Liposomal drug delivery system from laboratory to clinic

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ABSTRACT

The main objective of drug delivery systems is to deliver a drug effectively, specifically to the site of action and to achieve greater efficacy and minimise the toxic effects compared to conventional drugs. Amongst various carrier systems, liposomes have generated a great interest because of their versatility. Liposomes are vesicular concentric bilayered structures, which are biocompatible, biodegradable and nonimmumnogenic. They can control the delivery of drugs by targeting the drug to the site of action or by site avoidance drug delivery or by prolonged circulation of drugs. Amphotericin B (Amp B) remains the drug of choice in most systemic mycoses and also as a second line treatment for Kala azar. However, its toxic effects often limit its use. Although the liposome delivery system has been tried for several drugs, only a few have been used in patients due to the slow development of necessary large-scale pharmaceutical procedures. This paper reviews the development of the technique for liposomal Amphotericin B (L-Amp-LRC-1, Fungisome™) drug delivery system in our laboratory in collaboration with the department of Biochemistry, Delhi University in India and proving the safety and efficacy of this preparation in clinical practice. It also attempts to compare the efficacy and benefits of our product for Indian patients with those of similar products and it includes facts from the publications that flowed from our work. As compared to conventional Amp B, Fungisome is infused over a much shorter period requiring a smaller volume and no premedication. It was found to be safe in patients who had developed serious unacceptable toxicity with conventional Amp B. In renal transplant patients, Fungisome did not produce any nephrotoxicity. Fungisome is effective in fungal infections resistant to fluconazole, conventional Amp B and in virgin and resistant cases of visceral leishmaniasis. The cost of any drug is of great significance, especially in India. We have therefore devoted a section of our review to the relative costs of our product and those of other commercially available products. This patient-worthy formulation is safe, efficacious and cheaper than the commercially available formulation of liposomal amphotericin B. The product has been patented and technology transferred to a pharmaceutical company for marketing. Results of postmarketing study also document safety and efficacy as observed in premarketing studies. A brief review of this work is provided here.

KEY WORDS: Clinical trials, drug delivery system, liposomal amphotericin B (L-Amp-LRC-1)

Drug delivery systems have been envisaged and developed to deliver the drug to the site of action (targeted delivery) to avoid fluctuations in plasma drug levels (controlled release), slow release and in recent years to overcome cellular barriers and enzymatic degradation, which impede absorption. Development of new drug molecules is expensive and time-consuming. Improvement of the safety-efficacy ratio of "old" drugs is achieved by individualising drug therapy, dose titration and therapeutic drug monitoring. Developing drug delivery systems is another attractive option and is being pursued very vigorously to improve the safety and efficacy of drugs.

In India, pioneering work on liposomes was done by Dr. B. K. Bacchawat. He was remarkably far-sighted. He recognised the potential of liposomes in therapy in the mid-1980s and set up his laboratory in New Delhi. The development of the technique in India for preparing liposomes, successfully encapsulating amphotericin within them and proving the efficacy of this preparation in clinical practice owes much to him. The first two authors (NAK, SKP) had the good fortune of interacting with him and were thrilled when he offered us the fruits of his labours in the laboratory for use in clinical medicine. He pointed out to us that once this technique was perfected, other drugs could be similarly incorporated into liposomes.

Under his guidance and with his encouragement, we set up a liposome research laboratory in Seth G.S. Medical College and K.E.M. Hospital. The progress was slow but steady and by 1993 we were able to report on the pharmacological and preclinical tests of our product.[1]–[3] Our liposomal amphotericin B (L-Amp-LRC-1) has been used not only in the treatment of life-threatening systemic fungal infection but also in the treatment of leishmaniasis, especially in patients in whom conventional drugs prove to be ineffective.

This paper reviews the publications that flowed from our work and attempts to compare the efficacy and benefits of our product for Indian patients with those of other similar products. The cost of any drug is of great significance, especially in In-

dia. We have therefore devoted a section of our review to the relative costs of our product and those of other commercially available products.

Once the efficacy and safety of our liposomal amphotericin B had been proven in a statistically significant number of patients, we attempted to transfer our technology from laboratory to large-scale production and distribution. Our experiences may help others with similar interests to reduce the inordinate delay in such transfer.

As predicted by Dr. Bacchawat, we have now turned our attention to alternative routes for the administration of liposomes and other drugs that can be delivered via liposomes.[4]–[7] A brief review of this work is also provided.

Amphotericin B

Amphotericin B (Amp B) is a polyene macrolide antibiotic that is widely used for the 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 immuno-deficiency diseases.[8] The majority of these infections are caused by the species of *Candida* and *Aspergillus*. Despite the development of new classes of antifungal agents, Amp B 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. Amp B 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 Amp B develop acute infusion-related reactions such as fever, chills, nausea and vomiting.[9] This is in spite of the liberal use of premedications intended to prevent such side effects. Clements and Peaco[10] observed that nephrotoxicity is one of the most important chronic toxicities associated with Amp B usage because of its potential limiting effect on the total course of therapy. Nephrotoxicity is present in about 60–83% of patients. Another important and commonly encountered chronic toxicity is electrolyte disturbance secondary to renal wasting of potassium and magnesium. Ninety percent of patients on Amp B treatment require potassium supplementation.[10],[11]

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

Liposomal drug delivery system

The main objective of drug delivery systems is to deliver a drug effectively, specifically to the site of action and to achieve greater efficacy and minimise the toxic effects compared to conventional drugs. Amongst various carrier systems, liposomes have generated a great interest because of their versatility. Liposomes are vesicular concentric bilayered structures, which are biocompatible, biodegradable and nonimmumnogenic. They can control the delivery of drugs by targeting the drug to the site of action or by site avoidance drug delivery or by prolonged circulation of drugs.

Liposomal Amp B

Preclinical studies

Background information

Since the study by New *et al*.,[12] much interest has been centred on the use of liposomes as a drug carrier for Amp B in the treatment of several systemic fungal and parasitic infections. It was shown that L-Amp B was as effective as free Amp B in experimental histoplasmosis[13] and cryptococcosis[14] but much less toxic.[15] Lopez-Berestein *et al* carried out extensive studies on the use of L-Amp B in systemic candidiasis and paved the way for its clinical use.[15] They used multilamellar liposomes prepared form dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPC) in a 7 : 3 molar ratio. The toxicity of L-Amp B was far less than that of free Amp B, without any loss of activity against *Candida albicans*. L-Amp B also proved to be superior to Amp B in the prophylaxis and treatment of experimental candidiasis in neutropenic mice.[15]

Hopfer *et al*.[16] observed that the lipid composition of the liposomes played a major role in L-Amp B activity. The presence of a sterol component (like ergosterol and cholesterol) in liposomes decreased the antifungal activity by almost 50-fold. However, other workers found that the incorporation of cholesterol in liposomes did not result in any loss of activity.[17]–[20] Tremblay Gondal *et al*. and Szoka *et al*. postulated that in multilamellar vesicles (MLV), only about 10% of the lipid is on the external monolayer and the transfer of Amp B from the internal lamellae to the fungal cell cannot take place readily. While small unilamellar vesicles (SUV) containing cholesterol have about 50–60% of the lipids on the monolayer accounting for better transfer of Amp B.

With this concept, two SUV formulations were developed. Negatively charged small unilamellar vesicles made from hydrogenated soya phosphatidylcholine (SPC), cholesterol and distearoyl phosphatidylglycerol (DSPG) in 2 : 1 : 0.8 molar ratios were tested in murine candidiasis and cryptococcosis.[18] 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 stearylamin in 4 : 3 : 1 molar ratio.[21]

Indian studies

During the same time, Ahmad *et al*.,[22] in India, formulated small unilamellar liposomes from egg phosphatidylcholine (EPC), dipalmitoyl phosphatidyl ethanolamine (DPPE) and cholesterol. They showed that this liposomal intercalation of Amp B has reduced toxicity and improved therapeutic efficacy in murine aspergillosis compared to the free drug. They also demonstrated better delivery of Amp B to infected tissue. Liposomes grafted with mannose are taken up by macrophages because of the presence of mannose receptors on their sur-

face. These were more effective than nonmannosylated liposomes in the treatment of murine aspergillosis.[22],[23] Liposomes made from EPC and cholesterol were also effective in the treatment of murine aspergillosis.[24]

Interestingly, the tissue distribution of Amp B after incorporation into liposomes of various lipid compositions, size and surface charge was comparable.[15],[24]–[27] High levels of Amp B were detected in organs like liver, spleen and lungs, which are rich in macrophages. Amp B was detected in brain at potentially therapeutic concentrations showing good penetration through the blood–brain barrier.[18] The pharmacological basis for the enhanced therapeutic index observed with L-Amp B is not clearly understood. The efficacy of L-Amp B 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.[28]–[30] Liposomal Amp B retains antifungal potency without any toxicity to red blood cells.[31]

L-Amp-LRC-1: 'Patient-worthy" formulation for clinical use developed in India

Despite several promising studies in animals, liposomal drug delivery system did not progress. Any parenteral preparation must be sterile, pyrogen free, safe and stable; liposome-based formulations are no exception. Until mid-1990s, the clinical trials were limited to a few patients. This was partially due to the reluctance of large pharmaceutical companies to become involved with a not yet proven 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 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 the development of at least 10 different liposomal drugs for clinical trials which were able to overcome at least some of the initial problems.[32] In view of the favourable outcome observed in experimental animals, liposomal Amp B was used in patients.

Preclinical and pharmaceutical development

L-Amp-LRC-1 developed by Ahmad *et al.*[24] was further tested and developed at our hospital for clinical use. In the first series of experiments on murine aspergillosis by Ahmad *et al.*,[22] small unilamellar Liposomes prepared from EPC, DPPE and cholesterol were used. This preparation had LD50 of 8.1 mg/kg compared to 1.2 mg/kg with free Amp B. It was also found to be effective at doses of 0.5 mg/kg, with 70% of animals surviving on the 7th day compared to 13% with free Amp B. Although these formulations were easy to manufacture, the cost was exorbitant because of the use of DPPE and *p*-aminophenyl-D-mannopyranoside. Later, Ahmad *et al.*[24] showed that SUVs prepared from naturally occurring, inexpensive EPC/SPC and cholesterol were as effective as other formulations.

We studied the various formulations of L-Amp-LRC-1 using SPC and cholesterol to determine the ease of manufacturing while retaining reduced toxicity and improved efficacy against fungal pathogens.[1]–[3] We were able to encapsulate more than 90% Amp B into liposomes. Ahmad *et al.*[24] advocated the use of dialysis to remove unencapsulated Amp B 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 the free drug from that bound to liposome by dialysis, since the LD50 and efficacy were similar to that of dialysed and undialysed formulations. The toxicity of SUV was lower than that of MLV. Similar findings were observed by Szoka *et al.*,[20] SUVs were significantly more effective than MLVs as judged by the survival pattern and colony forming units (CFUs) in infected mice. Hence, it was decided to use small unilamellar liposomes without removal of free drug. However, to use such a 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 LD50 remained comparable to freshly prepared SUVs. The loss in efficacy of stored SUVs could be due to the leakage of Amp B 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, converted before use to SUV – undialysed (Table 1) was selected for clinical use.

Liposome-based systems cannot be sterilised by heat or ionising 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. We prepared several batches of L-Amp-LRC-1 under sterile conditions and tested for quality. The product was sterile and pyrogen free, with low batch-to-batch variation.[1],[2] 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 trials of Indian L-Amp-LRC-1

L-Amp-LRC-1 was tested for safety in 12 patients with suspected or proven systemic fungal infections. The trial was conducted with the approval of the Drug Controller General of India and the Ethics committee of our hospital.[2],[3] L-Amp-LRC-1 (FUNGISOME™) was given intravenously in three escalating doses of 0.1, 0.4, and 1 mg/kg 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 the laboratory. Mild rigors with fever occurred with 0.4-mg/kg dose in three out of 12 patients. The same three patients experienced moderate rigors with rise in temperature after a dose of 1 mg/kg. 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 with the injection of L-Amp-LRC-1 and in the post-treatment fol-

Table 1: Effect of various L-Amp-LRC-1 formulations on safety, efficacy and stability

Formulation	Day	LD50 (mg/kg)	Seven days after therapy (dose 0.5 mg Amp B/kg) Mean ± SE of three experiments	
			% Survival	CPU
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
MLV-undialysed	0	14.17	40.0 ± 2.8	968.3 ± 5.8
	15	13.7	43.3 ± 1.6	1003.3 ± 23.3
	30	13.9	41.6 ± 1.6	991.6 ± 11.6
SUV-dialysed	0a	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]
SUV-undialysed	0b	17.67	73.3 ± 1.6	11.6 ± 11.6
	15	17.35	55.0 ± 2.8***	123.3 ± 23.3***
	30	17.35	56.6 ± 1.6***	198.3 ± 11.6***
MLV-undialysed to SUV-undialysed	15	17.35	75.0 ± 0	0
	30	17.67	70.0 ± 2.8	11.6 ± 11.6

* $P < 0.001$; SUV-dialysed *vs* MLV-dialysed with respect to % survival and CFU; ** $P < 0.001$; SUV-undialysed *vs* MLV-undialysed with respect to % survival and CFU; *** $P < 0.001$ *vs* day 0 formulation; MLV, multilamellar vesicles; SUV, small unilamellar vesicles

low-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.

Phases II and III efficacy studies in patients with systemic fungal infections

After assessing safety and pharmacokinetics, efficacy of L-Amp-LRC-1 was investigated in patients suffering from systemic fungal infection. Initially, the Phase II study was carried out to assess safe and effective dose. Subsequently, Phase III study was carried out to compare safety and efficacy of L-Amp-LRC-1 with plain amphotericin.[33]–[35] Phase III B was carried out in patients not responding to standard treatment. In all these studies, standard protocol, which was approved by the Drug Controller General of India, and local Ethics committee, was used. Male and female patients of different age groups with clinically and or radiologically suspected and microscopically proven systemic fungal infections were included in these studies. Written informed consent of all the patients was taken. Patients were free to withdraw from the study at any time without giving reasons. Patients with proven systemic infections were excluded from the present study. So also if patient was pregnant or if a patient was moribund or other antifungal drugs were being co-administered. For patients suffering from renal disease, yet requiring administration of Amp B, dose of L-Amp-LRC Amp B was suitably adjusted in consultation with a nephrologist. The response to therapy was judged by assessing clinical signs and symptoms, radiological features and microbiological findings

Patients were judged to have complete response, partial response or as nonevaluable response based on standard criteria. The occurrence of and severity of adverse drug reactions was monitored daily and their temporal relation to drug administration was recorded.

In Phase II study, all patients received L-Amp-LRC-1. In Phase III comparative study, patients received either conventional amphotericin or L-Amp-LRC-1.

Drug administration

Fungizone (conventional Amp B). Initially, a small test dose (1 mg dissolved in 20 ml of 5% dextrose solution) was administered intravenously over 20–30 min. The temperature, pulse, respiratory rate and blood pressure were monitored every 30 min for 4 h. If no reaction was observed, then these patients were given 0.1 mg/kg of Amp B intravenously dissolved in 5% dextrose solution over a period of 2–6 h. This was increased by 5–10 mg/day to maximum of 1 mg/kg/day. All the doses were given using a sterile disposable intravenous infusion set.

L Amp LRC-1. This was given intravenously in three escalating doses 0.1, 0.4, and 1 mg/kg on 3 days after diluting these in normal saline I.P 20, 50, and 50 ml, respectively. On the first day, 1 mg (of the first dose of 0.1 mg/kg diluted with normal saline I.P) was given intravenously as the test dose. The temperature, pulse, respiratory rate and blood pressure were monitored every 30 min for 4 h. If no reaction was observed, then these patients were injected the rest of the first dose over 15 min. The remaining two doses were given over 30 min and 1 h, respectively. All the doses were given using disposable intravenous infusion sets. The dose of 1 mg/kg/day was administered for as long as necessary, generally for 3–8 weeks. If required, the dose was increased to a maximum of 3 mg/kg body weight based on the patients' response and adverse reactions, if any.

Other drugs

No other antifungal drug was coadministered. A detailed record was made of all the other drugs administered to these patients

Phase II study

Phase II study to assess dose, efficacy and safety was carried out in 77 patients suffering from systemic fungal infection. Their details are summarised in Table 2. Fifty patients were evaluable; complete response was observed in 32/33 cases of

Table 2: Response to L-Amp-LRC-1 in Phase II studies (total no. of patients: 77; evaluable cases: 50; Nonassessable cases: 27)

Infection	A	CR	PR	NR
Candidasis	23	23	-	-
Candiduria	8	7	1	-
Disseminated candidiasis	1	1	-	-
Oral candidiasis	1	1	-	-
Cryptococcal meningitis	7	6	1	-
Aspergillosis	7	4	1	2
Mucormycosis	2	-	1	1
Cladiosporosis	1	1	-	-
Total	50	43	4	3

A, assessable; RC, complete response; PR, partial response; NR, no response.

candida, 6/7 cases of *cryptococcal* meningitis, 4/7 cases of *aspergillosis* and 1/1 case of *cladiosporosis*. Four cases had partial response, while three failed to respond. Chills and fever occurred with 7.28% of infusion; nausea, vomiting, backache and excessive drowsiness occurred on 2.7% occasions, 19.48% received potassium supplements.

Thus L-Amp-LRC-1 was observed to be a safe and effective drug when used in the dose of 1 mg/kg/day. In two patients, the dose was required to be increased to 2 and 3 mg/kg/day; this dose was safe.

Phase III study

Group III A: Comparison of safety and efficacy of L-Amp-LRC-1 and Amp B

Patients selected for III A study were randomised into two treatment groups, one receiving commercial formulation [Fungizone (conventional Amp B)] and the other receiving L-Amp-LRC-1 after they were stratified by the infecting organism and site of infection and primary diseases. The patients in Group III A, treated with Fungizone therapy and showing poor tolerance or lack of response, were subsequently treated with L-Amp-LRC-1.

Group III B: Effect of L-Amp-LRC-1 in nonresponders or patients intolerant to Amp B

This was an open study to assess the safety and efficacy of L-Amp-LRC-1 in patients not responding to (as shown by histology or microbiology) or not tolerating conventional Amp B or other antifungals. Such patients were treated with L-Amp-LRC-1 after discontinuing the original antifungal drug.

Group III-C: L-Amp-LRC-1 in neonates

In this study, L-Amp-LRC-1 treatment was given to neonatal patients on specific request for L-Amp-LRC-1 from the physician in charge.[36],[37] These patients were suspected to be suffering from systemic fungal infection and were in critical condition. They were grouped separately since either their diagnoses were not proven or they were not randomised to two regimens of III A.

A total of 55 patients suffering from systemic fungal infection were enrolled in the study. Their demographic data, dose and number of infusions of Amp B or L-Amp-LRC-1 are given in Table 3. Thirty nine patients were enrolled in Group III A. Ninteen out of 39 received Amp B and 20 received L-Amp-LRC-1. Seventeen patients from each of these groups were evaluable; their ages ranged from 14 days to 36 years, 2 days to 65 years and body weight 1–60 kg, respectively.

Safety

For safety assessment, data on all the patients (in Groups IIIA, IIIB, and IIIC) who received plain amphotericin or L-Amp-LRC-1 are considered. It was noted that L-Amp-LRC-1 was better tolerated than Amp B. Out of the 695 infusions of Amp B, fever occurred on 25.4% occasions in 52% patients, despite pretreatment with paracetamol and hydrocortisone, while it occurred on 4.07% occasions out of 1309 infusions (in 25% patients) of L-Amp-LRC-1. Chills occurred on 16.8 and 2.0% occasions after Amp B and L-Amp-LRC-1, respectively. Other adverse effects were observed on 0.2–5% occasions, headache, nausea, vomiting, palpitations, and dizziness occurring more frequently in the Amp B group. Chest pain was observed in one patient and apnea was noted in two neonates given L-Amp-LRC-1. Tachyponea, tachycardia and dizziness were noted only in the Amp B group.

Bronchospasm. One patient from Group III A treated with Amp B developed bronchospasm and was shifted to L-Amp-LRC-1 group. He did not develop bronchospasm with L-Amp-LRC-1. Before shifting to L-Amp-LRC-1 treatment, the patient was given L-Amp-LRC-1 on one day and Amp B on the next day. The patient developed bronchospasm to Amp B but not to L-Amp-LRC-1.

Creatinine. Data on patients who received more than 40 doses of Amp B or L-Amp-LRC-1 were specifically analysed for effect on creatinine. Patient who received lower doses had no significant alteration in creatinine. Eleven patients received 44-147 infusions of L-Amp-LRC-1 (2.2–10.99 g). Three pa-

Table 3: Demographic data of patients entered in Group III comparative study

Treatment	IIIA Amp B	IIIA L-Amp	IIIB L-Amp	IIIC L-Amp
Total no.	19	20	5	11
Evaluable	17	17	5	–
Adults	6	8	4	0
Male	4	5	3	0
Female	2	3	1	0
Children	4	2	1	0
Male	2	2	1	0
Female	2	0	0	0
Neonates	9	10	0	11
Male	4	4	0	7
Female	5	6	0	4
Number of infusions	695	679	387	155
Dose (mg/kg)	0.5–1.0	0.5–2.0	1.0–2.0	0.5–1.0

Amp B = Amphotericin B, L-Amp = L-Amp B-LRC-1.
Five patients were enrolled in Group IIIB, four due to toxicity of Amp B and one due to nonresponse to Amp B. Eleven patients (Group IIIC) were treated empirically specially on Physician's request for L-Amp B-LRC-1 outside protocol.

Table 4: Response in III A patients

Infection	T	A	CR	PR	Fungizone Transferred to L-Amp B-LRC-1 due to NR or toxicity	Response to L-Amp B-LRC-1	L-Amp B-LRC-1 T	A	CR	PR	NR
Candidiasis	8	7	7	–	–	–	6	6	6	–	–
Candiduria	3	3	2	–	1 (Toxicity)	CR	5	4	4	–	–
Disseminated candidiasis	1	0	–	–	–	–	1	1	1	–	–
Oral candidiasis	–	–	–	–	–	–	1	1	1	–	–
Cryptococcal meningitis	2	2	1	–	1 (NR)	NA	2	1	1	–	–
Aspergillosis	2	2	1	–	1 (Toxicity)	NR	1	1	1	–	–
Mucormycosis	2	2	2	–	–	–	3	2	2	–	–
Histolasmosis	1	1	1	–	–	–	1	1	1	–	–
Total	19	17	14	–	3	–	20	17	17	–	–

T, total; A, assessable; CR, complete response; PR, partial response; NR, no response; L-Amp B-LRC-1, liposomal amphotericin B.

tients had renal disease, two having had renal transplant. In none of these patients was there any significant increase in creatinine. One patient was a case of multiple myeloma with chronic renal failure and renal transplant. His creatinine had risen to 6.7 mg% with 12 doses of Amp while no rise in creatinine occurred after 75 doses of L-Amp-LRC-1. Five patients had been treated with only plain Amp B. One of these was a case of chronic renal failure; his creatinine increased to 11.8 mg% and he required repeated dialysis to complete 40 doses of Amp B.

Potassium. Out of patients whose pretreatment potassium was normal, in 10/20 (50%) patients on L-Amp-LRC-1 and 10/13 (76.9%) on Amp B, serum potassium decreased from a pretreatment normal value below normal during treatment. These were restored to normal by treatment with an oral potassium supplement. Potassium was reduced by more than 10% of pretreatment value in 10/30 (33.3%) patients who received L-Amp-LRC-1 and 12/16 (75%) patients on Amp B. In 12 patients who received less than 10 infusions, post-treatment data are not available.

Haemoglobin. In the neonatal and paediatric group, 9/24 (37.5%) patients on L-Amp-LRC-1 and 4/13 (30.7%) patients on Amp B, haemoglobin decreased by >0.5 g% and eight were given blood transfusions during treatment. 4/13 (30.7%) patients on Fungizone and 4/24 (16.6%) on L-Amp-LRC-1 in the neonatal and paediatric group received blood transfusions.

SGOT and SGPT. SGOT and SGPT were elevated by more than 10% in 12/30 (30%) patients in L-Amp-LRC-1 and in 6/16 (37.5%) patients in the Amp B group. The rise was marginal in all patients but two patients in the Fungizone group the rise was substantial (to 577 and 377 IU/ml). In 12 patients who received less than 10 infusions, post-treatment data are not available.

Efficacy

Group III A patients

Thirty-nine patients were enrolled in Phase III A fungal diagnosis and response to the treatment is given in Table 4

Group III B patients

Group III B consisted of five patients. Four developed toxicity to Amp B and one did not respond to Amp B. Details of patients are given in Tables 5 and 6. Three patients in this group (1 disseminated candidiasis and 2 mucormycosis) responded completely. Two patients of aspergillosis failed to respond.

Effect of liposomal amphotericin in patients pretreated with fluconazole

Seven patients in the III A and III B groups who had been previously treated with fluconazole with or without Amp B did not respond. Three responded to treatment with L-Amp-LRC-1. Details of these patients are given in Table 7.

Safety of L-Amp-LRC-1 in patients intolerant to conventional Amp B

L-Amp-LRC-1 was given to nine patients who had not tolerated conventional Amphotericin and all of these tolerated L-Amp-LRC-1 well. These patients received 5–256 infusions of liposomal Amphotericin (10—11 230 mg). One patient, who had developed severe life-threatening bronchospasm leading to cynosis, was given L-Amp-LRC-1 without any adverse effects. Data on four patients who had developed rise in creatinine after 3–7 doses of conventional Amphotericin and who tolerated L-Amp-LRC-1 are given in Table 8.

Comparison of L-Amp-LRC-1, plain Amphotericin and Ambisome (marketed Liposomal preparation) in animals

Comparison of L-Amp-LRC-1 with marketed liposomal amphotericin could not be carried out in patients due to cost constraints. However, the study was carried out to compare efficacy and tissue distribution of L-Amp-LRC-1 to plain Amp

Table 5: Response to L-Amp-LRC-1 of Group IIIB patients

Infection	Total	A	CR	PR	NR
Disseminated Candidiasis	1	1	1	–	–
Aspergillosis	2	2	–	–	2
Mucormycosis	2	2	2	–	–
Total	5	5	3	–	2

A, Assessable; CR, Complete response; PR, Partial response; NR, No response.

Table 6: Details of patients in Group IIIB

Sex	Age	Fungal diagnosis	Effect of Amp B	Effect of L-Amp B-LRC-1
M	45	Mucormycosis sinus	Sever fever rigor, rise in creatinine	CR (115 doses)
M	40	CNS aspergilloma	No response	NR (99 doses)
M	75 days	Canidial ventriculitis	Toxicity (vomiting)	CR (27 doses)
M	53	CNS aspergilloma	Toxicity rise in creatinine in 12 doses (also received fluconazole for 4 months)	NR (75 doses)
F	60	Mucormycosis	Toxicity, rise in S. Cr. (three doses)	CR (80 doses)

CR, Complete response; NR, No response; L-Amp-B-LRC-1, Liposomal amphotericin B; CNS, Central nervous system

Table 7: Effect of L-Amp B-LRC-1 in patients pretreated with Fluconazole

Sex	Age	Fungal diagnosis	Fluconazole	Response to L-Amp B-LRC-1
F	55	Oral candidiasis	200 mg x 5	CR
M	30	Cryptococcal granuloma	(*)	CR
M	0.5 month	Candidemia	3.0 mg/kg BD x 14	NA three doses
M	40	*Aspergillus*	200 ´ 60 + (plain Amp B)	NR
M	40	Candidemia	3.0 mg/kg/day ´ 16	CR
F	Day 22	Candidemia	3.5 mg BD ´ (*)	NA two doses
M	53	Aspergillosis	200 ´ 180 oral and then IV + Fungizone	NR

*Details not available; CR, Complete response; NR, No response

Table 8: Creatinine levels in patients after plain amphotericin and L-Amp B-LRC-1

Serum creatinine after receiving plain Amp	Serum creatinine after receiving L-Amp B-LRC-1	No. of days of doses of L-Amp B-LRC-1
0.87–1.88 mg%	1.47 mg%	41 days: 1.0 mg/kg 213 days: 0.8 mg/kg
2.91 mg%	1.03 mg%	56 days: 1.0 mg/kg
4.44 mg%	2.25 mg%	33 days: 1.0 mg/kg
2.6 mg%	1.89 mg%	27 days: 1.0 mg/kg

B and AmBisome in *Aspergillus* murine model. In the *Aspergillus* murine model, established infection proved to be lethal to untreated control mice and the mortality was 100% by day five; a 0.5 mg/kg dose of L-Amp-LRC-1 (SUV) and AmBisome resulted in comparable survival and were more effective than same dose of plain Amp B. On increasing the dose of L-Amp-LRC-1 and AmBisome, the survival increased. The CFU counts in the lungs of control mice were significantly higher than lung CFUs treated with L-Amp-LRC-1 or AmBisome. CFUs with L-Amp-LRC-1 and AmBisome treated animals were comparable. In *Aspergillus* infected mice, the Amp B levels in lungs were higher but lower in kidney with L-Amp-LRC-1 than those in conventional Amp B. This explains the better efficacy and reduced toxicity of L-Amp-LRC-1. Concentration in lungs is higher, which may be because of greater entrapment of L-Amp-LRC-1 in lungs. In comparison to L-Amp-LRC-1, liver concentrations with Ambisome are high, which can be explained by prolonged circulation time and negative charge of AmBisome. Lung Amp B concentration of AmBisome is less than L-Amp-LRC-1, which may be because of smaller size of AmBisome. Although the concentration is less, efficacy is comparable which may be because of more sustained levels in blood. Lung Amp B concentration of SUV is less than that of MLVs (L-Amp-LRC-1), which may be because of the smaller size of SUVs. Thus, efficacy of L-Amp-LRC-1 and AmBisome is comparable and higher than conventional Amp B.

Efficacy and safety of L-Amp-LRC-1 in leishmaniasis

Ahmad *et al*.[22],[24] studied tissue distribution and antileishmanial activity of the Indian L-Amp-LRC-1 in BALB/c mice infected with *L. donovani* and noted that it was significantly more effective than the free form efficacy in patients. In clinical trials, L-Amp-LRC-1 was found to be effective in patients with visceral leishmaniasis resistant to antimony, pentamidine and interestingly even to Amp B. Bodhe *et al*.[34],[38] and Gokhale *et al*.[39] reported initially a case resistant to antimony and pentamidine, who responded completely to 21 doses of 1 mg/kg/bw of L-Amp-LRC-1. Since then, different dose schedules from 1 to 3 mg/kg/day given over 21–5 days have been investigated for efficacy. As expected, virgin cases (not having received any treatment) have responded to shorter courses of as low as 2 mg/kg for 10 days or even 3 mg/kg for 5 days.[34] Patients resistant to other drugs required higher doses. Bodhe *et al*. reported two cases resistant to Amp B successfully treated with L-Amp-LRC-1. One of the patients who had not responded to three courses of 20-mg/kg/day for 30 days of antimony and 48 doses of 50 mg Amp B was cured after receiving a total dose of 84 mg/kg of L-Amp-LRC-1. In another patient who had not responded to 26 days of 20 mg/kg/day antimony and 10 doses of 50 mg/day amphotericin B, responded completetly with 3 mg/kg/day of L-Amp-LRC-1. Fever, rigor and chills occurred in less than 10% of patients; no increase in creatinine was noted even in a patient with renal transplant. L-Amp-LRC-1 could be administered to a 7-year-old child with antimony-resistant visceral leishmaniasis with no adverse reactions except mild fever.[40] L-Amp-LRC-1 has other advantages such as administration of infusion over 1 h (compared to 4 h for Amp B), shorter courses (5–7 days) and lower per dose cost.

Quality control of L-Amp-LRC-1

Initially for early clinical trials, a lab-scale method was used. Later, a pilot-scale manufacturing plant was set up to cater to the requirement of Phase III study. The batch-to-batch variation and suitability for human administration of L-Amp-LRC-1 prepared by laboratory- and pilot-scale methods were tested using the following criteria.

- test for sterility,
- pyrogen test,
- Amp B content,
- phospholipid content,
- methanol content,
- LD50 and minimum lethal dose,
- test for efficacy,
- particle size measurement.

It was seen that pilot-scale batches passed quality control tests and the stability was 2 years

Comparison of Fungisome™ with marketed lipid formulations of Amp B

Apart from Fungisome, three other lipid formulations of Amp B exist: Amp B lipid complex (ABLC; Abelcet/Amphotec), Amp B colloidal dispersion (ABCD; Amphocil) and liposomal amphotericin (Ambisome). Commercially available liposomal amphotericin, i.e., Ambisome is recommended at the dose of 3–5 mg/kg/day whereas the recommended daily dose for Abelcet is 5 mg/kg/day and for Amphocil 3–4 mg/kg/day.[41] Fungisome is recommended in the dose of 1–3 mg/kg/day. These clinical recommendations are based on several clinical trials. The duration of therapy has to be individualised. Majority of clinical efficacy data related to the lipid-based Amp B are derived from compassionate use studies and small case series. Few randomised studies have been performed comparing various lipid formulations to Amp B. These studies show that lipid preparations are either superior to Amp B or equal to that in terms of outcome. The relative merits of these drugs are summarised in Table 9.

All commercially available lipid formulations have well-defined size range, C max, volume of distribution (Vd) and AUC. However, it is not clear whether these differences are clinically relevant.[42] The comparative properties of Amp B and commercially available lipid formulations of Amp B are outlined in Table 10. Fungisome and Ambisome are the only products that contains true liposomes. The major advantage of these lipid formulations compared to Amp B is a reduction in two forms of toxicity, which include infusion-related toxicities (nausea, vomiting, chills, and fever) and nephrotoxicity. Clinical experiences are now sufficient to state that lipid formulations of Amp B have a clear safety profile. The study of infusion-related reactions on the first day of infusion by Wingard *et al*. is the only study comparing several formulations of lipids. Ambisome was given at 3 and 5 mg/kg/day and Abelcet 5 mg/kg/day, which showed that there were no major differences between 3 and 5 mg/kg/day doses of Ambisome, but Abelcet led to significantly higher infusion-related reactions and nephrotoxicity. Thus, this prospective study shows that liposomal amphotericin B has a better profile with regard to day 1 infusion-related reactions and nephrotoxicity. Table 11 gives the frequency of adverse events attributed to lipid-based formulations of Amp B, based on available data from both open and comparative clinical trials as well as our own experience.
However, direct comparison of the safety and efficacy of these commercially available formulations in humans is difficult, since the treatment with each of the drug needs to be individualised. All formulations are better tolerated than conventionaL-Amp-B and at the same time are effective in the treatment of systemic fungal infections. The acquisition cost of Fungisome is less than that of the other marketed lipid preparations in India. The total cost of treatment for a person weigh-

Table 9: Comparative properties of Amp B and commercially available lipid formulation of Amp B

Formulation	Size (nm)	Structure	Dose (mg/kg)	C_{max}	AUC (mg h/l)	V_d (l/kg)
Amp B	0.035	Micelles	0.6	1.1	17.1	5.1
Ambisome	<0.080	Liposomes	3-5	83	555	0.11
Abelcet	1.6–11	Ribbon like	5	1.7	9.5	131
Amphocil	0.11–0.014	Disc like	5	3.1	43	4.3
Fungisome (Gokhale *et al*. 1999)	0.01 ± 0.014	Liposomes	1-3	1.012	11.426	2.285

V_d, volume of distribution.

See Ref. 42.

Table 10: Relative merits of commercial lipid preparations

Preparations	Amphotec[41],[43] ABCD	Abelcet[41],[43],[46] ABLC	Ambisome[41],[43],[45]	Fungisome[33],[51]
Dose	3–4 mg/kg/day	5 mg/kg/day	3–5 mg/kg/day	1–3 mg/kg/day
Efficacy*				
Aspergillosis	34%	46%	61%	64%
Candidiasis	59%	75%	80%	82.6%
Cryptococcosis	45%	67%	67–85%	85%
Mucormycosis	NA	71%	NA	72.7%
Febrile neutropenia	50%	33%	58.7%	NA

* Efficacy based on noncomparative clinical trials.

ABCD, amphotericin B colloidal dispersion; ABLC, amphotericin B lipid complex.

ing 50 kg (for 4 weeks of treatment) is approximately Rs. 165,200.00 whereas other marketed preparations cost more: Ambisome [Rs. 873,600.00, Ampholip Rs. 514,500.00, Amphocil Rs. 962,080.00 (Table 12)]. Efforts are going on to further reduce the cost by pharmaceutical manufacturers. In comparison to the acquisition cost of the marketed liposomal preparations in India, the cost of Fungisome is 8–10 times less. Fungisome is thus a safe and cost-effective option for Indian physicians.

Problems encountered

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

Cost of L-AMP-LRC-1 (Fungisome)

With a few exceptions, liposome research has been done 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. Any attempts to further reduce the cost can only be possible if the raw materials, particularly SPC, are made in India.

Industry partner

We initially thought that taking drugs from laboratory to clinic will be the most difficult task given that liposomal drugs had to be sterile, stable, pyrogen free, safe and effective. However, as can be seen from the studies reported above, we successfully overcame these difficulties and developed a product that will save lives and reduce foreign exchange. Marketing of this drug is certainly a major boost to liposome research in India.

Suggested solutions to our problem

The laboratory research scientists in India should interact closely and frequently with clinical researchers, to identify the needs and to take the benefit of their research to the patients. Industry partners should be involved in research with potential to develop marketable products at early stages in research. The funding agencies in the country should actively and quickly respond to promising research development.

Summary

Steps in Fungisome development

1. Liposomes are concentric multilamellar (or unilamellar) bilayered structures made from lipids and aqueous layers. Liposomes are biocompatible and biodegradable. They are therefore a very promising mode of drug delivery.
2. The incidence of systemic infection is increasing worldwide on account of increase in immunodeficient disease/conditions, viz.; AIDS, use of cancer chemotherapy and use of immunosuppressants in patients receiving organ transplants. Visceral leishmaniasis is a major problem in Bihar and in other states. It also occurs in other countries in Africa and Latin America.
3. Conventional Amp-B is in use for the past 40 years for the treatment of systemic fungal infection. It is used as a second line treatment for kala azar. The limiting factor in the treatment with Amp B is toxic effects associated with the drug and resistance especially in kala azar.
4. Liposome made of soya lecithin and cholesterol can act as carriers for Amp B. These components occur naturally and are nontoxic. A laboratory method to prepare patient-worthy, sterile, pyrogen free L-Amp-LRC-1 formulation (Fungisome) was developed.[1],[2] Quality control specifications were defined and batches prepared were tested in conformation to these.
5. L-Amp-LRC-1 (Fungisome) prepared at the Liposome

Table 11: Adverse effects of Amp B products

Infusion-related adverse events	Amphoteticin B	Abelcet (ABLC)	Amphocil (ABCD)	Ambisome (L-Amp)	Fungisome (L-Amp-LRC-1)[33]
Fever	>50%	26–50%	>50%	11–25%	21.74%
Chills	>50%	26–50%	>50%	11–25%	13.04
Nausea	26–50%	<10%	11–25%	11–25%	8.70%
Vomiting	11–25%	<10%	<10%	10%	4.35%
Headache	<10%	<10%	<10%	<10%	4.35%
Dyspnea	11–25%	<10%	<10%	<10%	4.35%
Hypotension	<10%	NA	11–25%	<10%	NA
Tachycardia	11–25%	11–25%	<10%	<10%	0%
Hypertension	11–25%	11–25%	NA	<10%	NA
Hypoxia	<10%	11–25%	11–25%	<10%	NA

See Ref. 43 (www.nfid.org/publications/clinical updates/fungal/june1999 table4.gif).

Table 12: Cost comparisons of different lipid preparations of Amp B for a person weighing 50 kg

	Dose	Duration	Cost/vial (Rs) (50 mg)	Cost/day (Rs)	Totol cost of treatment (Rs)
Ambisome	3 mg/kg	4 weeks	10 400	31 200	8 73 600
Abelcet (ABLC) Ampholip	5 mg/kg	4 weeks	3675	18 375	5 14 500
Amphocil (ABCD)	4 mg/kg	4 weeks	8590	34 360	9 62 080
Fungisome	1 mg/kg	4 weeks	5900	5900	1 65 200
Conventional Amp B	1 mg/kg	4 weeks	280	280	7846

Clinical Pharmacology Centre, Seth G.S. Medical college, K.E.M. Hospital, Mumbai 400 012, developed in collaboration with the Department of Biochemistry, Delhi University, has higher LD50 than conventional Amp B and has better efficacy in animal models of fungal infection and leishmaniasis.

6. The MLV of Fungisome prepared are stable for two years when stored at 4^0C. These are required to be converted to SUV by a process of ultrasonication for 45 min. All the patient studies done with L-Amp-LRC-1 are with ultrasonicated L-Amp-LRC-1.
7. Phase I study was carried out with L-Amp-LRC-1 in 12 patients suffering from systemic fungal infection (proven or unproven), it was found to be safe for administration.[1],[47] Following the encouraging results from Phase I study, Phase II efficacy and dose-range finding studies were taken up in patients suffering from systemic fungal infection and also in leishmaniasis to decide effective, convenient and safe dose.[38]. In Phase III comparative study, L-Amp-LRC-1 was found to be better tolerated than Amp B and equieffective with Amp B. It was safe in patients who developed unacceptable toxicity with plain Amp B.
8. In order to cater to the need of L-Amp-LRC-1 for an increased number of patients, scaling up of the production of L-Amp-LRC- 1 was undertaken. The scaling of production was also necessary to have a viable ethos for production for the purpose of marketing. On completion of all the studies, the product has been patented and the technology is transferred through the National Research Development Corporation (NRDC) to pharmaceutical companies. L-Amp LRC-1 (Fungisome) is manufactured and marketed by Lifecare Innovations Pvt. Ltd., and is approved for systemic fungal infections and visceral leishmaniasis. The drug is available in 10 mg, 25 mg and 50 mg vials.

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