Development and therapeutic application of liposomal amphotericin B

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A formulation of liposomal amphotericin B suitable for use in human patients has been prepared and tested successfully. Phase I clinical trial of its safety has been completed and phase II studies of its efficacy in systemic fungal infection in patients is under way and shows promising results. It has been used safely in immuno-compromised patients, those with impaired renal function and seriously ill individuals.

ONE of the basic aims of therapeutics is to deliver drugs efficiently and specifically to the site of disease or disorder with minimal toxic effects. In spite of the recent developments in molecular and cell biology, progress in the design of selective drugs has been slow. On the other hand, during the past two decades, significant advances have been made in the area of controlled release as an alternative aproach to conferring selectivity and specificity on drugs. Targeted drug delivery was conceptualized as early as the beginning of this century by microbiologist, Paul Ehrlich. However, only recently has this concept been exploited with the use of sophisticated carrier systems for drug molecules. Among the various macromolecular, cellular or synthetic carriers for drugs, liposomes generated a great interest because of their versatility.

Liposomes, described as concentric bilayered structures made of amphipathic phospholipids, were first discovered by Alec Bangham in 1965. Depending on the number of bilayers, liposomes are classified as either multilamellar (multilamellar vesicles, MLV) or unilamellar (small unilamellar vesicles, SUV and large unilamellar vesicles, LUV). These vesicles range in size from less than 0.025 μm to 10 μm in diameter. The size and morphology of the structure are regulated by the method of preparation.

Since the discovery, liposomes have been extensively studied as model membranes to obtain information on the physicochemical organization of lipid bilayers and to study the interaction of various membrane-active drugs with lipids. The possible application of liposomes in the field of medicine, based on their biodegradability, non-immunogenicity and capability of encapsulating both hydrophilic and hydrophobic substances, was first proposed by Gregoriadis and Ryman[1]. They used liposomes as a carrier to treat lysosomal storage disorders. This led to the development of liposomes of different compositions, size and surface charge, displaying good therapeutic promise as carriers for anticancer[2], antibacterial[3], antiviral[4], antiparasitic[5] and antifungal[6] drugs.

The encapsulation and delivery of a drug in liposome has the potential to improve drug therapy by: (i) controlled drug delivery, (ii) targeted drug delivery, and (iii) site avoidance drug delivery.

Liposomal cytosine arabinoside appears to act as circulating controlled release delivery system. Normally, cytosine arabinoside is rapidly degraded (deaminated) in blood. Liposomes protect the compound until it is released[7]. A portion of liposomal drug is released in the vicinity of tumour. Although the efficacy of the compound is not usually improved by such a system, the decrease of adverse effects, improved patient convenience and decreased frequency of drug administration significantly benefit the patient.

Liposomes have been administered through a variety of routes: intravenous, intramuscular, intraperitoneal, intranasal and oral. The intravenous route is the most widely used. Liposomes, following intravenous administration, are rapidly cleared from the circulation by the mononuclear phagocytic system[8], thus, making liposomes an attractive vehicle for the treatment of infections of the reticuloendothelial system such as leishmaniasis and malaria. Alving *et al.*[9] observed marked increase in efficacy in the treatment of experimental leishmaniasis in mice and hamsters by the use of antimonial drugs encapsulated in liposomes. Another distinct advantage is the activation of macrophages to a tumoricidal or virucidal state by encapsulating muramyl dipeptides or other immunostimulatory compounds in liposomes and delivering them to the macrophages[10].

Since liposomes do not readily concentrate in organs

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such as the kidney and heart, drugs such as amphotericin B (AmpB) and doxorubicin intended to act on other parts of the body, produce little or no toxic effects on them when encapsulated in liposomes. The enhanced therapeutic index of primaquine phosphate is due to reduced toxicity from modified distribution of drug rather than drug targeting[11].

In spite of these promises offered by liposomes, the progress towards its clinical use has been slow. An important milestone in the journey of liposomal dosage forms from the laboratory to clinic has been with the use of liposomal amphotericin B. We review here the development of various liposomal amphotericin B (L-Amp B) formulations in preclinical and clinical studies focusing on the problems and promises of liposomal amphotericin B (L-AmpB-LRC) L-AmpB developed and tested in India.

Amphotericin B

AmpB is a polyene macrolide antibiotic that is widely used for treatment of systemic fungal infections. Disseminated fungal infections are a major cause of morbidity and mortality in patients with leukaemia receiving chemotherapy and in a variety of immunodeficiency diseases[12]. The majority of these infections are caused by species of *Candida* and *Aspergillus*. Despite the development of new classes of antifungal agents, AmpB remains the drug of choice. Its antimicrobial activity results from its ability to bind to the sterol component of the cell membrane, leading to the formation of transmembrane pores that allow the leakage of vital cellular constituents. AmpB binds preferentially to ergosterol, a major component of the fungal cell wall. Unfortunately, the drug also interacts with cholesterol in mammalian membrane which probably is the basis for its profound acute and chronic toxicity. Approximately 20–50% patients treated with AmpB develop acute infusion-related reactions such as fever, chills, nausea and vomiting[13]. This is in spite of the liberal use of premedications intended to prevent such side effects. Of the chronic toxicities associated with AmpB usage, nephrotoxicity is one of the most important because of its potential limiting effect on the total course of therapy. Nephrotoxicity is reported in about 60–83% of patients[14]. Another important and commonly encountered chronic toxicity is electrolyte disturbance secondary to renal wasting of potassium and magnesium. Ninety per cent of patients on AmpB treatment require potassium supplementation[14].

Attempts to investigate the various preparations of AmpB with the aim of reducing side effects while maintaining its antifungal activity led to the successful incorporation of AmpB into liposomes.

Liposomal amphotericin B

Preclinical studies

Since the study by New *et al.*[15], much interest has been centered on the use of liposomes as a drug carrier for AmpB in the treatment of several systemic fungal and parasitic infections. It was shown that L-AmpB was as effective as free AmpB in experimental histoplasmosis[16] and cryptococcosis[17] but much less toxic. Lopez-Berestein *et al.*[18] carried out extensive detailed studies on the use of L-AmpB in systemic candidiasis and paved the way for its clinical use.

Lopez-Berestein *et al.*[18] used multilamellar liposomes prepared from phosphatidylcholine dimyristoyl (DMPC) and phosphatidylglycerol dimyristoyl (DMPG) in 7:3 molar ratio. The toxicity of L-AmpB was far less than that of free AmpB, without any loss of activity against *Candida albicans*. L-AmpB also proved to be superior to AmpB in the prophylaxis and treatment of experimental candidiasis in neutropenic mice[18].

Hopfer *et al.*[19] observed that the lipid composition of the liposomes played a major role in L-AmpB activity. The presence of a sterol component (like ergosterol and cholesterol) in liposomes decreased the antifungal activity by almost fifty-fold. However, other workers found that the incorporation of cholesterol in liposomes did not result in any loss of activity[20–24]. Szoka *et al.*[21] postulated that in MLV, only about 10% of the lipid is on the external monolayer and the transfer of AmpB from the internal lamellae to the fungal cell cannot take place readily. Whilst SUV containing cholesterol have about 50–60% of the lipid on monolayer accounting for better transfer of AmpB.

With this concept, two SUV formulations were developed. Negatively charged small unilamellar vesicles made from hydrogenated soya phosphatidylcholine (SPC), cholesterol and phosphatidylglycerol distearoyl (DSPG) in 2:1:0.8 molar ratio were tested in murine candidiasis and cryptococcosis[24]. The efficacy was found to be comparable with conventional Amp B on an equal dose basis. The other formulation is positively charged, prepared from SPC, cholesterol and stearylamine in 4:3:1 molar ratio.

During the same time, Ahmad *et al.*[25], in India, formulated small unilamellar liposomes from egg phosphatidylcholine (EPC), phosphatidylethanolamine dipalmitoyl (DPPE) and cholesterol. They showed that this liposomal intercalation of AmpB had reduced toxicity and improved therapeutic efficacy in murine aspergillosis compared to the free drug. They also demonstrated better delivery of AmpB to infected tissue. Liposomes grafted with mannose are taken up by macrophages because of the presence of mannose receptors on their surface. These were more effective

than nonmannosylated liposomes in the treatment of murine aspergillosis[26]. Liposomes made from EPC and cholesterol were also effective in the treatment of murine aspergillosis[27].

Interestingly, the tissue distribution of AmpB after incorporation into liposomes of various lipid compositions, size and surface charge were comparable[23,27,28]. High levels of AmpB were detected in organs like liver, spleen and lungs which are rich in macrophages. AmpB was detected in brain at potentially therapeutic concentrations showing good penetration through the blood-brain barrier[24].

The pharmacological basis for the enhanced therapeutic index observed with L-AmpB is not clearly understood. The efficacy of L-AmpB may be due to tissue targeting, altered interactions with yeast and mammalian cells and intracellular delivery to phagocytic cells. Immunostimulation and capillary leakage secondary to fungal endothelial invasion may also play a vital role[29]. L-AmpB retains antifungal potency without any toxicity to red blood cells[30].

Formulation for clinical use

Any parenteral preparation must be sterile, pyrogen free, safe and stable. Liposome-based formulations are no exception.

Until recently, the clinical trials were limited to a few patients. This was partially due to the reluctance of large pharmaceutical companies to become involved with an yet unproven system. Not many academic research teams have the resources to bring a new liposomal dosage form to the clinic. The efforts of a few groups have led to the use of liposome encapsulated drugs in preliminary clinical trials. In the eighties a number of liposome drug companies were formed which has resulted in development of at least 10 different liposomal drugs for clinical trials (Table 1) and overcoming of at least some of the initial problems.

In view of the favourable outcome observed in experimental animals, liposomal AmpB was used in patients. At present three different liposomal preparations have undergone clinical trials with one preparation being marketed in Europe. Their characteristics are described in Table 2.

Liposomal amphotericin B developed by Ahmad *et al.*[27] was further tested and developed at our hospital for clinical use.

In the first series of experiments on murine aspergillosis by Ahmad *et al.*[25], small unilamellar liposomes prepared from EPC, DPPE and cholesterol were used. This preparation had LD_{50} of 8.1 mg kg^{-1} compared to 1.2 mg kg^{-1} with free AmpB. It was also found to be effective at doses of 0.5 mg kg^{-1}, with 70% of animals surviving on 7th day compared to 13% with free AmpB. Subsequently, they showed[26] that grafting mannose onto these liposomes provided twice the therapeutic benefit with LD_{50} also increasing to 9.3 mg kg^{-1}.

Although these formulations were easy to manufacture, the cost was exorbitant because of the use of DPPE and p-aminophenyl-D-mannopyranoside. Later they showed that SUVs prepared from naturally occurring, inexpensive EPC and cholesterol were as effective as other formulations[27].

We studied the various formulations of liposomal amphotericin B using SPC and cholesterol to determine the ease of manufacturing while retaining reduced toxicity and improved efficacy against fungal pathogens. We were able to encapsulate more than 90% AmpB into liposomes. Ahmad *et al.*[27] had advocated the use of dialysis to remove unencapsulated AmpB in order to reduce toxicity. In practice however, the dialysis procedure is subject to contamination by pathogens and is cumbersome (requiring about 4–5 h). We did not find any advantage of removing free drug from that bound to liposome by dialysis, since the LD_{50} and efficacy were similar with dialysed and undialysed formulations (Table 3).

The toxicity of SUV was lower compared to that of MLV. Similar findings were observed by Szoka *et al.*[21]. SUVs were significantly more effective than MLVs as judged by the survival pattern and CFUs in infected

Table 1. Liposome-based products in clinical development

Indication	Drug
Anticancer	Daunorubicin Doxorubicin Diamino cyclohexane platinum compound Muramyl tripeptide phosphatidylethanolamine
Antibacterial	Gentamicin
Antifungal	Amphotericin B
Bronchodilator	Orciprenaline Salbutamol
γ-Imaging agent	^{111}Indium

Table 2. Formulations of L-AmpB in clinical development

Formulation	Lipids	Size
MLV	Dimyristoyl phosphatidylcholine Dimyristoyl phosphatidylglycerol (7:3 molar ratio)	0.5–6.0 μm
SUV*	Hydrogenated soya phosphatidylcholine Cholesterol Distearoyl phosphatidylglycerol (2:1:0.8 molar ratio)	<100 nm
SUV	Egg phosphatidylcholine Cholesterol Stearylamine (4:3:1 molar ratio)	60–250 nm

MLV-Multilamellar vesicles
SUV-Small unilamellar vesicles
*Marketed in Europe.

Table 3. Effect of various L-AmpB-LRC formulations on safety, efficacy and stability

Formulation	Day	LD_{50} (mg/kg)	7 Days after therapy (dose 0.5 mg AmpB/kg) Mean ± S.E. of 3 experiments	
			% Survival	CFU
1. MLV-dialysed	0	14.4	41.6±1.6	910.0±35.0
	15	14.2	40.0±0	875.0±11.6
	30	14.4	40.0±0	910.0±35.0
2. MLV-undialysed	0	14.17	40.0±2.8	968.3±50.8
	15	13.7	43.3±1.6	1003.3±23.3
	30	13.9	41.6±1.6	991.6±11.6
3. SUV-dialysed	O[a]	19.07	75.0±2.8	0
	15	20.4	71.6±1.6	11.6±11.6
	30	19.0	61.6±2.8[c]	93.3±23.3[c]
4. SUV-undialysed	O[b]	17.67	73.3±1.6	11.6±11.6
	15	17.35	55.0±2.8[c]	123.3±23.3[c]
	30	17.35	56.6±1.6[c]	198.3±11.6[c]
5. MLV-undialysed to SUV-undialysed	15	17.35	75.0±0	0
	30	17.67	70.0±2.8	11.6±11.6

[a] $P < 0.001$, SUV-dialysed vs. MLV-dialysed with respect to % survival and CFU.
[b] $P < 0.001$, SUV-undialysed vs. MLV-undialysed with respect to % survival and CFU.
[c] $P < 0.001$, vs. day 0 formulation.
Statistical significances were calculated by Chi-square test and ANOVA for % survival and CFU respectively.

mice. Hence it was decided to use small unilamellar liposomes without removal of free drug. However to use such preparation in patients, it must be stable for at least a month.

It was found that the efficacy of SUV decreased with storage while the LD_{50} remained comparable to freshly prepared SUVs. The loss in efficacy of stored SUVs could be due to the leakage of AmpB from the liposomes. The encapsulation efficiency of SUV after 30 days was found to be 80% while it was >90% for MLVs. It is commonly accepted that the size stability of MLVs is greater than that of small unilamellar liposomes. Based on these results, formulation MLV-undialysed to SUV-undialysed (Table 3) was selected for clinical use.

Liposome-based systems cannot be sterilized by heat or ionizing irradiation after manufacture. The manufacturing process itself has to be meticulously carried out in a positive pressure sterile room equipped with HEPA filters to generate a sterile product. Several batches of L-AmpB-LRC were prepared under sterile conditions and tested for quality. The product was sterile and pyrogen free with low batch to batch variation[31]. The particle size of MLV (tested using laser light scattering technique) was found to be $1.53 \pm 0.329\ \mu m$ ($n = 10$, mean ± SD).

Phase I clinical trial of L-AmpB-LRC

L-AmpB-LRC was tested for safety in 12 patients with suspected or proven systemic fungal infections. The trial was conducted with the approval of Drug Controller of India and the Ethics Committee of our hospital[32].

L-AmpB-LRC was given intravenously in three escalating doses of 0.1 mg kg^{-1}, 0.4 mg kg^{-1}, and 1 mg kg^{-1} on three days using a syringe infusion pump. Subjective adverse drug reactions were graded as mild (not requiring treatment), moderate (requiring treatment) and severe (requiring discontinuation of therapy). Biochemical parameters were recorded as abnormal if the deviation from pretreatment values was more than 10% and if the values were outside the normal range of that laboratory.

Mild rigors with fever occurred with 0.4 mg kg^{-1} dose in three out of 12 patients. The same three patients experienced moderate rigors with rise in temperature after the dose of 1 mg kg^{-1}.

The changes in biochemical parameters seen in five patients could be explained by disease-related causes since all had advanced disease with multiple concurrent therapies. No cardiac, pulmonary or neurological toxicity was observed during injection of L-AmpB-LRC and in the post treatment follow up.

Of the eight patients receiving conventional Amp B, six had developed moderate to severe fever and chills in spite of pretreatment with hydrocortisone, 100 mg intravenously and/or pheniramine maleate, 50 mg intravenously.

Adverse effects of liposomal AmpB described by other workers[33–36] include somnolence, hyperkalemia, hypokalemia, nausea, vomiting and arthralgia. As mentioned in these reports there were always several other possible explanations related to disease for these manifestations.

Pharmacokinetics

Plasma concentrations of AmpB were measured using

HPLC (sensitivity of the method is 0.05 mcg ml^{-1}). The peak plasma concentration in all our patients was in the range of 0.747 to 1.429 mcg ml^{-1} (1.012 ± 0.055, mean $\pm$ SE) and trough levels at 24 hours ranged from 0.148 to 0.363 mcg ml^{-1} (0.237 ± 0.017, mean $\pm$ SE).

These findings are different from the observations on other liposome preparations. Similar L-AmpB preparations (SUVs) but with either negative or positive charge resulted in higher plasma concentrations (our preparation is neutral)[35,37]. The comparative pharmacokinetic parameters are given in Table 4. This may indicate a wider tissue distribution of our liposomes. However, the interpretation of the pharmacokinetic data is difficult because HPLC method determines total AmpB (viz. free, protein bound and liposomal bound).

Curiously, more recently, Collette *et al.*[38] found that there was no difference in the tissue distribution of AmpB in patients after injecting positively charged SUVs and conventional AmpB, in spite of major difference in their C_{max} and volume of distribution. They also found that the proportion of diffusible, nontissue bound AmpB, which is microbiologically active, was similar using both formulations.

Efficacy studies

Six patients were evaluated for safety and efficacy after treatment with IL-AmpB-LRC. Two patients had been previously treated with free AmpB and had either histologic, culture or radiologically evident progressive fungal disease. The patient characteristics and response to therapy are given in Table 5. Four patients were cured while in two patients the response to therapy was not evaluable as the drug was given irregularly and for shorter duration. The drug was found to be well tolerated by these patients and no limiting toxicity was observed.

Table 4. Comparative pharmacokinetic parameters of different L-AmpB formulations in clinical use

Pharmacokinetic parameters (dose 1 mg kg^{-1})	Reference		
	32*	35**	37†
C_{max} (μg/ml)	0.75–1.43	10.0–20.0	6.0–12.0
$t_{1/2}\,\alpha$(h)	1.18	0.7	–
$t_{1/2}\,\beta$(h)	17.2	25.3	–
AUC (μg h/ml)	11.4	217.0	–
CL (ml/h/kg)	91.7	6.2	–
Vd (L/kg)	2.28	0.19	–

All the formulations are small unilamellar liposomes.
Pharmacokinetic parameters of multilamellar liposomes not evaluable because of rapid clearance from circulation.
Liposome composition:
*Soya phosphatidylcholine : cholesterol, 7 : 3
**Egg phosphatidylcholine : cholesterol : stearylamine; 4 : 3 : 1
†Hydrogenated soya phosphatidylcholine : cholesterol : distearoyl phosphatidylglycerol; 2 : 1 : 0.8
C_{max}–peak plasma concentration; $t_{1/2}\,\alpha$–Distribution half-life; $t_{1/2}\,\beta$–Elimination half-life; AUC–Area under the plasma concentration time curve, CL–Total body clearance; Vd–Volume of distribution.

Problems encountered

We faced the following difficulties in developing our formulation for wide patient acceptability.

Cost of L-AmpB-LRC

With a few exceptions, liposome research has been done

Table 5. Characteristics of patients and response to therapy after treatment with L-AmpB-LRC

Patient no. (age/sex)	Underlying disease	Fungal disease: Diagnosis	Site	Method of diagnosis	Treatment with L-AmpB (mg)	Duration (days)	Response
1. 31, F@	ALL	*C. albicans*	Liver, spleen kidney	Kidney biopsy	3000	60	CR
2. 56/M	CRF	*H. capsulatum*	Liver, spleen, adrenal, kidney	Liver, kidney adrenal, biopsy	1000		*
3. 67/F	CRF with kidney transplant rejection and MI	Mucor	Maxillary sinus	Biopsy	675	8	*
4. 27/M	CRF with kidney transplant rejection	*C. neoformans*	CSF	Culture	1300	60	CR
5. 3/M	Nephrotic syndrome	*C. albicans* casts	Urine	Culture	320	15	CR
6. 9/M	Nephrotic syndrome	*C. albicans* casts	Urine	Culture	330	15	CR

M–Male; F–Female; ALL–Acute lymphoblastic leukaemia; CRF–Chronic renal failure; MI–Myocardial infarction; CSF–Cerebrospinal fluid; CR–Complete response; *–Not evaluable.
@–Patient was refractory to conventional AmpB (fungizone).

in small animals requiring small amounts of lipids to prepare liposomes. In man, the cost of the finished liposomal preparations is prohibitively high at present, placing them beyond the means of most of our patients in India. Our formulation is prepared from less expensive lipids but is as effective as other formulations. In doing so, we were able to reduce the cost of production of liposomes many fold.

Lopez-Berestein *et al.*[33] used synthetic lipids, viz. DMPC and DMPG to prepare liposomes. This results in the cost to the patient in excess of US $12,000 for the recommended therapeutic regimen. AmBisome(R), an L-AmpB preparation marketed in Britain, costs approximately £150 for a 50 mg vial. The cost of a full course of such a preparation is £6000. The cost of our liposomes, excluding the overheads, is approximately Rs 1000 for a 50 mg vial. The complete course of treatment costs Rs 30,000. Although the cost of our liposomes is much less than that of formulations produced abroad, it is obviously still prohibitive for most Indians.

Any attempt to further reduce the cost can only be possible if the raw materials, particularly SPC, are made in India.

Stability of L-AmpB-LRC

Lopez-Berestein *et al.*[34] studied the stability of their MLV preparation in terms of phospholipid and AmpB analysis and found it stable for a year. Heymans *et al.*[39] also reported similar stability for positively charged ampholiposomes on measuring the bioactivity of the preparation.

Our liposomal formulation was tested for stability see Table 2) by checking the biological end point LD_{50} and efficacy) in murine model. As established at present, it has been found to be stable for one month. We are conducting further studies for checking the stability of our preparation over at least one year.

Large scale manufacture of L-AmpB-LRC

The most important aspect is the manufacture of liposome-based drug system on a large scale. We prepare liposomes by the traditional method of thin film hydration to form MLV. This method, although easy, is very time-consuming with the rate limiting step of solvent evaporation.

Pilot plant studies are required to study the various methodologies, viz. spray drying and the use of the solvent injection method for preparing liposomes on large scale. The process must not only assure sterility, nonpyrogenicity and safety but also reproducibility. We are developing methods for overcoming the rate limiting step and are also considering other methods of preparation.

Conclusions

Liposomal drug delivery system is a promising new method to improve drug efficacy and reduce toxicity. Liposome encapsulated AmpB is an effective and non toxic way of treating fungal infections in animals and in man.

L-AmpB, the first liposomal preparation marketed, has been found to be beneficial in several aspects. Its cost remains a major drawback. In India, Bachhawat and coworkers developed liposomal formulation using egg phosphatidylcholine and cholesterol and this preparation was further developed and tested in patients by us. The preparation is easy and has low batch to batch variation. It has been found to be stable over at least one month when stored as MLVs. More studies are under way to improve the formulation to increase the stability over a year.

L-AmpB-LRC was found to be well tolerated and accepted by the patients. There is a major difference in the pharmacokinetics of our preparation from those reported in the literature. The volume of distribution of amphotericin B with our preparation is greater than that noted by other workers (Table 4) and may indicate better tissue delivery.

Our preparation was also found to be effective in a patient with systemic candida infection and safe in a patient with chronic renal failure resistant to AmpB. Further studies are being carried out to establish its efficacy against resistant cases and in patients tolerating conventional AmpB poorly. Another promising application of L-AmpB-LRC is in the treatment of visceral leishmaniasis. Prospective trials with this formulation in leishmaniasis are being planned.

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