other properties (Zhank and Tan 1997). The intercalation efficiency of andrographolide was found to be 46% in MLV liposomes.

The subcutaneous injections of infected hamsters with free andrographolide at intervals of 3 days in 4 different doses showed 100% survival. From the same treatment, the EC_{50} of the parasite (drug concentration needed to reduce the parasite load of spleen to 50%) was found to be 2.5 mg/kg body weight, calculated from the exponential curve drawn by plotting parasite burden in spleen against various drug doses (Figure 1).

The antileishmanial activity of the drug was tested in vivo against experimental leishmaniasis in the hamster model (Table 1). The results presented reveal the efficacy of andrographolide tested against experimental leishmaniasis in hamsters in three different forms: free, liposome-intercalated, and mannose-grafted liposome-intercalated forms. Highly improved efficiency was observed using mannose-grafted liposome with intercalated andrographolide, causing 86% reduction of splenic parasitic burden. In a separate experiment, in which the 8-dose treatment was followed, the mannose-grafted liposome-intercalated form reduced the splenic parasitic burden to the extent of 94% (data not shown).

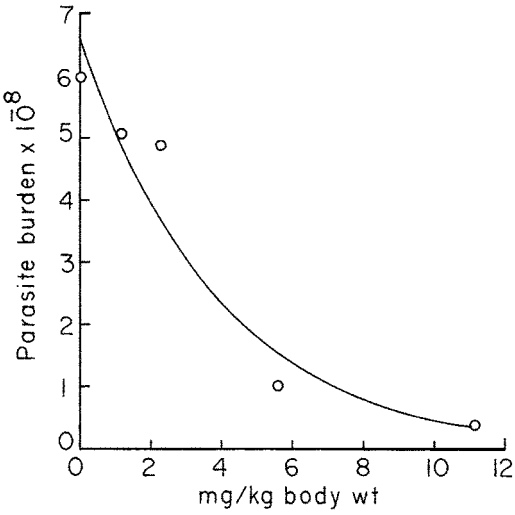

FIG. 1. Dose-dependent reduction of parasitic burden of spleen of hamsters undergoing experimental leishmaniasis.

TABLE 1
Effect of mannose-grafted liposome-encapsulated andrographolide in the 30-day infected hamster model undergoing experimental leishmaniasis

Group	Parasite load in the spleen $\times 10^8$	Percent suppression of spleen parasite load
Infected control	6.14 ± 0.78	—
Free-drug treated	3.86 ± 0.50	37
Liposome-intercalated drug treated	2.04 ± 0.19	67
Sugar-grafted liposome-intercalated drug treated	0.88 ± 0.05	86

The values are expressed as mean $\pm$ *SD* ($n = 3$).

Levels of alkaline phosphatase and glutamate pyruvate transaminase in serum were measured in different treatment conditions (Table 2). For free drug treatment, the level of alkaline phosphatase and serum glutamate pyruvate transaminase showed a significant increase, but reduced further and reached the normal level in animals when treated with regular liposome-intercalated and mannose-grafted liposome-intercalated drugs, proving the drug to be nonhepatotoxic in nature.

In assessment of nephrotoxicity, the levels of serum urea and creatinine were examined and compared (Table 3). Elevated levels of urea and creatinine were observed in free-drug treated animals and found to be nearly normal in animals treated either with regular liposomal or mannose-grafted liposomal drug, indicating a nonnephrotoxic nature.

TABLE 2
Effect of andrographolide on specific enzyme levels related to normal liver function in sera of hamsters undergoing experimental leishmaniasis

Groups	Serum glutamate pyruvate transaminase[a]	Alkaline phosphatase[b]
Infected control	72.14 ± 5.65	13.48 ± 0.62
Free-drug treated	98.18 ± 7.89	17.91 ± 9.62
Liposome-encapsulated drug treated	78.08 ± 8.74	12.00 ± 1.87
Sugar-grafted liposome-encapsulated drug	62.46 ± 13.18	10.81 ± 1.83

Values are expressed as mean $\pm$ *SD* ($n = 3$).

[a] μmol of sodium pyruvate released/min/L sera. Normal serum glutamate pyruvate transaminase was 49.6 ± 11.63 U.

[b] μmol of *p*-nitrophenol released/min/dL sera. Normal value of alkaline phosphatase was 10.77 ± 2.35 U.

TABLE 3
Effect of andrographolide on serum urea and creatinine levels related to normal kidney function of hamsters undergoing experimental leishmaniasis

Groups	Urea[a]	Creatinine[a]
Infected control	45.70 ± 2.33	0.61 ± 0.11
Free-drug treated	46.90 ± 0.48	0.75 ± 0.11
Liposome-encapsulated drug treated	37.46 ± 1.37	0.44 ± 0.03
Sugar-grafted liposome-encapsulated drug treated	30.14 ± 0.76	0.42 ± 0.03

Values are expressed as mean ± *SD* ($n = 3$).

[a]Values are expressed as mg/dL. Normal values of serum urea and creatinine are 26 ± 13.5 and 0.76 ± 0.21, respectively.

The drug also was nontoxic to the host macrophages when examined with the Trypan blue exclusion test. Results, when compared with untreated controls and heat-killed macrophages (100% mortality), showed negligible macrophage mortality on treatment with drug at concentrations of 50, 250, and 500 μg/ml.

Some positive histologic changes were observed in comparison with untreated infected controls. In untreated infected controls (Figure 2a), the hyperactivity of white pulp region was evident with an increase in the size of white pulp region. Monocyte migration as groups of 2–5 were seen in the splenic blood vessels. In free drug treatment (Figure 2b), healthy appearance of lymphocyte could be observed without any pycnotic appearance of nuclei. Red pulp region also was found to be healthy. Monocytes decreased significantly confirming a reduction in infection. In mannose-grafted liposome intercalated drug treated animals (Figure 2c), a normal and healthy appearance of red pulp and white pulp region could be seen. There was no appearance of monocyte migration, confirming a reduction of the infection.

A labdanic diterpene [(4S,9R,10R)-methyl-18-carboxy labda-8, 13(E)-diene 15 oate] (Figure 3B), isolated from *Polyalthia macropoda*, shows antileishmanial activity (Rhicomme et al. 1991). Close inspection of the structure of andrographolide (Figure 3A) and the labdenic diterpene reveals a very similar structural resemblance between the two molecules, both of which possess the same labdane diterpene skeleton with similar substitution pattern. The main difference between the two molecules is the presence of γ-lactone in andrographolide and that of an open chain ester in labdanic diterpene. Since both the compounds show antileishmanial activity, it is highly probable that the structural moiety containing ring A and ring B is critical for the biological activity. Again, the presence of lactone group, as in polyene antibiotics, may further increase the microbicidal property of the compound. Now, the chemical reduction of andrographolide results in deoxyandrographolide, containing double bonds at C_{10}–C_{14} positions in the lactone moiety, whereas mild oxidation of andrographolide yields a compound in which

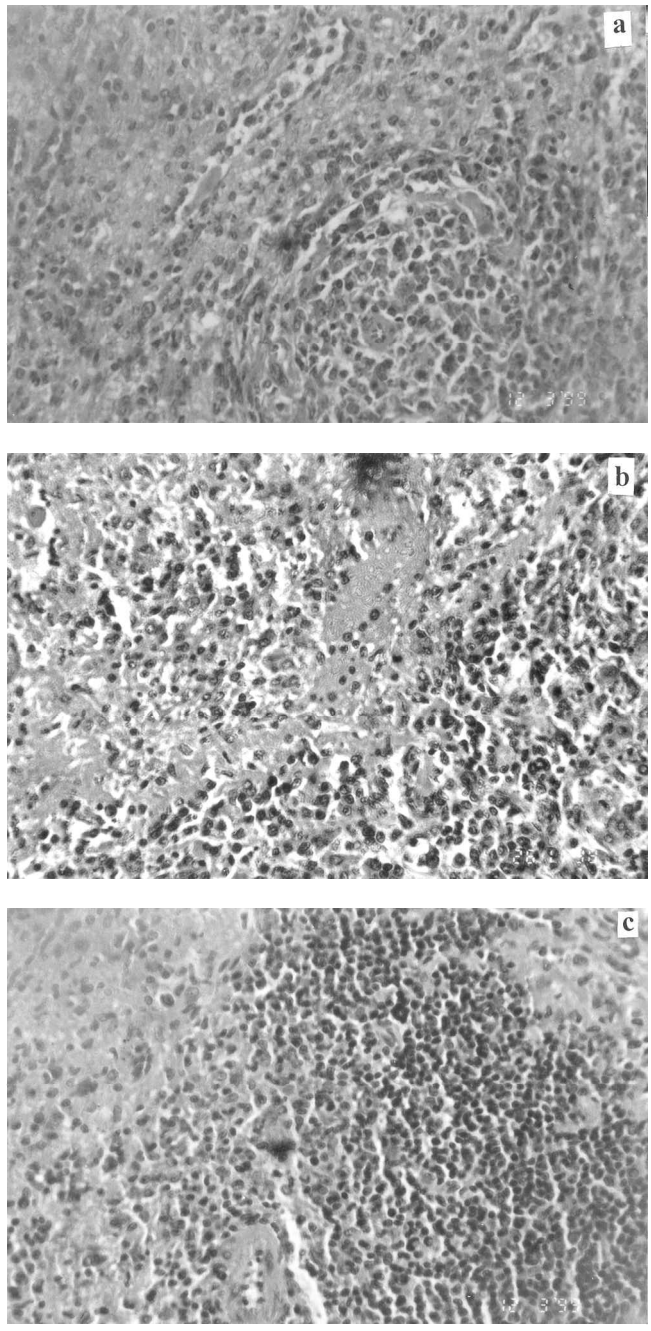

FIG. 2. Histopathologic examination of eosin–hematoxylin-stained splenic sections: (a) infected control; (b) free-drug treated; (c) sugar-grafted liposome-encapsulated drug treated.

-CH_2OH group at C_4 position changes into -CHO group that again converts into carboxylic groups on prolonged oxidation.

Comparing the structure of andrographolide (Figure 3A) and labdanic diterpenoid (Figure 3B), both of which are antileishmanial in nature, it appears that andrographolide changes into deoxyandrographolide in the presence of some reductases. Moreover, CH_2OH groups at C_4 position in andrographolide are oxidized to -COOH group with some oxidases present in the

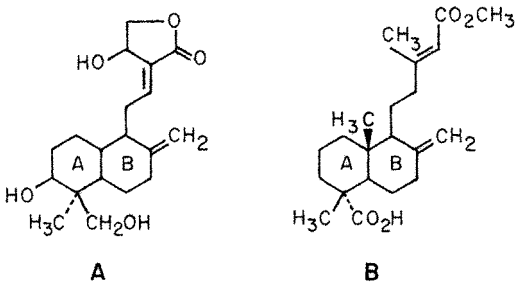

FIG. 3. Structure of andrographolide (A) and labdanic diterpenoid (B) showing homology in their "A" and "B" rings.

system. Moreover, andrographolide in acidic pH results in a compound containing dihydrofuranoid structure, but it is not clear yet whether the furanoid compound is the real active molecule. This hypothesis needs further verification by preparing the dihydrofuranoid molecule from andrographolide by successive treatments with oxidizing and reducing agents followed by acid treatment. The activity of these molecules were tested both in vivo and in vitro.

In addition, andrographolide and some other related and interconvertible compounds showed potent growth inhibitory activity (Matsuda et al. 1994) against M1-tumor cell line proving their cytostatic activity. Andrographolide and its derivatives may have similar cytostatic and growth inhibitory activity against *L. donovani* amastigotes, causing a reduction in splenic parasitic burden. Thus, being of indigenous origin and nontoxic to the host, liposomal andrographolide may have possible clinical application.

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