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Delivery *in vivo* of 14-deoxy-11-oxoandrographolide, an antileishmanial agent, by different drug carriers

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An antileishmanial compound, 14-deoxy-11-oxo-andrographolide, a derivative of andrographlide, isolated from the Indian medicinal plant *Andrographis paniculata* was evaluated for efficacy in free form and in different vesicular delivery modes on hamster model of Leishmaniasis. The subcutaneous injection of free drug reduced the spleen parasite load by 39%, whereas for drug incorporated in liposomes, niosomes and microspheres, reductions in the parasite load were 78%, 91% and 59%, respectively. Moreover, the drug in various delivery modes, particularly in liposomal and niosomal forms, showed no apparent immediate toxicity. Although an inverse linear relationship between the size of carriers and per cent efficacy in reduction of spleen parasite load was established, involvement of other factors such as drug release profiles or rates remains an open question. Because of greater efficacy and lesser toxicity, liposomal, niosomal and possibly microsphere-incorporated 14-deoxy-11-oxo-andrographolide might have clinical application to combat visceral Leishmaniasis.

Key words: *Andrographis paniculata,* antileishmanial compound, 14-deoxy-11-oxoandrographolide, drug carriers, *in vivo* delivery, liposomes, microspheres, niosomes

Leishmaniasis is a macrophage-associated parasitic disease, which affects an estimated 10 million people worldwide[1]. Chemotherapy of the disease is still a major challenge. The most potent drugs, the pentavalent antimonials are restricted in use due to their toxicity[2,3] and ineffectiveness against several strains of *Leishmania* spp., while the second line of drugs, such as pentamidine and amphotericin B have low efficacy with toxic side effects[4]. Therefore, it is desirable that new drugs as well as new delivery systems be developed to enhance the efficacy and to reduce the toxicity. Earlier, we have reported a study on andrographolide, a labdane diterpenoid[5,6] extracted from the leaves of an Indian medicinal plant *Andrographis paniculata (*Kalmegh plant*)*, against *in vivo* experimental model of Leishmaniasis[7]. The present study was designed to evaluate and compare the efficacy of its derivative, 14-deoxy-11-oxoandrographolide (Fig. 1) isolated from the same plant, in free form as well as in different delivery modes on hamster models of Leishmaniasis.

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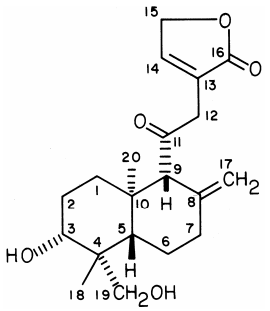

Fig. 1—Structure of 14-deoxy-11-oxoandrographolide

Materials and Methods

Egg phosphatidyl ethanolamine (PE), cholesterol, dicetyl phosphate (DCP), poly-lactide (PLA) and gelatin were obtained from Sigma Chemicals (St. Louis, MO, USA), *p*-nitrophenyl phosphate (PNPP), *p*-nitrophenol, DL-alanine and pyruvic acid from SRL (Mumbai, India), α-oxoglutaric acid, aniline and citric acid from Merck (Darmstadt, Germany), eosin and haematoxylin from Loba Chemie (Mumbai, India), 2,4-dinitrophenyl hydrazine from Aldrich (Milwaukee, WI, USA) and Sorbitan monopalmitate (Span 40) from Fluka Chemie AG (Switzerland). The drug 14-Deoxy-1-oxoandrographolide (purity 99.6%)

prepared from *A. paniculata* was supplied by the Medicinal Chemistry Department of our Institute.

Preparation of liposomal and niosomal 14-deoxy-11-oxoandrographolide

Multilamellar liposomes were prepared by the method as described earlier[9]. In short, PE, cholesterol and DCP were taken in the molar ratio of 7:2:1 and dissolved in a chloroform methanol mixture (2:1 v/v) in a round bottom flask. To the resulting solution 14-deoxy-11-oxoandrographolide (4 mg/20 mg PE) was added and dissolved. A thin lipid film was formed by evaporating organic solvents under nitrogen and was desiccated overnight. The thin dry film was swollen in 0.025 *M* phosphate buffered saline (PBS, *p*H 7.2) for 1 hr and sonicated for 30 sec. The suspension was ultracentrifuged at 10,000 × *g* for 30 min in a Beckman ultracentrifuge and washed to remove excess drug. For preparation of niosomes, a non-ionic surfactant sorbitan monopalmitate (Span 40) was used as a substitute for phospholipids. For preparation of niosomal 14-deoxy-11-oxoandrographolide, Span 40:cholesterol: DCP in molar ratio 1:1:0.1 were dissolved as before in chloroform:methanol (2:1 v/v) mixture. To it, the drug was added (40 mg/20 mg of Span 40). The dry film was swollen, sonicated and centrifuged as before[10,11].

To estimate the incorporated drug, total pellet for liposomal or niosomal drug was centrifuged out and supernatants were collected. Equal volume of methanol was added to a known volume of supernatant, and the optical density was measured at Z35 nm ($\in_m = 28.7 \times 10^3\ M^{-1}\ cm^{-1}$), using methanol as blank. Total drug intercalation (%) was determined from the total amount of drug added for interaction in liposomes or niosomes and the amount of unincorporated drug found in the supernatant. Again, the total amount of drug to be added was determined by taking increasing amounts of drug during preparation and selecting the amount at saturation level of incorporation. The incorporation of drug in liposomes and niosomes was about 10% and 1%, respectively. The amounts of the drug added for liposomal and niosomal preparations were determined on the basis of percentage of intercalation and the optimum dose, as mentioned hereafter.

Preparation of 14-deoxy-11-oxoandrographolide-incorporated microspheres

14-Deoxy-11-oxoandrographolide loaded microspheres were prepared by oil in water solvent evaporation. technique[12,13]. In short, 20 mg (approx.) PLA along with 4 mg drug (dissolved initially in minimum volume of DMSO) was dissolved in 2 ml of dichloromethane. This was gently added into 40 ml of 0.025 *M* PBS (*p*H 7.2) containing 1% gelatin as dispersing agent. The solution was emulsified by stirring on a magnetic stirrer for 2 hr, resulting in the evaporation of dichloromethane. The gelatin suspension was centrifuged at 12-14K rpm for 25 min and pellet was washed with PBS. The microsphere pellet was collected and resuspended in PBS.

The percentage of incorporated drug was determined by dissolving the lyophilized pellet in 2 ml DMSO and keeping for 3 days. An aliquot of 100 µl was taken and diluted with PBS to make 1 ml. The optical density was read at 235 nm and total drug incorporated was determined from the amount of drug in total pellet divided by total amount of drug added during microsphere preparation. The incorporation of drug in microsphere was found to be about 10%. The amount of drug used for microsphere preparation was determined on the basis of percentage of incorporation and optimum dose, as mentioned hereafter.

Animal Experiment

Our colony of golden hamsters (*Mesocristatus auratus*) originally from Haffkine Research Institute (Mumbai, India) was used to maintain *L. donovani* strain AG83 by intracardial passage every 6 weeks. Amastigotes were isolated from hamster spleens according to the method of Looker *et al.*[14] modified by Das *et al*.[15] Each animal was injected intracardially with 2×10^6 amastigotes.

To study dose response, 12 animals were infected, and treatment was started 30 days post-infection. They were divided into four groups with 3 animals in each and were subcutaneously injected with different doses of the drug — 1.25, 2.5. 4.75 and 9.0 mg/kg body wt, given six times to each animal at intervals of 3 days. The optimum mean therapeutic dose (MTD) was determined to be 2 mg/kg body wt. For chemotherapy, a total of 15 animals were infected each time and treatment started 30 days post infection as before. The animals were divided into five groups of three animals each, having average body wt of 90-100 g. A multiple dose treatment was followed. The animals of the five groups were treated as follows: i) no drug (infected untreated control); ii) free drug; iii) liposome-incorporated drug; iv) niosome-incorporated

drug and v) microsphere-incorporated drug. For free drug treatment, 200 μg of 14-deoxy-11-oxoandrographolide dissolved initially in minimum volume of DMSO and diluted to 0.5 ml with PBS was injected subcutaneously into each hamster every 3 days for a total of 6 doses in 15 days. Likewise, for liposomes, niosomes and microspheres incorporated drug treatement, 200 μg of drug incorporated in 10 mg PE or 10 mg Span 40 or 20 mg PLA, respectively, each suspended in 0.5 ml of PBS, was injected subcutaneously each time. The animals were killed by cervical dislocation after 7 days from the last injection. The splenic impression smears were taken on slides, fixed in absolute methanol and stained with Giemsa (1:4 v/v) before microscopic examination. The parasitic load in the spleen was calculated using the Stauber formula[16] :

Total no. of amastigotes = No. of amastigotes/host cell nucleus × (weight of spleen in mg) × 2×10^5.

Investigations on drug toxicity

The parameters such as spleen histology, specific enzyme levels related to normal liver function and biochemistry related to kidney function were considered for determination of drug toxicity. Fresh blood was collected from the hamsters just before sacrifice and sera were procured by centrifugation. Serum alkaline phosphatase and serum glutamate pyruvate transaminase (SGPT) were assayed, according to published protocols[17,18]. Briefly, for the assay of alkaline phosphatase, serum was incubated with *p*-nitrophenyl phosphate in alkaline buffer (*p*H 10.4) for 30 min at 37°C. The reaction was stopped with 0.02 *N* NaOH and absorbance was measured at 410 nm, to determine the amount of *p*-nitrophenol released.

For SGPT, the serum was incubated with phosphate buffer (*p*H 7.5) containing DL-alanine and α-oxoglutaric acid, for 30 min at 37°C. The mixture was further treated with aniline citrate for 20 min at 37°C, followed by 2,4-dinitrophenyl hydrazine hydrocholoride for 20 min. The reaction was stopped by adding 0.4 *N* NaOH and absorbance was measured at 520 nm. The amount of sodium pyruvate released was determined from a standard curve.

Urea and creatinine were assayed using standard kits (Preccugent, Pinnacle Marketing, Mumbai, India), for assessing nephrotoxicity. Splenic histopathology was examined from 5 micron microtome sections stained with eosin-haematoxylin.

Determination of carrier size by dynamic light scattering and micrometry

The average carrier diameters of the niosomes, liposomes and microspheres were determined through dynamic light scattering instruments of Otsuka Electronics, Japan, using Neon laser of $\lambda_{632.2}$ nm and taking measurements at 90° angle[19,20]. The parallel measurements for all the three carriers were also done through light micrometry for comparison.

Results

In vivo anti-leishmanial activity of 14-deoxy-11-oxoandrographolide

The single dose response curve of hamsters infected for 30 days with *L. donovani* and treated with free 14-deoxy-11-oxoandrographolide at doses of 1.25, 2.5, 4.75 and 9.0 mg/kg of body wt. is shown in Fig. 2. No death occurred over a period of 15 days with any dose. The optimum dose, as judged from reduction in parasite burden in spleen was 2 mg/kg of body wt., calculated from the exponential curve drawn by plotting parasite burden in spleen against various drug doses.

The effects of chemotherapy on hamsters infected for 30 days with *L. donovani* are shown in Table 1. Experiments were performed using the same equivalent drug concentration for free, liposomal, niosomal and microsphere-incorporated forms, viz. 2 mg/kg of body wt for each animal injected subcutaneously every 3 days for 6 doses. The SC injection of free drug reduced the spleen parasite load

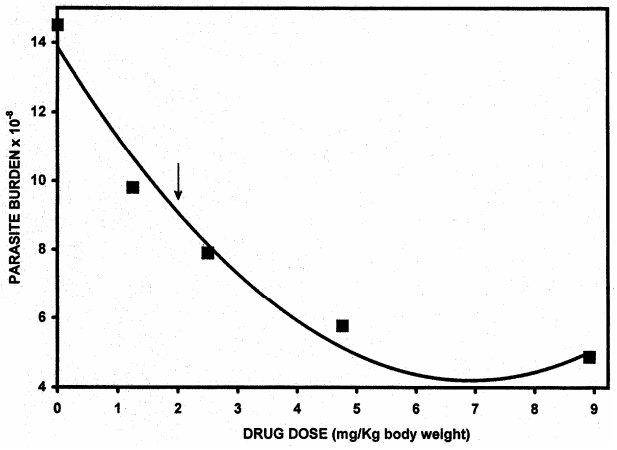

Fig. 2—Mean Therapeutic Dose (MTD) determination curve of 14-deoxy-11-oxoandrographolide, [MTD was assessed through single dose treatment using varying amounts viz. 1-10 mg/kg body wt; a therapy of six such doses were given through subcutaneous route at an interval of 3 days]

Table 1— Effect of 14-deoxy-11-oxoandrographolide on 30-days infected hamster models undergoing experimental Leishmaniasis

[Values are expressed as mean ± SD (n=3). Free drug was dissolved in minimum quantity of DMSO and diluted with PBS, to desired concentration. Percent suppression of spleen parasite load for empty carriers was found to be in the range of 12-15% at the most]

Group	Parasite load in spleen × 10^8	Suppression of spleen parasite load (%)
Infected control (no drug)	14.50 ± 1.16	
Free-drug treated	8.82 ± 0.72	39
Liposome intercalated drug-treated	3.13 ± 0.10**	78
Niosome-intercalated drug-treated	1.30 ± 0.15*	91
Microsphere- incorporated drug-treated	5.90 ± 0.08	59

*p<0.001, **p<0.002 compared to no drug control

Table 2 — Effect of 14-deoxy-11-oxoandrographolide on specific enzyme levels related to normal liver function in sera of hamsters models undergoing experimental Leishmaniasis

[Values are expressed as mean ± SD (n=3)]

Group	Serum glutamate Pyruvate transaminase[a]	Alkaline phosphatase[b]
Infected control (no drug)	71.80 ± 1.50	14.90 ± 0.15
Free-drug treated	111.65 ± 1.36	28.56 ± 3.14
Liposome intercalated drug-treated	74.08 ± 2.27	17.74 ± 0.38
Niosome-intercalated drug-treated	65.00 ± 2.27	19.66 ± 0.48
Microsphere- incorporated drug-treated	88.50 ± 1.82	24.96 ± 2.73

[a]μmol of sodium pyruvate released/min/L sera, normal value 49.6 ± 11.63, [b] μmol of *p*-nitrophenol released/min/dL of sera. Normal value of alkaline phosphatase was 10.77 ± 2.35]

by 39%, whereas for the drug incorporated in liposomes, niosomes and microspheres, reductions in the parasite load were 78%, 91% and 59%, respectively. Thus, the efficacy in lowering of spleen parasite load was in the following order: niosomal >liposomal > microsphere-incorporated drug.

Toxicity of 14-deoxy-11-oxoandrographolide

Hepatoxicity of the drug was assessed by the assay of serum alkaline phosphatase and SGPT. Both

Table 3—Effect of 14-deoxy-11-oxoandrographolide on serum urea and creatinine levels related to normal kidney function of hamsters undergoing experimental Leishmaniasis

[Values are expressed as mean ± SD (n=3)]

Groups	Urea[a] (mg/ml)	Creatinine[b] (mg/ml)
Infected control (no drug)	61.47 ± 3.41	0.48 ± 0.12
Free-drug treated	100.50 ± 7.93	0.58 ± 0.08
Liposome intercalated drug-treated	33.85 ± 5.26	0.48 ± 0.12
Niosome-intercalated drug-treated	33.22 ± 0.02	0.41 ± 0.08
Microsphere- incorporated drug-treated	68.20 ± 2.4	0.50 ± 0.05

Normal value: [a] 33.9±2.31; [b] 0.37 ± 0.06

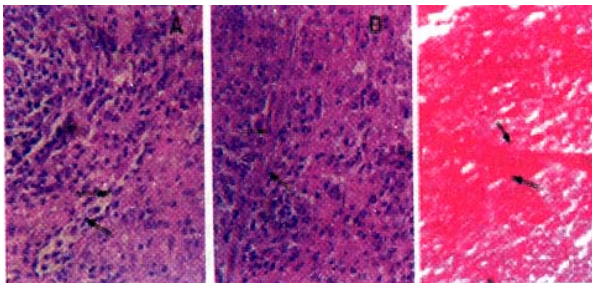

Fig. 3—Histological examination of microtome sections of spleen under different experimental conditions.[(**A**), Infected untreated control; (**B**), free drug treated; and (**C**), niosomal drug treated spleen (magnification × 400).Circulating monocytes in the sinusoids are shown with the arrows. No circulating monocytes in the sinusoid could be seen in (C) indicating reduced infection]

activities increased on free drug treatment, but decreased towards normal levels on treatment with drug incorporated microspheres, liposomes and niosomes in the same order as their efficacies (Table 2). Similarly, serum urea and creatinine, as indicators of nephrotoxicity increased significantly on free drug treatment, but reduced on treatment with liposomal, niosomal and microsphere incorporated drugs, simulating the earlier order (Table 3). These results indicate that three delivery modes used were successful in reducing both hepatotoxicity and nephrotoxicity of the drug again in the following order: niosomes > liposomes > microspheres.

Histopathological investigations have revealed positive changes in comparison to infected controls (Fig. 3). In infected controls, hyperactivity of white

pulp was indicated by its expanded size. The central vein was prominent, while the red pulp appeared regressed. The sinusoids were found to contain migrating monocytes, and the cell nuclei were moderately pycnotic. On free drug treatment, the white pulp diminished in size, red pulp became normal, the number of migrating monocytes decreased in the sinusoids, while the cell nuclei became non-pycnotic. On treatment with niosomal, liposomal and microsphere-incorporated drugs, predominant changes such as progressive regression of white pulp to normal dimensions, expansion of red pulp to normal, gradual decrease in number of circulating monocytes in sinusoids and reversion of cell nuclei to the non-pycnotic state could be seen.

Sizes of carriers and their possible relationship with efficacy

The mean diameters of different vesicles as determined by dynamic light scattering show that the size of microspheres is much greater (14,000 nm), compared to liposomes (2250 nm) and niosomes (526 nm). The size *vs.* efficacy curve obtained by plotting percentage of reduction of parasite burden against log of mean diameter in nm is shown in Fig. 4. Efficacy is found to have an inverse relationship with the size of carriers. Thus, microsphere-incorporated drug was found to have the least efficacy, followed by liposomal and niosomal drugs.

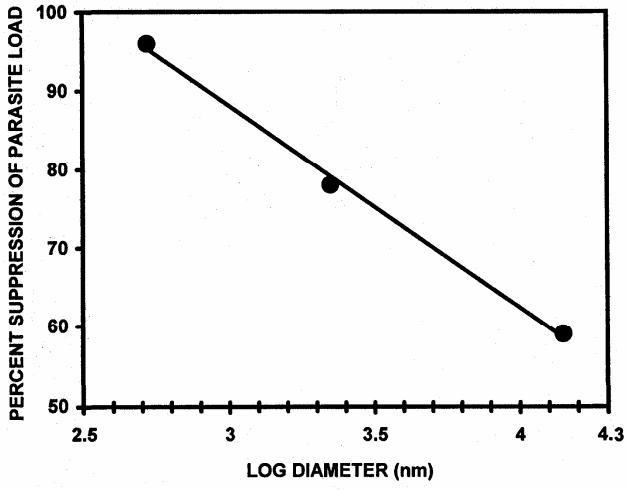

Fig. 4—Relationship between size of different carriers and percentage efficacy in the reduction of spleen parasite load [Diameters of different delivery systems e.g. niosomes, liposomes and microspheres were measured by dynamic light scattering]

Discussion

Chemotherapy of Leishmaniasis by conventional drugs is severely restricted by toxicity of the drugs. In general, toxicity is usually due to two main reasons: (i) the diffused delivery to uninfected tissue; and (ii) the inherent toxicity of the drug molecule. The first line of drugs, the trivalent and pentavalent antimonials, are reported to be highly toxic,[21] with increased levels of various marker enzymes and hepatomegaly[22] and other typical skin reactions of heavy metals[23]. The second line of drugs, pentamidine and amphotericin B, were effective against antimonial unresponsive cases and relapses[24], but still proved to be highly toxic with cumulative effects of nephrotoxicity, anemia and many other complex disorders[25]. Antileishmanial activity of primaquine, both in free[26] and liposomal form[27], formycin B[28] and sinefungin[29] have also been reported. Besides, andrographolide[7], piperine[30], amarogentin[31], and bacopasaponin C[12] are also known for their antileishmanial activity, but none of them was taken for even pre-clinical toxicity test level. Although, amphotericin B and a few other antileishmanial compounds e.g. imofosine, miltefosin and allopurinol are known to be in clinical trials, but the details are still awaited[32].

Andrographolide and some other related and interconvertible compounds also showed potent growth inhibiting activity against M-1-tumor cell line, proving their cytostatic activity[33]. The drug under study, 14-deoxy-11-oxoandrographolide isolated from *Andrographis paniculata***,** which is widely used in Indian indigenous medicine for its various therapeutic properties, such as antimicrobial, stimulant, liver protectant etc. has shown better efficacy, in lowering of spleen parasite burden in free and liposomal forms, compared to the parent compound i.e. andrographolide[7]. The free drug at the optimum dose could reduce the parasite burden to 39% after six injections. However, the efficacy could be greatly enhanced by using drugs incorporated in different carriers. The maximum efficacy was shown by niosomal drug at 91% after six injections, followed by liposomal and microsphere-incorporated drug (Table 1). The efficacy is found to be inversely related with the size of carriers[12]. Also, the carriers with the least size showed the least toxicity. However, unless other possible factors e.g. drug release kinetics are carefully studied, attempt to correlate only with the sizes may be erroneous. Thus, because of their greater efficacy

and lesser toxicity, niosomal, liposomal, or even microsphere-incorporated 14-deoxy-11-oxoandrographolide could have clinical application in the treatment of Leishmaniasis.

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