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In Vitro Studies with Liposomal Cryptolepine

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ABSTRACT

The antifungal activity of cryptolepine when encapsulated in liposomes was compared with free cryptolepine. Multilamellar and unilamellar liposomes with positive and negative charge on their surface were prepared and extruded through polycarbonate membranes to obtain a liposome size of 400 nm. Cryptolepine liposomes were then tested in vitro for their antifungal activity against C. albicans, U. maydis *and* S. cerevisiae. *Results showed that negatively charged cryptolepine liposomes were at least 2-4 times more active than free cryptolepine whereas positively charged liposomes were comparable to the activity of free cryptolepine. However, none of the liposome preparations were superior to amphotericin B in their antifungal activity.*

INTRODUCTION

Cryptolepine is the methyl quindolanol alkaloid of the West African climbing plant *Cryptolepsis sanguinolenta* (Fig. 1) (1,2). The extracts of this plant have found several applications in the folklore of African traditional medical practice. Traditional healers in West Africa have claimed success in treatment of several infectious diseases, including fevers, bronchitis, venereal diseases, skin disorders, and worm infestations (4-6). This alkaloid was also reported to produce a variety of pharmacological effects. Some of these effects include hypotension, vasoconstriction of the rabbit perfused ear, α-adrenoreceptor blockade, and antagonism of carrageen-induced edema of the rat hind paw. In a previous study, cryptolepine inhibited platelet aggregation both in vitro and in vivo, disaggregated platelets, and stimulated fibrinolysis (7). It was also reported that inhibition of platelet aggregation by cryptolepine may be due to elevation of platelet cAMP, probably through the stimulation of platelet adenylate cyclase (8).

Biological evaluation of cryptolepine confirms the use of the plant as an antimicrobial and an antimalarial agent by West African natives. While the potency of cryptolepine is less than desired for many of its actions, cryptolepine may serve as a lead compound for struc-

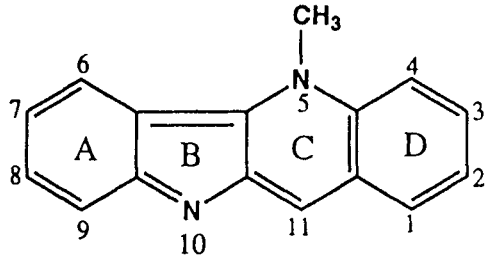

Figure 1. Structure and numbering system of cryptolepine.

ture modification. Recently, this compound was studied for its antifungal activity in our laboratory and showed activity against selected fungal isolates such as *C. albicans* and *C. neoformans* (3). These initial studies have indicated that although the zones of inhibition of cryptolepine in agar well diffusion assays were on average wider than those of amphotericin B, the minimum inhibitory concentration (MIC) values were reversed. For example, against *C. neoformans*, cryptolepine has a zone of inhibition of 13 mm and amphotericin B has 7 mm. However, the MIC for cryptolepine against *C. neoformans* is 12.5 μg while that of amphotericin B is 0.20 μg. In an effort to explain the anomaly, we decided to evaluate the biological activity of liposomal preparations of cryptolepine with a view to ascertaining whether transport phenomena have a role in the apparent decrease in activity.

Liposomes have been successfully used to enhance the antifungal activity of several drugs, e.g., amphotericin B, nystatin, gramicidin S, and haymycin (9–12). In most cases these antifungal agents are lipophilic and hence liposomal formulations were prepared to overcome the water insolubility. For a long time amphotericin B (Fungiozone®) was used as the drug of choice for most offending fungal pathogens. However, recent years have seen the advent of various azoles: ketoconazole, fluconazole, and itraconazole. Although fluconazole is water soluble and well received by patients, it has recently been reported to interact with various drugs such as cyclosporine, oral hypoglycemics, phenytoin, and rifampin. Fluconazole has also been reported to induce thrombocytopenia, hypokalemia, and suppression of neutrophil function.

Liposome formulations of water-insoluble azoles (viz. ketoconazole) have been reported and tested with encouraging results (13,14). Recently a liposomal formulation of a water-soluble azole, fluconazole, was also prepared with significant antifungal activity (15). Currently liposomal amphotericin B (AmBisome, Vestar Inc.) and Econazole (Pevaryl lipogel, Cilag AG, Schaffhausen) are being sold commercially in Europe. Despite all the advances made in this area, there is still need of newer antifungal agents which are less toxic and more active in vivo.

The objective in our laboratory was twofold: (a) to prepare derivatives of cryptolepine which will be more potent than cryptolepine itself and comparable to better in activity than amphotericin B in antifungal activity; (b) to prepare liposomal formulations of cryptolepine. It is expected that liposomal formulations of cryptolepine will be more potent than cryptolepine itself and may also be comparable in activity to currently available antifungal agents.

MATERIALS AND METHODS

Synthesis of Cryptolepine:

Cryptolepine, a 3:4 benz-δ-carboline derivative, was synthesized from quindoline. Briefly, quindoline (800 mg, 3.7 mmol) was treated with dimethyl sulfate (3 ml) and refluxed for 3 hr. Ether was added to the resulting reddish solution to precipitate the sulfate salt of cryptolepine. To obtain the free base form, the salt was dissolved in water, basified with ammonia, and exhaustively extracted with ethyl acetate. The organic phase was dried with anhydrous sodium sulfate and the solvent was removed to yield a violet powder of cryptolepine. Column chromatography on silica gel produced the pure compound; m.p. 178–180°C.

The nuclear magnetic resonance (NMR) spectra of cryptolepine was obtained on a Varian VXR 300 spectrometer operating at 300 mHz and 75 mHz for proton and carbon, respectively, standard Varian pulse programs were utilized for the COSY, APT, HECTOR, and LRHECTOR. $CDCl_3$ was used for all NMR determinations with TMS as internal standard.

Lipids and Reagents:

Dipalmitoyl phosphatidyl choline (DPPC), stearyl amine (SA), cholesterol (Chol), and phosphatidic acid were obtained from Sigma Chemical Co. (St. Louis, MO). All solvents were obtained from Fisher Scientific (Atlanta, GA) and were glass distilled.

Preparation of Liposomes

Multilamellar liposomes were prepared with either a positive or a negative charge by the method of Mezei

and Nugent (16). Briefly, positively charged liposomes consisted of lipid composition DPPC:Chol:SA with the molar ratio 5:3:1. The negatively charged liposomes consisted of DPPC:Chol:Phosphatidic acid (PA) with the molar ratio 5:3:1. The liposomes were then extruded through 0.4μ polycarbonate membranes. The unentrapped cryptolepine was removed by centrifugation at 20,000 rpm. The amount of drug entrapped in liposomes was estimated by reading the absorbance at 500 nm using a liposomal blank. The size of liposomes was estimated with the Brookhaven particle sizer (BI-90, Brookhaven Instruments, NY). The stability of liposomes was studied in phosphate-buffered saline and human serum at 37°C.

In Vitro Antifungal Activity

Organisms

Candida albicans (ATCC 102310), *Ustilago maydis* (ATCC 14826), and *Saccharomyces cerevisiae* (ATCC 9763) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and stored frozen at -80°C in 20% glycerol.

Microbiological Assay

The antifungal activity of free compounds and the liposomal formulations were tested by the microbroth dilution method using YM broth (Difco 0711). Overnight culture was adjusted to a density of $1–5 \times 10^5$ cfu/ml and 5 μl were added to 100 μl of YM broth containing the appropriate dilutions of the formulation such that the final drug concentration ranged from 0.06 to 128 μg/ml. Plates were incubated at 28°C for 20–48 hr. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic which prevented turbidity. Amphotericin B was used as a reference standard. Blank liposomes were also used as controls to test for any toxicity with phospholipids.

RESULTS AND DISCUSSION

Cryptolepine prepared in the laboratory had >95% purity as ascertained by high-performance liquid chromatography (HPLC) analysis. The NMR data as obtained in our laboratory were consistent with already published results (4,17). The encapsulation efficiency of cryptolepine in liposomes was 11% for positively charged liposomes and 15% for negatively charged liposomes. No attempts were made to vary the lipid composition or the drug amount to enhance entrapment. The liposomal size was found to be 390 nm as ascertained by the particle sizer. The liposomes were found to be stable in phosphate-buffered saline (PBS) and less stable in human serum (Table 1). The negatively charged liposomes were found to be more stable in serum as compared to the positively charged liposomes.

The antifungal activity of free cryptolepine and liposomal cryptolepine is given in Table 2. Cryptolepine (free) appears to be more active against *Ustilago maydis* and *Saccharomyces cerevisiae* than *Candida albicans* after 18- to 20-hr incubation, but the difference in activity was not substantial after 48 hr incubation. Al-

Table 1

Stability Profile of Cryptolepine Liposomes

Liposome Type	Incubation Time (hr)	Percentage of Cryptolepine Retained in the Pellet	
		PBS	Serum
Positive liposomes	12	100	100
	24	100	90
	48	100	70
	72	100	65
Negative liposomes	12	100	100
	24	100	100
	48	100	80
	72	100	78

Table 2

Antifungal Activity with Cryptolepine Liposomes (MIC, μg/ml)

Organism	*C. albicans*	*U. maydis*	*S. cerevisiae*
Amp. B	0.25 (1)	0.03 (8)	0.25 (4)
Cryptolepine	8 (16)	1 (16)	1 (8)
Positive cryp. liposomes	4 (4)	1 (2)	2 (2)
Negative cryp. liposomes	1	0.25 (0.25)	0.25 (0.25)

Note: The numbers in parentheses indicate incubation of 48 hr.

though amphotericin B was severalfold more active than free cryptolepine against all three strains tested, the liposome-encapsulated cryptolepine appeared to substantially reduce the difference in activity. The liposomal cryptolepine was 2–8 times more active than free cryptolepine, the negatively charged liposomal formulation being more favorable than the positively charged one. The 48-hr MICs convincingly suggested that the liposomal cryptolepine produced extended growth-inhibitory effect at much lower concentration than the free drug. The liposomal formulation may be either stabilizing the drug or enhancing its transport into the cell.

This is the first study involving liposomal preparations of cryptolepine. Not much is known about the mechanism of action of cryptolepine liposomes but it is apparent that negatively charged liposomes were more active than positively charged liposomes. Cryptolepine has a pK_a of 11.4. This implies that at physiological pH of 7.4, cryptolepine will be completely ionized with a positive charge on the N-5 atom. This can decrease transportation across cell membranes and consequently decrease potency. Negatively charged liposomes would reduce the overall positive charge density on cryptolepine, which would reduce the hydrophilicity and probably help cryptolepine to cross the cell membranes in culture systems or in vivo to exert its physiological activity. Another interesting finding in this study has been that cryptolepine is very fungi specific. It was found to be most active against Cryptococcus and least active against Candida (personal communications). This species-specific behavior could not be altered by encapsulating it in liposomes.

The liposomal size in these experiments was below 400 nm. The liposomes as observed by an electron microscope were both unilamellar and multilamellar, with more liposomes being in the unilamellar range. However, other workers have obtained favorable results both with multilamellar vesicles (MLVs) and with small unilamellar vesicles (SUVs). Amphotericin B has been incorporated in both unilamellar and multilamellar liposomes (18,19), with significant antifungal activity in each case; the commercial product AmBisome (Vestar Inc.) is a unilamellar product with a diameter of 55–75 nm. Our intention was to have a limited heterogeneous size distribution to maximize the in vitro potency. Experiments were also carried out with MLV preparations of cryptolepine in vitro which showed no improvement of antifungal activity (results not shown).

The present studies indicate that the activity of cryptolepine can be increased by encapsulating the drug in liposomes, but the activity was not better than amphotericin B, a well-known antifungal agent. These findings also indicate that the antifungal activity of cryptolepine is limited, as compared to current antifungal agents available, even after encapsulating in liposomal drug delivery systems. Currently, the research emphasis in our laboratory is in structure–activity relationship (SAR) studies in an effort to identify structural features present in cryptolepine which contribute to its antifungal activity. Another modification of interest being pursued is to modify the electronic features associated with N^5 nitrogen (Fig. 1).

CONCLUSIONS

The study has provided information for the activity of cryptolepine when incorporated in positively and negatively charged liposomes. The in vitro studies indicate that though the activity of cryptolepine was significantly increased by over 10-fold by entrapping it in liposomes, its activity was still no better than amphotericin B. Current studies are being made to prepare more active chemical derivatives of this drug.

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