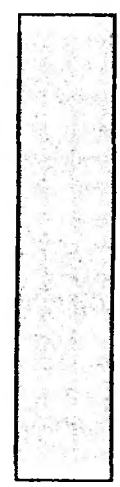
SUMMARY AND CONCLUSIONS



The present investigation deals with the development of a novel hydroalcoholic method for preparation of liposome derived from proliposome containing amphotericin-B (AmB) complexed with different chemically modified β-cyclodextrin (β-CD's), this approach is selected to reduce toxicity and to enhance therapeutic efficacy of AmB in-vivo. The method involves initial preparation of inclusion complex. (AmB-βCDs) intercalated proliposmes, which is subsequently converted into liposome dispersion by two-stage dilution method. The AmB-liposome derived from proliposome exhibited superior entrapment stability as compared to liposomes prepared by employing conventional solvent-based techniques.

The next objective was to ascertain as well as examine, whether reduction in toxicity and improvement of therapeutic efficacy is due to altered pharmacokinetics and tissue distribution of amphotericinB when delivered through liposomal AmB vesicles derived from proliposmes, This is further extended by studying its biodistribution in infected as well as normal mice. Finally while studying of pharmacokinetics, an attempt was made for prolonging the retention of AmB in circulation by carrying out saturability of RES

macrophages via concurrent increasing lipid infusion. The influence of varying lipid dose on the serum concentration and tissue distribution of amphotericin B by comparing the different liposomal amphotericin B in treated rats.

The research work carried out in the present thesis work is summarized here in below:

1) Preparation of sterically stabilized liposomal AmB derived from proliposome by hydroalcoholic method.

(a) Formulation of inclusion complexes :

The preparation involved the complexation of amphotericin B (AmB) with different β-cyclodextrin derivatives by co-grinding method and characterized the formation of inclusion-complexes by phase solubility method and verified AmB: β-CD complex formation by differential scanning calorimetry (DSC).

(b) Entrapment of inclusion complex into liposomes :

The preparation of liposomes derived from proliposome technique was used to entrap inclusion complex (AmB-βCD) into the aqueous phase of liposomes. The method involved the production of an initial proliposome mixture, which was subsequently converted to standard liposome dispersion by two-stage dilution method.

Structural organization of proliposome mixtures were determined by phase solubility technique & ^{31}P-NMR and ascertained proliposome mixture composition containing lipid: ethanol: water (500:400:1000) w/w/w for EPC and (500:300:800) w/w/w for DOPC liposomes. Structure of the proliposome mixtures varied with the relative amount of lipid, ethanol, & water (buffer) present in the mixture, the nature of the lipid used, temperature maintained and concentration of different β-cyclodextrin. The entrapment efficiency of AmB-HPBCD/SBEBCD inclusion complex was found to be 51.8 to 66.7% for liposome prepared from hydroalcoholic EPC proliposome in the ratio of lipid: ethanol: water (buffer) 500:400:1000 w/w/w and whilst average size of liposomal vesicle is around 4–5μm. Whereas, entrapment efficiency of inclusion complex AmB-HPBCD/SBEBCD into DOPC liposome (in the ratio of lipid: ethanol: water (buffer) 500:300:800 w/w/w) was 41.8 to 46.5 % and average size of vesicle was centered around 3-4μm.

2) <u>In-vitro</u> drug release studies :

Drug release pattern from liposomes in terms of amphotericin-B loss was evaluated by determining the amount of AmB released after 6h,which is found to be 2-4% from EPC liposomes and 14-27% from DOPC liposome entrapped

SBEBCD/HPBCD, prepared by proliposome method. The effect of different pH on AmB loss was maximum at low and high pH as compared to nearly neutral pHs (6.0 to 8.0).

3) **Stability Studies: -**

(a) In- Vitro drug leaching studies : -

The extent of drug loss due to leaching from various proliposome-based liposomal formulations during storage in refrigerator upto 2 months was studied. The results showed a minimum 1.6 –2.9 cumulative % drug leached from EPC liposome comprising AmB-SBEBCD/HPBCD in concentrated form. Whereas, 5.53 – 7.22 cumulative % drug leached from DOPC liposome comprising AmB-SBEBCD/HPBCD in similar form

(b) Product Shelf Life Determination :

The shelf life of product was also been determined at different accelerated conditions based on arrhenious chemical degradation kinetics and study results revealed the EPC proliposomal product exhibited more stability in the concentration form (310-404 days). Whereas, DOPC proliposomal product showed poor stability in the similar form (217-232 days). Thereby clearly indicating superior shelf life of EPC proliposomal form of AmB.

4). **Toxicity studies :**

The in- vitro toxicity of different PBLV containing varying amounts of AmB was compared and the effect of AmB_{Doc} on erythrocyte lysis was considered as 100%. The significant reduction in the hemolysis effect may infact be attributed to the reduced rate of amphotericinB release from the EPC liposomes. Further, consistent with reduced nephrotoxicity was evident in all PBLV- treated mice after administration of single dose upto 19 mg/kg.

Acute LD_{50} of AmB_{DOC} as well as different liposomal incorporated AmB was determined in Balb/c mice by intravenous route. The LD_{50} of AmB was increased from 2.0 mg/kg to 18.6 mg/kg in mice when AmB was administered in EPC liposomal form prepared by proliposome technique, similarly LD_{50} was 7.8 mg/kg in mice when AmB was administered in DOPC liposomes. EPC liposomal AmB (Proliposome method) treated mice also exhibited comparatively mild toxic reactions (viz. Pyrexia, preconvulsive symptoms etc) as compared to those treated with DOPC liposomal AmB.

5) Comparative studies on therapeutic efficacy of various liposomal preparation of AmB on the aspergillosis mice model:

After 24h of spore (A.fumigatus 1.8×10^7I.V.) challenge the survival of the animals after therapy with different liposomal preparations of AmB (0.5mg/kg,I.V.) was observed for a period of 7 days. The pathogen load was determined by culturing the processed left lung from killed animals on sabour and dextrose agar plates and then counted for colony forming units (CFU) after 48h incubation at 37^0C.

The CFU values (fungal counts) in infected animals were increased significantly in lung. Infected animals treated with EPC liposomal AmB (HPBCD/SBEBCD) showed significant reduction in CFU values, whereas infected animals free AmB showed insignificant reduction in CFU. A marked increase in the percent survival (80% and 90% survived after 7 days of infection) was observed in the case of animal treated with EPC based liposomal formulation (AmB-HPBCD/SBEBCD).

6) Tissue distribution pattern of different liposomal AmB:

Amphotericin B disposition profile in various tissues following intravenous administration of free as well as different liposomal AmB preparation (12mg/kg, I.V.) is compared after 1h and 48h. Animals receiving PBLV demonstrated high level of amphotericinB in liver with 52.2% uptake in 1h and even after 48h 40.6%

retention was observed with the EPC liposome entrapped AmB-SBEBCD. There were reduced levels in plasma (4.3%) and kidney (3.5%), the major target organ of toxicity. In contrast, after AmBDOC administration there was a significant reduction in AmB retention in liver (only 18.6% retained till 48h) and enhanced levels in kidneys and plasma (2-3 fold) were observed. It was glaringly seen, an increased uptake of drug by macrophage rich organs such as lung, liver and spleen, when either liposomal AmB (proliposome based) was administered in infected mice .The results suggest that prolonged retention of higher concentration of PBLV in the liver and increased accumulation at the infected sites may be responsible for the improvement in therapeutic efficacy.

The other important observation of the present finding indicates that, in contrast to the liposome-entrapped inclusion complex where splenic drug value remained virtually higher at 24h after injection in infected mice, corresponding value for AmB incorporated as such in the AmB_{DOC} were reduced by about 78% (spleen). Therefore it appears that slow complex dissociation rather than rate of vesicle disintegration or rate of elimination of AmB content from these tissues following the initial 24h period in mice injected with proliposome based liposome comprising AmB-SBEBCD. On the other hand, hepatic drug value only slightly

declined at 24h in the infected liver as compared to AmB value in non-infected liver.

The preferential uptake of liposomal vesicles entrapped inclusion complexes (AmB-HPBCD and AmB-SBEBCD) by the RES (Reticuloendothelial systems) may partly explain minimal disposition of liposomes derived from proliposome in infected and non infected kidney which would result in reduced nephrotoxicity.

The pharmacokinetic characteristics with single I.V. doses of liposomal vesicles (AmB-βCD) were conversely different with that of free AmB after the administration of increasing lipid dose, as compared to without the administration of lipid dose. The results suggest that decreased the clearance of AmB from blood stream and decreased L-AmB concentration in the kidney and lung, as lipid dose was increased. This can be attributed largely due to saturability of the reticuloendothelial system (RES).

The following conclusions are drawn from the present research findings:

The sterically stabilized liposomes produced by modified proliposome technique is simple, avoids the use of unacceptable organic solvents (i.e.

chloroform and methanol etc.) and exhibited high degree of entrapment efficiency 46-66 % with PBLV entrapped AmB-SBECD inclusion complex. The proliposome approach described in this investigation also avoids involving sonication steps and it is eminently suitable for scaling up for production purposes.

Incorporation of chemically modified β-cyclodextrin has shown that high binding affinity and high specificity to accommodate water insoluble molecules ("guest") in the hydrophobic milieu of the cavity present in β-cyclodextrin structure, effectively circumvented liposomal shelf life problems such as poor drug retention during storage and a poor stability in biological fluids such as serum.

Liposomal AmB (proliposome based liposomal preparation) on the mice model of aspergillosis was showed significant protection both in terms of percent survival, fungal load in the vital organ like lung. Proliposome based liposomal vesicles (PBLV) had significantly higher therapeutic efficacy than free amphotericin B in the treatment of invasive aspergillosis in Balb/c mice.

Toxicity of amphotericin-B was significantly reduced when delivered through liposomal vesicles comprises inclusion complex of AmB/HPBCD and

AmB/SBEBCD as evidenced by reduced nephrotoxicity in Balb/c mice, lower in vitro toxicity to erythrocytes and higher LD_{50} (it increased from 2 mg/kg for free AmB (AmB_{DOC}) to 18.6 mg/kg for proliposome liposomal AmB/SBEBCD.

The altered pharmacokinetic behavior of the present liposomal AmB preparation by proliposome approach suggest that reduced level of amphotericin-B in plasma and kidney after PBLV AmB/HPBCD and PBLV-AmB/SBEBCD administration is the reason for the reduced toxicity. Moreover, prolong retention of high concentration of PBLV-AmB/HPBCD and PBLV-AmB/SBEBCD in the liver localization at the infected sites may be reason for achieving higher therapeutic efficacy.

The influence of varying lipid dose on the concentration in serum and distribution in tissue of free amphotericin-B and different PBLV entrapped inclusion complexes (AmB/βCD) demonstrated a decrease volume of distribution and saturable, nonlinear elimination from plasma via reticuloendothelial organ uptake at higher lipid level.