CHAPTER 2

MATERIALS AND METHODS
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II.1. Chemicals:

Amphotericin-B (Amp-B), Stearylamine (SA), dipalmitoyl phosphatidyl ethanolamine (DPPE), yeast mannan, p-aminophenyl α-D-mannopyranoside, and glutaraldehyde, were purchased from Sigma chemical Co. St. Louis U.S.A. Trinitrobenzene Sulphonic acid (TNBS) was obtained from Fluka, Switzerland. Phosphatidyl Serine was purchased from Avanti polar Inc. U.S.A. Egg phosphatidyl Choline (EPC) and cholesterol (Chol) were purchased from CSIR, Centre for Biochemicals Delhi, India. Fungizone the deoxycholate preparation of Amp-B, was obtained from Sarabhai Chemicals, India and was reconstituted in 5% dextrose before use. Concanavalin A was prepared according to the method of Surolia et al. (125). All other reagents used were of the highest purity available.

II.2. Animals:

Male BALB/c mice (body weight, 20-30 gm) were purchased from the Laboratory Animal Facility of Central Drug Research Institute, Lucknow India, and maintained in our animal house.

II.3. Fungus:

Fungus used in course of this study was Aspergillus fumigatus Strain VP 256. The Strain VP 256, isolated from an Indian patient, was kindly provided by Dr. Z.U. Khan of VP Chest Institute, Delhi.
II.4. Preparation of medium used for culturing Aspergillus:

The Sabouraud dextrose medium used for Aspergillus culture was prepared as follows. Agar 20gm, Glucose 20gm and Peptone 10 gm were diluted to 1 lit with distilled water and the solution was autoclaved. 0.05 gm chloramphenicol was added aseptically to the solution and the final broth was poured in tubes to make slant cultures. The organism was cultivated in these slants by incubation at 37 C for 2-3 days. The grown spores of Aspergillus fumigatus were suspended in 0.15 (M) sterile saline and counted on a hemocytometer.

II.5. Animal Model:

Since Aspergillus is a systemic fungal infection among the immunocompromised patients, it was felt, that immune status of the mice should first, be suppressed and then followed by the injection of Aspergillus fumigatus spores, in order to get the proper fungal infection in the lungs of experimental mice. The drug used to suppress the immune status of the mice was cyclophosphamide. Different concentrations of cyclophosphamide (50-150 mg/kg body wt) were used. Groups of animals were infected after 24 hr of cyclophosphamide treatment with 0.17 ml of 0.15 M Saline containing varying number of spores (15 x 10^7, 7.5 x 10^7, 3.5 x 10^7) via the tail vein. In all these experiments no animal survived after the first day of injection of A. fumigatus. Varying number of fungal spores (15 x 10^7,
7.5 \times 10^7, 3.5 \times 10^7, 1.8 \times 10^7 were also injected without prior injection of cyclophosphamide. It was found that all the animals infected with $15 \times 10^7, 7.5 \times 10^7, 3.5 \times 10^7$ died within 2-3 days, but a spore dose of $1.8 \times 10^7$ caused disseminated fungal infection and the animals survived for 2 days providing sufficient time for evaluation of therapeutic efficacy.

**II.6. Preparation of liposomes:**

Liposomes were prepared from phospholipids with or without cholesterol. Amphotericin-B (Amp-B) was intercalated into liposomes, wherever needed. Different formulations of liposomal Amp-B were used. The molar ratios of phospholipid with the types of liposomal formulation of Amp-B are mentioned below:

<table>
<thead>
<tr>
<th>Types of formulation</th>
<th>Molar lipid ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPC</td>
<td>1</td>
</tr>
<tr>
<td>EPC:Chol</td>
<td>7:3</td>
</tr>
<tr>
<td>EPC:SA</td>
<td>7:1</td>
</tr>
<tr>
<td>EPC:Chol:SA</td>
<td>4:3:1</td>
</tr>
<tr>
<td>EPC:PS</td>
<td>9:1</td>
</tr>
<tr>
<td>EPC:Chol:PS</td>
<td>6:3:1</td>
</tr>
<tr>
<td>EPC:Chol:DPPE</td>
<td>6:3:1</td>
</tr>
<tr>
<td>EPC:DPPE (Mannosylated)</td>
<td>9:1</td>
</tr>
<tr>
<td>EPC:CHOL:DPPE (Mannosylated)</td>
<td>6:3:1</td>
</tr>
</tbody>
</table>
In general, 45 mg of lipid was dissolved in 2:1 chloroform : methanol in a round bottom flask. 1 mg Amp-B dissolved in methanol (40 ug/ml) was then added. The solvent was then removed under reduced pressure at 37 C and the flask was kept in vacuum dessicator overnight to remove residual solvent.

The thin dry film was resuspended in 0.15 M sterile saline and sonicated for 45 min at 4 C under nitrogen atmosphere using water bath sonicator (Model-decon FS-200, Ultrasonic Ltd. England). The liposomal suspension thus obtained was dialyzed at 4 C against 400 volumes of 0.15 M NaCl which was changed two times over a period of 12 hr, to remove non intercalated Amp-B. Liposomes without intercalated Amp-B were also prepared according to the same procedure as described above.

II.7. Covalant Coupling of p-aminophenyl \(\alpha\)-D manno-pyranoside to dipalmitoyl phosphatidylethanolamine:

Covalant coupling of p-aminophenyl \(\alpha\)-D manno-pyranoside to dipalmitoyl phosphatidyl ethanolamine liposomes was done according to the method of Torchilin et.al. (126) with slight modification. Phosphatidylethanolamine liposome suspension (20-30 mg lipid) in 0.15 M NaCl was mixed with 5 mg (contained in 1 ml) p-aminophenyl-\(\alpha\)-D mannopyranoside. Glutarddehyde was added slowly to the liposome suspension upto 15 mM final concentration and the mixture was allowed to react for 5 min at 20 C. Uncoupled p-aminophenyl
- D manno-pyranoside and glutaraldehyde were removed with two washings by ultracentrifugation.

II.8. Monitoring of coupling of p-aminophenyl-\(\alpha\) - D manno-pyranoside to dipalmitoyl phosphatidylethanolamine liposomes.

The coupling of the mannoside on liposomes was monitored by:

Titration of amino group of phosphatidyl ethanolamine in liposomes with trinitrobenzene sulfonic acid:

To 1 ml of liposomal suspension (0.5-1 mg total lipid) were added 1 ml of 4% NaHCO (pH 8.5) and 1 ml of 10% SDS. The mixture was kept at room temperature for 20 min and then 1 ml of 0.1% TNBS solution was added. The reaction mixture was allowed to react at 40 C for 2 hr.

After 2 hr, the reaction was terminated by adding 0.5 ml of 1N HCl. The absorbance of the solution was read at 335 nm against a blank treated as above but with 1 ml of water instead of the liposome suspension (126). About 80-85% of phosphatidyl ethanolamine amino groups were found to be modified, in all cases, by the above treatment.

II.9. Preparation of Mannose grafted liposomes:

Liposomes (total lipid 45 mg) without Amp-B were prepared having a molar ratio EPC:Chol:DPPE-Man (6:1:3) and EPC:DPPE-Man (9:1) respectively. The lipid suspension was then lyophilized and dissolved in chloroform in a round bottom
flask, 1 mg of Amp-B dissolved in methanol was then added to it. The lipid Amp-B mixture was concentrated to dryness in rotatory evaporator and kept in dessicator overnight to remove residual solvent from the flask. The thin dry film was resuspended in 0.15 M NaCl and sonicated for 45 min under nitrogen atmosphere using bath type sonicator. The non-associated Amp-B was removed by extensive dialysis against 0.15 M saline at 4°C. The presence of mannose on surface of liposomes has been confirmed by agglutination of intact liposomes with Con A at 660 nm as described by Ghosh et al. (89).

II.10. Assay of liposome intercalated Amp-B:

The drug concentration in the liposome suspension was determined by two methods:

(i) Spectrophotometric Method

(ii) HPLC Method

(i) Spectrophotometric Method:

The amount of Amp-B intercalated into liposomes was determined before and after dialysis by diluting a sample of the final preparation of 20 ul to 3000 ul in methanol, measuring the absorbance at 405 nm, and comparing the absorbance with standard curve prepared form Amphotericin-B in methanol; the absorbance blanks consisted of equivalent amount of lipid dissolved in methanol.
HPLC Method:

The Amp-B content in liposome was also confirmed by HPLC analysis. The HPLC used for the analysis of Amp-B consisted of Gynkotek Model (Munich, FRG) 300 C high precision pump with a solvent delivery system, an injector with 20 ul loop and dual wave length UV detector (Gynkotek UVD-160). A 0.005 M EDTA Methanol (3:7 vol/vol) was used as chromatographic eluent. A C reverse phase column (250 mm X 4.6 mm) was used for separation and the absorbance was recorded at a wave length of 345 nm. The analysis of the peak area was performed with the help of a PC-integrator attached with HPLC. To determine the retention time and peak area of Amp-B, 20 ul (in duplicate) of Amp-B from stock of 2.5, 5, 10 ug/ml methanol was injected into HPLC column. The peak area obtained after injection of liposome lysed with methanol was compared with that of Amp-B standards to calculate the percentage of intercalation of Amp-B.

II.11. Standard curve for Amp-B in various tissues:

The recovery of Amp-B from various organs by methanol extraction method was determined as follows: Tissue samples (0.5 g, in duplicate) from BALB/c mice were homogenised after adding 10 ug of Amp-B in 2 ml methanol. The samples were centrifuged at 9000 X g for 15 min. The supernatant was taken for HPLC analysis. An aliquot (20 ul) of supernatant was injected into column separately and the peak area obtained in each case was compared with that of standard Amp-B to
calculate the recovery of Amp-B for each tissue. The recovery of Amp-B in liver and spleen was found to be 61% and 75%, while the recovery of Amp-B in lung and kidney was 74% and 61% respectively.

II.12. Determination of LD : 50

Free Amp-B (Fungizone), commercially available form (0.5-4 mg/kg) and liposome associated Amp-B (2.5-30 mg/kg) were injected into BALB/c mice (weight: 20-30 g, 10 animals in each group) via tail vein and survival was checked over a period of 4 days. In all these studies various liposomal formulation of Amp-B were used as described in sections II.6 and II.9. Median lethal doses (LD ) were calculated by the formula:

\[ \log (LD_{50}) = \log \text{dose (minimum + log ratio (f+1)) as described by Weil (127).} \]

II.13 (a) Treatment of mice infected with Aspergillus fumigatus:

The antifungal activity of free and liposome intercalated Amp-B was tested in BALB/c mice infected with \( 1.8 \times 10^7 \) Aspergillus fumigatus spores as described in animal model section (II.5). BALB/c mice were injected via the tail vein with \( 1.8 \times 10^7 \) fungus spores. After 24 hr of spore challenge, infected animals were randomly divided into four groups, each group containing 15 animals. Animals in the first group received either 0.25 mg/kg or 0.5 mg/kg of free
drug, and those in the second group were treated with either 0.25 mg/kg or 0.5 mg/kg liposomal Amp-B. The control group received only liposome having same lipid content as in second group but without Amp-B. The fourth group was left untreated.

The efficacy of the drug was evaluated on the basis of survival of animals along with number of colony forming unit (CFU) in lung. Survival was checked for 7 days after therapy.

To study the fungal load, the number of CFU in lung was determined as follows: On various days, animals were sacrificed, left lungs taken out aseptically, and weighed and homogenised in sterile 0.15 M saline. A 10 fold serial dilution of lung homogenate was made and aliquots were plated on sabouraud dextrose medium. After incubation of the plates at 37 °C for 48 hr, colonies were counted.

II.13 (b) Statistical analysis of CFU and survival:

The CFU data were statistically evaluated by the method of analysis of variance of one way classification with unequal frequencies as given by Snedecor & Cochran (148). Heterogeneity of means was tested by the F-ratio of treatment variance to the experimental error variance. The latter was used to find the standard error of difference between the pairs of means by calculating the observed t-value and comparing it with the tabular t-value against degrees of freedom due to error.
Data pertaining to survival were studied by using the CHI-square technique of 2 X k table as given by Goulden (149). Since our interest was mainly on the outcome of the results on the 7th day (Final day) detailed examination of the results pertaining to the remaining days have not been presented although the data was analysed completely. The comparison between paired sets were analysed and difference between pairs at p <0.05 level were considered.

II.14. Tissue distribution of free and liposome intercalated Amp-B:

The recovery of Amp-B from various organs by methanol extraction method was measured as follows: Tissue samples (0.5 g, in duplicate) from BALB/c mice were homogenised after adding 10 ug of Amp-B in 2 ml methanol. The samples were centrifuged at 9000 X g for 15 min. The supernatant was taken for HPLC analysis. An aliquot (20 ul) of supernatant was injected into column separately and the peak area obtained in each case was compared with that of standard Amp-B to calculate the amount of Amp-B in each tissue.

At 1 hr and 24 hr after intravenous challenge of either free or liposomal Amp-B, groups of six mice were sacrificed and different organs namely lung, liver, spleen and kidney were taken out, excised and frozen until analysis. The tissue samples were pooled, homogenised in methanol, centrifuged and the supernatant was taken for HPLC analysis, as described earlier. The amount of Amp-B in various organs
was calculated on the basis of drug assayed by HPLC method after methanol extraction followed by multiplication by recovery factor.

Tissue distribution of Amp-B intercalated into mannose grafted liposomes: Mannan inhibition study:

For mannan inhibition study, mannan in saline (80mg/kg body weight of animals) was injected intravenously 5 min prior to injection of Amp-B intercalated into liposomes. After 15 min challenge of liposomal Amp-B, the animals were sacrificed and various organs such as lung, liver, spleen, and kidney were taken out, and processed for Amp-B detection as described (II.14).