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Evaluation of the in-vivo activity and toxicity of amarogentin, an antileishmanial agent, in both liposomal and niosomal forms

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The antileishmanial property of amarogentin, a secoiridoid glycoside isolated from the Indian medicinal plant *Swertia chirata*, was examined in a hamster model of experimental leishmaniasis. The therapeutic efficacy of amarogentin was evaluated in free and two different vesicular forms, liposomes and niosomes. The amarogentin in both liposomal and niosomal forms was found to be a more active leishmanicidal agent than the free amarogentin; and the niosomal form was found to be more efficacious than the liposomal form at the same membrane microviscosity level. Toxicity studies involving blood pathology, histological staining of tissues and specific enzyme levels related to normal liver function showed no toxicity. Hence, amarogentin incorporated in liposomes or niosomes may have clinical application in the treatment of leishmaniasis.

Introduction

The therapy of leishmaniasis, which is estimated to affect around 10 million people worldwide,[1] poses problems because the toxicity of drugs that are often used in treatment. Clinical reports[2] indicate that a large proportion of cases are becoming unresponsive to chemotherapy. In view of the present clinical scenario it is desirable that new drugs as well as new macrophage-specific delivery systems are developed. Our laboratory has reported the use of plant glycosides in liposomes as delivery systems for the purpose of targeting specific cellular sites.[3,4] The interesting structural feature of the plant glycosides in general is that a hydrophilic sugar moiety is attached to a hydrophobic aglycone. The hydrophilic sugar moiety remains at the surface of liposomes, facilitating adsorption and attachment to the tissues. The novelty of amarogentin (Figure 1), a glycoside isolated from the indigenous plant *Swertia chirata*, is that it could be used both for targeting specific cellular sites and for chemotherapy. The antileishmanial activity of amarogentin originates from its capacity to inhibit DNA topoisomerase I,[5] a vital enzyme for the survival of the parasite *Leishmania donovani*, the causative agent of visceral leishmaniasis. In this paper, we describe the effective use of amarogentin as an antileishmanial agent both free and incorporated in liposomes or niosomes. Non-ionic surfactant vesicles (niosomes) were used as an alternative to liposomes.

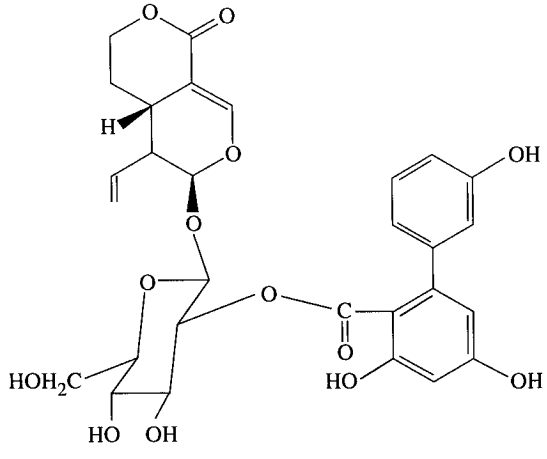

Figure 1. Structure of amarogentin.

Materials and methods

Chemicals

Egg phosphatidyl choline (PC), cholesterol (Chol), phosphatidic acid (PA), *p*-nitrophenol phosphate and 6-carboxyfluorescein were obtained from Sigma (St Louis, MO, USA; DL-alanine from SRL (Bombay, India); α-oxoglutaric acid, aniline and citric acid from Merck (Darmstadt, Germany); eosin and haematoxylin from Loba Chemie (Bombay, India); 2,4-dinitrophenyl hydrazine from

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Aldrich (Milwaukee, WI, USA; sorbitan monolaurate (Span 20) from Fluka Chemie AG (Switzerland). Amarogentin was isolated and purified from a methanol extract of *S. chirata.*

Preparation of liposomal and niosomal amarogentin

Liposomes were prepared essentially by the method of Gregoriadis and Ryman.[6] For preparation of liposomal amarogentin, the lipids in the proportions PC:Chol:PA 7:4:1 were dissolved in a chloroform–methanol mixture (2:1 v/v). To the resulting solution amarogentin (500 μg in methanol) was added. The thin dry film that formed after evaporating the solvents was swelled in phosphate-buffered saline for 1 h and sonicated for 30 s. For the preparation of niosomes, the non-ionic surfactant of the span series, e.g. sorbitan monolaurate (Span 20), was used as a substitute for phospholipids.[7] For niosomal amarogentin, the reagents, e.g. Span 20:Chol:PA 1.0:0.5:0.1, were dissolved as before in a chloroform–methanol mixture and amarogentin (500 μg in methanol) was added. The dry film was swelled, sonicated and centrifuged (100,000**g**) for 30 s to remove excess amarogentin.

The intercalation of amarogentin in liposomes and niosomes was around 33 and 24%, respectively. The stability of liposomes and niosomes, as judged from the leakage of 6-carboxyfluorescein when incubated *in vitro* with hamster blood plasma, was 3 and 5 h, respectively. The composition of the two vesicle types was adjusted so that the membrane microviscosity, measured with a fluorescence depolarization technique with diphenyl hexatriene as the fluorescent probe,[8–12] was similar.

Efficacy of amarogentin in liposomes and niosomes in a hamster experimental leishmaniasis model

Our colony of golden hamsters (*Mesocricatus auratas*), originally from the Haffkine Research Institute (Bombay, India), was used to maintain *L. donovani* isolate AG83, from an Indian kala-azar patient, by intracardial passage every 6 weeks. Amastigotes were isolated from the infected spleen by the method of Looker *et al*.[13] with some modifications.[14] Amastigote numbers were determined by use of a haemocytometer. Each animal was infected intracardially with 2×10^6 amastigotes. After 30 days the hamsters were selected for chemotherapy and were distributed in groups of four for the testing of amarogentin in free, liposomal and niosomal forms at the same equivalent concentration. The dose given to each animal was 2.5 mg/kg bodyweight. In practice, 2 mg of amarogentin intercalated in 0.5 mL liposomal or niosomal suspension was injected into each hamster subcutaneously every 3 days for a total of six doses over 15 days. Free amarogentin was also administered to one group. The animals were killed 5 days after the last injection. Parasite burden in the spleen was assessed microscopically from stained impression smears taken on slides after fixing in absolute methanol and Giemsa staining. The total number of amastigotes was determined by using Stauber's formula.[9]

Investigation of drug toxicity

Various factors including tissue histology, blood pathology and specific enzyme levels related to normal liver function were examined to determine the toxic effects of the drug delivered both free and in liposomal and niosomal forms. Fresh blood was taken from both untreated and treated hamsters just before they were killed and was collected in EDTA tubes. The red blood cells, white blood cells and haemoglobin were examined by established clinical procedures. The spleens of the animals were removed and processed for histological examination after staining with eosin and haematoxylin.[15] The sera of the animals undergoing drug treatment were assayed for serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase by published procedures.[16,17] In brief, for assay of alkaline phosphatase, the serum was incubated with *p*-nitrophenol phosphate for 30 min at 37°C. The release of *p*-nitrophenol was measured by absorbance at 410 nm. For SGPT, the serum was incubated with 10 mL phosphate-buffered saline (pH 7.5) containing 0.5 g DL-alanine and 2 mg α-oxoglutaric acid. The mixture was further treated with aniline citrate for 20 min at 37°C followed by the incubation with 2,4-dinitrophenyl hydrazine hydrochloride solution for 20 min. The reaction was stopped by addition of 0.02 M NaOH and the absorbance was measured at 520 nm.

Results

Leishmanicidal activity of amarogentin in vivo

The single dose–response curve for survival of hamsters infected for 30 days with *L. donovani* and treated with free amarogentin (1.25 mg/kg, 2.5 mg/kg, 5.5 mg/kg and 11.0 mg/kg bodyweight) was examined. No death occurred over a period of 15 days with any dose. The optimum dose, as judged from reduction in parasite burden in the spleen (Figure 2), was 2.5 mg/kg bodyweight.

The effects of chemotherapy on hamsters infected for 30 days with *L. donovani* are shown in Table I. Experiments were performed with the same equivalent drug concentration for free, liposomal and niosomal forms. The dose given to each animal was 2.5 mg/kg every 3 days. The sc injection of free amarogentin reduced the parasite load by 34%, whereas the intercalation of free drug in the liposomal and niosomal forms reduced the parasite load by 69 and 90%, respectively. Thus niosomal amarogentin showed better efficacy than liposomal amarogentin. Reduction of spleen parasite load with empty liposomes or niosomes was 12–14%.

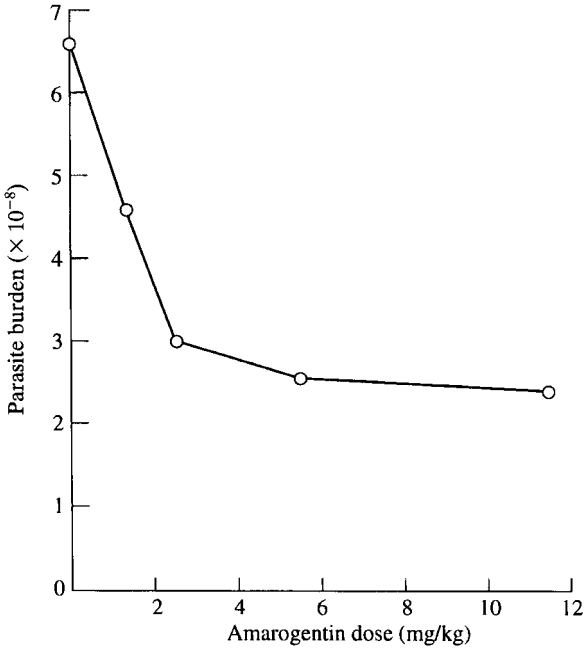

Figure 2. Amarogentin dose dependence of reduction in parasite burden in the spleen of hamsters infected with *L. donovani.*

Table I. Effect of amarogentin on hamsters infected for 30 days with *Leishmania donovani*

Treatment	Parasite load in the spleen ($\times 10^{-8}$; mean ± S.D.[a])	Suppression of parasite load in the spleen (%)
No drug (control)	7.4 ± 0.5	–
Free drug	4.86 ± 0.6	34
Drug-intercalated liposomes	2.3 ± 0.20	69
Drug-intercalated niosomes	0.72 ± 0.31	90

[a] $n = 4$.

Toxicity of amarogentin

Liver function was tested by examination of specific enzyme activity in sera of hamsters with experimental leishmaniasis. Both the SGPT and alkaline phosphatase activity increased on free-drug treatment but were close to normal levels when liposomal or niosomal amarogentin was used, indicating no apparent toxicity in either of the two vesicular forms (Table II).

To check kidney function, the concentrations of urea and creatinine were examined in the sera of hamsters undergoing chemotherapy. Although the concentrations of both increased initially on infection and further on free-drug treatment, they came down to normal when amarogentin was applied in either liposomes or niosomes. The change in haemoglobin levels was not significant both before and after chemotherapy.

Table II. Enzyme activity in sera of hamsters with experimental leishmaniasis

Group	Alkaline phosphatase[a] (mean ± S.D.[b])	SGPT[c] (mean ± S.D.[b])
Uninfected	6.0 ± 0.7	46 ± 4.0
Infected	11.0 ± 0.64	103 ± 5.9
Infected + free drug treated	12.5 ± 1.3	131 ± 6.7
Infected + liposomal drug treated	7.0 ± 1.3	44 ± 6.6
Infected + niosomal drug treated	6.5 ± 1.9	44 ± 8.1

[a] Number of μmol of *p*-nitrophenol liberated/min/L serum.
[b] $n = 3$.
[c] Number of μmol of sodium pyruvate released/min/dL serum.

Histological examination of the spleen was made after staining with eosin and haematoxylin.[15] Although some changes were noticed in comparison with the untreated controls, the cells looked healthy and no toxic effects were evident.

Discussion

Although the leishmanicidal properties of crude extracts of the Indian medicinal plant *S. chirata* have long been known,[18] the mechanism of its action was not clear. Recently the active component, amarogentin, a secoiridoid glycoside isolated from a methanol extract of *S. chirata*, has been reported[5] to inhibit the activity of DNA-topoisomerase I of *L. donovani*. The compound exerts its inhibitory effect by binding to the enzyme and thus preventing binary complex formation between topoisomerase I and DNA, resulting in the killing of the parasites. This potent inhibitory effect may provide a lead for the design of more-effective drugs against leishmaniasis. In our studies with experimental leishmaniasis in hamsters we observed 90% parasite killing with only six doses. However, at the same membrane fluidity and at an equivalent drug concentration, the niosomal form was found to be more effective than the liposomal form. At comparable toxicity levels, amarogentin was found to be very active in either of the two vesicular forms. Hence, amarogentin could possibly have clinical application in the treatment of leishmaniasis.

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