CHAPTER 1

INTRODUCTION
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The term "fungal infection" signifies the sum of biological processes which take place in the macro-organism upon the penetration of pathogenic fungus (micro-organism) into it, independent of whether the penetration will entail the development of an obvious or latent disease or whether the macro-organism will only become a temporary carrier of the causative agent. This whole series of event constitutes the infectious process and one of the extreme degrees of manifestation of infection process is designated as infectious disease (1). Fungal infections(2) can be classified into:

i) Superficial fungal infections,
ii) Cutaneous fungal infections,
iii) Subcutaneous fungal infections and
iv) Systemic fungal infections

Superficial Fungal Infections

These include diseases in which a cellular response of the host is lacking. Invasion of fungi occurs only on the outer layers of the skin e.g. Tinea versicolor, Tinea nigra, White piedra, Black piedra etc.

Cutaneous Fungal Infections

Cutaneous fungal infections are common in the keratinized tissue of the host, like hair and nails. Fungi invades only epidermis and its appendages. The lesion of
infected skin is roughly circular and usually expand in all directions. Some examples of infectious cutaneous fungi are Tinea capitis, Tinea corporis, Tinea cruris and Tinea pedis.

Subcutaneous Fungal Infections

Such mycotic infections are usually common in the inner layer (dermis) of the skin. The infection is initiated by the penetration of fungi associated with contaminated splinters, thorns or soil into the skin. Invasion of fungi may also occur by direct implantation of soil fungus into the affected areas of the skin. Once established, these infections tend to extend to bone and lymphatic system.

Systemic Fungal Infection

These are most common and life threatening amongst immunocompromised patients. The infection is initiated by the direct inhalation of fungal spores in the lung with subsequent hematogenous spread to other viscera and the central nervous system. The increased incidence of systemic fungal infection that has been noticed during the last decade can be attributed to a number of factors, such as treatment with broad spectrum antibiotics, corticosteroids, antineoplastic drugs, open surgery, and other conditions that lead to impaired host natural resistance (3-7).

Considerable efforts have been made in treating fungal infections but successful treatment of systemic fungal
infections has remained problematic because the therapeutic armamentarium has been limited in variety and efficacy. Most of these infections are caused by species of Aspergillus, Candida, Blastomyces, Cryptococcus, Histoplasma especially in the immunocompromised and neutropenic host (8-10). Among these, candida and Aspergillus species are most common pathogens. Our work mainly concerns systemic Aspergillosis and hence will be taken up in slightly more detail.

Candida Albicans

These are opportunistic pathogens giving rise to local inflammation under a wide variety of conditions. The inflammatory reaction is normally mild and superficial with polymorphonuclear exudate but systemic invasion may occur in drug addicts and patients on immunosuppressive or cytotoxic therapy. The common sites of the infection are mouth, intestine, lower respiratory tract, and vagina (11). The infection of the mucosa is known as “thrush”. The commonest candida infection is vaginitis or vaginal thrush. Pregnant women are very susceptible to candida infection. There is an acidic whitish discharge and microscopically some pus cells and many yeasts, including pseudomycelial forms, are visible.

Aspergillosis

Aspergillosis is a granulomatous, necrotising and cavitory disease of the lungs, often with hematogenous spread (12). It is one of the most common airborne systemic fungal
infection which can be fatal for immunodebilitated patients such as those suffering from AIDS, cancer, tuberculosis etc. The causative agent is the most commonly isolated species *Aspergillus fumigatus*. Species of aspergillus are ubiquitous in nature and particularly abundant in soil, farm houses, barns, grain dust and decaying vegetation. The conidiophore of *Aspergillus fumigatus* is a single stalk with large terminal vesicles containing many sterigmata which bear long chains of conidia. The spores are small in diameter (2.5-3 um) and are readily inhaled. The infection is initiated by direct inhalation of fungal spore in the lungs from where it may disseminate to different parts of the body. *Aspergillus fumigatus* invades the lung parenchyma in immunocompromised patients and can colonise in pre-existing or healed tuberculous pulmonary cavities (13). The threat assumes alarming proportions in individuals who have undergone renal transplantation or have haematological neoplasia or chronic granulomatous disease. In addition *Aspergillus* enters through the respiratory tract, it subsequently invades blood vessels and causes thrombosis, and infarcts, causing manifestation of the disease in like brain, liver, spleen, kidney or other organs. Categories of lung infection caused by aspergillus are divided according to distinct clinical syndromes.

1. **Hypersensitivity lung diseases:** Extrinsic Asthma, Extrinsic Allergic Alveolitis, Allergic bronchopulmonary Aspergillosis.
2. **Non-invasive infection**: Pulmonary aspergilloma, Suppressive Bronchitis.

3. **Invasive infection**: Chronic necrotizing, Acute necrotizing.

The clinical manifestations are usually overlapping and the aspergillus lung infections can not always be placed under different categories. Persons suffering from aspergilloma may develop bronchopulmonary Aspergillosis. Similarly pneumonia manifests as aspergilloma. Therapy with corticosteroids for invasive aspergillosis can impair the accumulation of phagocytes at inflammatory sites, bactericidal and candidacidal activity of monocytes, cell mediated immunity (14-16) and also the natural resistance. The increasing incidence of aspergillosis in chronic granulomatous patients impairs phagocytic action against aspergilli. Until 1970 it was not clear, whether phagocytes can kill spores or mycelia of *A. fumigatus* in vitro but the evidence was there that polymorphonuclear granulocytes (PMN) can damage the hyphae (17).

It has been shown that natural immunity to resting spores of *A. fumigatus* is independent of PMN, T lymphocytes and humoral immunity but the mononuclear phagocytes, and neutrophils can kill the fungus in its conidial stage very efficiently. Andreas Schaffner et al (1982) demonstrated, (18) by *in vivo* as well as *in vitro* studies that spores of *A. fumigatus* are readily ingested by macrophages.
Polyene Antibiotics

Since the discovery of Nystatin, the first member of this class of compounds, by Hazen and Brown in 1950, (19), many other polyene antibiotics have been described. Most of these are produced by soil actinomycetes of the genus Streptomyces.

At present more than 200 polyenes are known and of these, 40 have known structures (20). Among the large list of polyene antibiotics that have been isolated and described, some are most effective antifungal agents but the problem associated with solubility, stability, absorption and toxicity limits their maximal exploitation in clinical medicine. Polyenes can be divided into three main categories on the basis of their structure:

1. Polyenes containing sugar residue
2. Polyenes without sugar residue
3. Aromatic polyenes

Polyenes with sugar moiety are effective antifungal agents. Amp-B and nystatin are important members of this group. Amp-B forms small pores in the membrane ranging from 8-10 Å in diameter (21-23). The best example of polyenes which do not have sugar moiety is filipin. The pore formed by these polyenes are bigger in diameter for e.g. filipin forms a pore of 150-250 Å in diameter (24-25). Aromatic Polyenes are very promising antifungal agents. The activity of these aromatic polyenes is higher against yeast cells as compared to non aromatic polyenes. The distinctive feature of this
class of polyenes is that they contain a p-aminophenyl or p-N-methylaminophenyl group in their structure e.g. hamycin, perimycin A, candidicidin D, particin A, vacicidin A.

Physico chemical properties of Polyene antibiotics

Elemental analysis of polyene antibiotics shows that they contain carbon, hydrogen, oxygen and in some instances nitrogen (26). The major characteristic feature of polyene antibiotics is the presence of the large macrolide ring in its structure. Polyenes are classified according to the number of double bonds in the principal chromophore as either trienes, tetraenes, pentaenes, hexaenes or heptaenes. None of the trienes contain carbohydrate units but many tetraenes contain the amino sugar, mycosamine. The macrolide ring contains three to eight conjugated double bonds with one sugar moiety linked through a glycosidic linkage. The ring size varies from 12-14 to 35-37 carbon atoms. Macrolide confers a characteristic peak on infrared spectra of these polyenes. In 1971, Ganis et al (27) showed by X-ray crystallographic studies that all the double bonds of Amp-B were in trans configuration. The polyene antibiotic has a large number of hydroxyl groups usually distributed on the macrolide ring on alternate carbon atoms. The number of hydroxyl group varies from 6-14. The presence of polar hydroxyl groups and multiple hydrophobic double bonds in the macrolide of polyenes confers the additional property of their being amphipathic. This amphipathic feature of polyene
antibiotic plays a major role in its interaction with biomolecules in various biological systems. The hydrophobic and hydrophilic components reside opposite each other in the macrolide ring. The carbohydrate in all polyenes was found to be mycosamine. The structure was established in 1961 by Von Saltze et al. (28) when they chemically synthesized the mycosamine molecule. This is a 3 amino analogue of D-rhamnose or 6-deoxy-D mannose. The -pyranose ring form of mycosamine is more stable than the -furanose ring structure.

The most distinctive physical characteristic of polyene antibiotics is the UV absorption spectrum. The absorption spectra ranges from 280-405 nm. Oroshnik and Mebane (1963) hypothesized (29) that the characteristic absorption spectra of polyenes is due to the conjugated double bond system. The presence of several distinct classes of chromophores i.e. tetraenes, pentaenes, hexaenes and heptaenes have been indicated from the analysis of different UV absorption spectra.

Due to the amphipathic nature of the polyene antibiotics they show limited solubility in both water and in organic solvents such as alcohols, esters or ethers. Polyenes form suspension of micelles rather than true solutions in aqueous solvents. Polyenes may be dissolved in very polar organic solvents, such as dimethylsulphoxide (DMSO) or dimethyl formamide (DMFD). Solutions of antibiotics
in DMFQ or DMSO are stable if they are protected from light and oxygen to which they are very susceptible.

Polyenes exert inhibition against many species of fungi such as yeasts (e.g. Candida), dimorphic fungi (e.g. Histoplasma), dermatophytes (e.g. Trichophyton) and moulds (e.g. Aspergillus) (30). Interestingly they have no activity against intact bacteria.

Polyenes are also toxic to protozoa many of which are of medical importance such as Entamoeba, Trypanosomes and Leishmania species. Polyenes alter membrane permeability due to which there is a loss of intracellular constituents leading to the lysis of the cells. It has been seen that as the number of double bonds increase in the structure, the polyenes become more toxic. Kitahara et al. 1976 studied various factors which effect the inhibition of polyenes. The factors which may affect the minimum inhibitory potency were inoculum size, temperature, duration of incubation and medium composition (31). The presence of serum reduces the activity of the polyenes.

Mechanism of action of Polyene antibiotics

In the early 1960's, several laboratories reported that polyene antibiotics can increase the cell membrane permeability of various organisms. The increase in cell membrane permeability results in the leakage of several vital + + + metabolites viz K , Na , H ions. leading eventually to
cell lysis and death. Kinsky et al. (32-33) observed that polyenes caused a rapid decrease in the dry weight of mycelial material, followed by leakage of aminoacids, sugars and other metabolites into culture media. This effect was evident even at very low concentrations (1uM) of the polyene. The effect was specific for polyene antibiotics but other metabolic inhibitors or non-polyene type of agents were ineffective (32-33). These studies indicate that polyene antibiotics act at the membrane level. Since the plasma membrane is responsible for maintenance of cell membrane permeability, it is logical to think that plasma membrane is the site of action of polyenes.

As stated earlier, intact bacteria are not sensitive to polyenes and hence unaffected by them. On the contrary, fungi, leishmania species and human red blood cells are susceptible. Bacteria do not have sterol in their membrane whereas fungi/mammalian/protozoal membranes do have it. These observations led various scientists to propose a hypothesis that polyenes interact with the membrane sterols which are present in eukaryotic cells but are not found in bacterial cell walls or membranes. In 1960, Lampen et al. (34) observed that addition of sterol to an aqueous solution of polyene decreased the absorbance of the antibiotic and there was an increase in absorbance when the antibiotic was taken in an organic solvent. Such a change in absorbance value is a clear indication of the antibiotics interaction with sterol. Regarding the sterol selectivity of polyenes, it has been
observed that ergosterol is preferred over cholesterol (35). Fungal cells do have ergosterol whereas in mammalian cells ergosterol is replaced by cholesterol and thus polyene antibiotic preferentially lyse fungus cells. Several attempts have been made to correlate the resistance strains of various fungi such as S. Cerevisiae, candida albicans in the light of sterol content specificity. Molzahn and Woods (1972) reported alteration in the sterol content in nystatin resistance S. Cerevisiae cells (36). It has been suggested that zymosterol may have replaced ergosterol here. The in vitro analysis of polyene resistant yeast mutant reveals a lower or negligible ergosterol content than the corresponding wild type (37-39) although the total sterol content may increase (40). It should be mentioned here that the formation of methylated sterol has been reported in the resistance strain of S. cerevisiae (105). Thus it appears that modification of sterol may decrease the abrogate binding affinity for polyene to sterol, rendering the fungus resistant. Reports are also available in which it has been shown that resistance is not only limited to sterol content but lipid composition may also play an important role (38). The specific enrichment of phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine in the S. cerevisiae cells can offer protection from the polyene antibiotic action (41-43).

There are a number of models which have been used to study the mechanism of action of polyene antibiotic. These
models (44-47) include Acholeplasma laidlawai cells, planar bilayers and liposomes (SUV, LUV and MLV).

Acholeplasma laidlawai cell serve as a suitable model system because of the fact that they do not have any sterol on their membrane but can incorporate sterol when grown in a sterol rich media. Filipin and Amp-B could inhibit the growth of A. laidlawai cells only when they are grown in a media containing sterol. A large number of groups reported (48-49) that sterol added to the medium of A. laidlawai (having incorporated sterol in the membrane) could protect the cells from the antibiotic (50-52). It has been observed that ergosterol containing A. laidlawai cells are much more sensitive to Amp-B and its ester derivative than are the cholesterol containing cells. Studies with liposomal model membrane have been done to understand the sterol specificity of polyenes but unfortunately results are not so clearcut and sometimes appear to be controversial. The selectivity could be demonstrated using MLV as model membrane and Amp-B as the antibiotic (53). When SUV was used as model membrane, such selectivity for sterol could not be demonstrated in the case of Amp-B (54) or nystatin (55). It is thus apparent that such controversies may root from the selection of a particular model membrane or the methodologies used for demonstrating the selectivity. In fact, it has been observed that in SUVs where Amp-B is directly incorporated, such specificity for sterol is poorly expressed as studied by cation/proton exchange method (56). But the transfer of Amp-B from DPPC
vesicles to another SUVs could express such specificity. Thus, selection of a particular type of model membrane and parameters used to study selectivity and permeability may pose problems to elucidate the present concept regarding the mechanism of action of polyene antibiotic. It should be noted here that the present concept regarding the mode of action of polyene antibiotic is that it interacts preferentially with the fungal sterol, ergosterol and forms transmembrane channel through which the essential components of the cell leak causing cell death. The conclusion has been arrived from physical studies such as spectrophotometric, CD and measurement of cation permeability by the exchange method using various model membranes such as planar membranes, MLV and SUV. A limited amount of work in the same direction has been done with LUV as the model. At this point, it should be emphasised that one should take care in choosing a particular model membrane which could mimic the fungus/RBC membrane. Bolard et al. (57) have pointed out that SUV, because of the strong curvature of the bilayers could not be used as ideal model for interaction studies. They also mentioned that although MLVs have large diameter but because of broad light scattering, there is an impairment in optical measurement. In addition to that, in MLVs the internal leaflets are not directly accessible to the antibiotic which may raise a question regarding the validity of quantitative measurements (20). In a recent paper, Bolard’s group (58) suggested that LUVs could be ideal for studies of such interactions and studied extensively the
binding of Amp-B and filipin with LUVs and its after effect such as permeability changes to gain an insight into the mechanism of polyene antibiotic action. Applying CD techniques, they showed that the Amp-B interaction with LUV is different from that with SUV. Furthermore they obtained completely different spectra for Amp-B interaction with cholesterol and ergosterol containing LUVs, respectively. Such specificity has not been observed with SUVs (59). The interaction of filipin with LUV occurs at much higher concentration of antibiotic (equal or higher than 10 uM) than that of Amp-B (around 1 uM) (58). The CD spectra for filipin binding to LUVs remains unaltered irrespective of the nature of the sterol (58). Moreover filipin binds to sterol free liposomes (without sterol in liposomes). Binding of filipin with LUVs is observed which in turn indicates the binding of filipin with phospholipids. Thus in liposomes with sterol, there is competition between sterol and phospholipid of liposomes for binding with filipin. Existence of such type of phospholipid-Amphotericin-B complex in EPC LUVs is however not possible to demonstrate (58).

While addressing the Amp-B induced permeability of LUV, Bolard's group, using EPC/Chol LUVs observed the cation leakage (Ca$^{2+}$) at Amp-B concentrations as low as $3 \times 10^{-7}$ M; 90% leakage was observed at 10 sec. When sterol is incorporated into liposomes, similar events occurred, but at an Amp-B concentration almost 10 times lesser (58). The incubation of Amp-B at a concentration higher than 1 uM
with ergosterol-containing EPC LUV at 30 C for long periods leads to the appearance of ionic channels with enlarged diameter which enable the release of larger molecules like glucose (61).

Thus the importance of physical studies on the interaction of polyenes with LUVs as model membrane could explain sterol selectivity of the antibiotic and also the formation of ionic channels in sterol containing membranes which may form the basis of the mechanism of antibiotic action.

Studies with various model membranes indicate the formation of ionic channels after the polyene antibiotic binds to membrane sterol. In this, attempts have been made to elaborate the nature of pores and the possible interaction of the antibiotic with membrane in such pores.

The hydrophobic channel of a pore formed after polyene binding to membrane has a radius of 5 A (21-23). Interaction between polyene antibiotics and sterol have some common characteristic features (i) Within these complexes the interaction is mainly hydrophobic (ii). Polyene antibiotics can make complexes only with membrane sterols having 3β-DH group, a planar ring system and hydrophobic side chain at C17 (62) (iii). The number of cholesterol molecules per mole of polyene antibiotics in the complex is of the same order.
Despite these common characteristic features, there are remarkable differences among the various polyene antibiotics in inducing membrane permeability. The filipin–cholesterol complex forms an aggregate of 150–250 Å in diameter within the membrane (24–25) and leads to release of cytoplasmic components. The complex formed by cholesterol with Amphotericin-B, Nystatin functions as an aqueous channel traversing the membrane while the pimaricin–cholesterol complex does not alter permeability of A. laidlawii cells and liposomes (63-64). Dekruyff, et al. (1974) demonstrated important features of Amphotericin-B, cholesterol and distearyl lecithin complex by space filling models (65). The length of the double bond system of Amphotericin-B is equal to the length of the cholesterol molecule. The carboxyl group and the mycosamine residue are located at one side of the molecule, making it amphipathic. The only hydroxyl group of the ring system not aligned with the other hydroxyl group is surrounded by 3 methyl groups. The length of lecithin molecule is almost same as the length of the Amphotericin-B molecule as measured from charged phosphate group to terminal methyl group. The charged group of amphotericin-B and hydroxyl group of cholesterol is located on the same side of the complex. This is a characteristic feature of Amp-B cholesterol complex in which the cholesterol molecule complements the ring of the Amp-B molecule. The Amp-B (A), cholesterol (C) complex model has shown that (A-C) can form a half pore through the lipid bilayer. Two such half pores on either side of the lipid bilayer are necessary for complete
pore formation. The hydroxyl group of Amp-B may be involved in anchoring the two half pores and stabilizing the conducting channel.

In 1974 Andreoli et al. gave a tentative hypothesis based on experimental data for Amp-B cholesterol pore (44). Schematic representation of this hypothesis shows C-1 - C-13 and C-20 - C-33 segment of Amp-B pictured as a rod like array, 20-24 Å long. The C-15 hydroxyl group, C-16 carboxyl group, and C-19 mycosamine group are at the end of the rod with these hydrophilic groups located at the water bilayer interface, the rod being situated in the membrane interior, parallel to the hydrophobic chains of the phospholipid, the planar cyclopentano phenanthrene skeleton and the C-17 acyl residue of cholesterol. According to this hypothesis two types of hydrogen bondings are involved. One is the hydrogen bonding between 3-OH proton of cholesterol and C-16 carboxyl group of Amp-B, and the second involved between the 3-OH oxygen of cholesterol and the C-17 hydroxyl proton of Amp-B.

Amphotericin-B

Amp-B is the major drug of choice for the treatment of aspergillosis. Amp-B is a polyene antibiotic derived from a strain of *Streptomyces nodosus* (66). It is a low molecular weight compound (Mol Wt 924.1). The molecule is roughly about 24 Å long. The glycosidically linked aminosugar mycosamine and carboxyl side chain give the compound its
amphoteric nature. It is insoluble in water and shows greatest antifungal activity between pH 4.2 and 6.5. The drug is available commercially as a topical cream, oral suspension, tablets and in powder form suspended in deoxycholate. The commercial name of the latter form is fungizone which contains 50 mg Amp-B and 41 mg of sodium deoxycholate with 25.2 mg sodium phosphate as buffer.

The deoxycholate form is widely used intravenously to treat several fungal infections including Aspergillosis. As stated earlier the antifungal activity of Amp-B lies in its preferential binding to ergosterol, a fungal sterol found in the membranes, forming transmembrane channels of approximately 10 Å in diameter. This causes the release of vital metabolites from the fungal cell and ultimately its lysis. However, the drug can also interact with cholesterol found in membranes of mammalian cells (especially red blood cells) leading to a similar effect. This makes it highly toxic and thus has severely restricted the maximal exploitation of Amp-B as a therapeutic agent in clinical medicine. Amp-B also accumulates in the cells of kidney and produces nephrotoxicity (68), which causes an unavoidable side effect of Amp-B leading to renal dysfunction and is manifested as azotaemia, renal tubular acidosis along with loss of potassium. Several other adverse reactions of Amp-B are now well established. These include anorexia, nausea, fever, headache and vomiting.
LIPOSOME

Phospholipid vesicles (liposomes) were first described two decades ago by Bangham et al. (69). It has been shown that phospholipids spontaneously form closed structures when hydrated in aqueous solution. These vesicles, composed of one or more phospholipid bilayer membranes, can carry drugs either in the aqueous phase or in the lipid phase depending on the nature of the drug. Liposomes vary in charge (positive, negative), size (MLV-size range 0.1-5 um, SUV-0.02-0.05 um, LUV size is about 0.06 um) depending on the method of preparation and the lipids used. A large number of general procedures are available for the preparation of liposomes as described below:

PREPARATION OF LIPOSOMES

Multilamellar liposomes (MLV)

The simplest method for preparation of liposomes was originally described by Bangham (1965). In this, phospholipids are dissolved in a mixture of organic solvents, which is then evaporated to make a thin dry film. This film of lipid is then dispersed in water at a temperature above the phase transition temperature (Tc) of the highest melting component in the lipid mixture.

The time of hydration and the conditions of agitation play a major role in the encapsulation. Longer the duration of hydration, higher will be the encapsulation.
Small Unilamellar Liposome (SUV)

Multilamellar vesicles prepared above are sonicated either by probe type sonicator or by bath type sonicator to give a clear suspension of liposomes. Small unilamellar vesicles can be separated from MLVs by ultracentrifugation or by column chromatography on Sepharose 2B, Sepharose 4B or Sephadex G-50. The encapsulation efficiency is however very low (4-7%). This method gives a mixture of homogeneous vesicles.

Detergent dialysis Method

This method was developed by Racker et al. (70). In this method detergents like cholate, deoxycholate, n-octylglucoside etc. are used to solubilize the lipid and the detergent is removed by dialysis in a controlled manner. A number of liposome preparation systems are available for this purpose. The ratio of lipid to detergent is an important parameter for the type of liposomes. About 12% of solute can be encapsulated by this method. The major advantage of this method is that liposomes are homogeneous.

Ethanol injection method

In this method phospholipids dissolved in ethanol are rapidly injected into a buffer solution where they spontaneously form small unilamellar vesicles. Nearly all vesicles formed by this method are unilamellar. The major drawback
is that the dispersion is diluted and the encapsulation efficiency is poor.

French Press method

Here, MLVs are prepared according to Bangham’s procedure and instead of sonication, the dispersion of MLVs are extruded through a French Press Cell at 20,000 Lbs/in at 4°C. This dispersion has vesicles in the size range of 250-500 Å. Further extrusions yield more homogeneous preparations of SUVs.

Reverse phase evaporation

By this method LUVs can be prepared from a mixture of phospholipids and organic solvents. Diethylether or a mixture of diethylether and methanol is injected into an aqueous solution of the material to be encapsulated at 55-65°C under reduced pressure. The solvent is removed by evaporation and large unilamellar vesicles of mean size 1500-2500 Å are formed. This method is suitable for encapsulation of macromolecules. The disadvantages of this method include exposure of material to organic solvents and to high temperature, and production of heterogeneous population of liposomes. The entrapment efficiency is quite high (up to 65%). This method is however not appropriate for encapsulation of proteins that get denatured on exposure to organic solvents.

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Rehydration dehydration Method

This method developed by Kirby and Gregoriodis in 1984 is employed for the large scale preparation of liposomes which are prepared by dissolving lipids in organic solvents, followed by the removal of solvent under reduced pressure and subsequently dispersing the lipid film in aqueous phase. The liposome preparation can be freeze dried and can be stored frozen without any major leakage of entrapped material for more than a year. Liposomes can be regenerated from this freeze dried lipid by adding water whenever required.

The major advantage of this method is that the lipid powder could be stored for a long time and is suitable for diagnostic purposes as it is easy to prepare when desired.

Application of liposomes

Since the discovery of liposomes by Bangham, there has been tremendous activity in the use of liposomes in diverse areas of research.

Initially liposomes had been exploited as a model membrane for numerous physico chemical studies because intact cell is a very complicated system and the study on a molecular level is very difficult, if not impossible. These studies are better done with liposomes as they can give good clues regarding possible role of a particular component in relation to cell ligand interaction under defined experimental conditions. These studies are based on the
interaction between the ligand and the receptor. In 1975, Surolia et al. devised a simple model system where receptor ligand interaction could be studied under defined experimental conditions. In these studies, GM containing liposomes (MLV, SUV) were made and their interaction with a specific lectin from Ricinus communis beans was investigated. On the basis of various kinetic parameters e.g. association constant and rate of cluster formation they concluded that liposomes could be used as a model system to study the receptor ligand interaction (60).

Since the last decade, there has been a spurt in the activity of the use of liposome as drug delivery system and also in immunology. In 1971, Ryman and her coworkers were first to recognize the potential medical application of liposomes as drug carrier and emphasized the possibility of their use in the treatment of various metabolic and physiological disorders (72).

The potential application of liposomes in immunology was first described by Allison and Gregoriadis in 1974. They showed the immuno-adjuvant effect of liposomes for the antigen diptheria toxoid. Since then, the exploitation of liposome as adjuvant, immunomodulator and also as an immunodiagnostic tool has been a subject of an increasing number of studies. To cover all the above areas is beyond the scope of the discussion. Since the present investigation has been directed towards the use of liposomes as drug carriers, the emphasis will be given on this particular aspect.
Liposome as drug carrier

Since the pioneering work of Ryman et al. (72) in the area of liposomes for drug delivery system, liposomes have been extensively used as a drug delivery system because most of the drugs which are in use for the treatment of several diseases, are not free from adverse side effects. Moreover, their specific targeting and proper tissue distribution is not feasible because of random uptake by tissues. Much research has been conducted to target the drug specifically to the disease affected site and to develop drug formulations which could reduce drug toxicity. Liposomes, are easily biodegradable and can be made immunologically inert. Liposomes can accommodate both hydrophillic and hydrophobic drugs without any chemical modification in the structure of the drug molecule. There are a number of parameters which can be conveniently altered like size, lipid composition, surface charge and the membrane fluidity in order to deliver liposomes to the appropriate site. Several workers have reported the use of liposome as a drug carrier in the treatment of leishmaniasis (73), metabolic disorders and fungal diseases (74). It has also been demonstrated that liposomally delivered drug can reduce the toxicity of the drug. Besides, by careful manipulation and suitable tailoring of liposomes, drugs may be targeted to specific organ(s).
In vivo fate of liposome

The clearance of liposomes from the circulation is controlled by various factors like liposomal size, surface charge and lipid composition.

Studies have shown that majority of the liposomes injected intravenously are retained in the liver and spleen regardless of liposome size, structural class or composition. The retention of liposomes injected i.v. in these organs are primarily due to their uptake by phagocytic cells in these organs.

Studies have shown that large liposomes are cleared more rapidly from circulation as compared to small liposomes. This was inferred from the biphasic rate of clearance of liposomes which were heterogeneous. Juliano and Stamp in their studies showed a more linear clearance suggesting that the two phase clearance might be due to size heterogeneity (75).

There are many factors which govern the retention of the solute in the liposomes e.g. the type of the liposome (MLV’s retain more than SUV), temperature (At 37 °C there is more leakage than at 4 °C) and lipid composition (saturated phospholipids retain more than unsaturated phospholipids). Experiments done in rats showed that liposomes containing proteins were rapidly cleared from the circulation following intravenous injection, and were mainly taken up by the liver and spleen (76). Subcellular studies showed that the radioactive enzyme used as marker was mainly localized in the
mitochondrial lysosomal fraction. By density gradient centrifugation the liposomes were found to be associated with the lysosomal fraction (77).

Intravenously injected liposomes can be successfully targeted to cells in different organs only if the liposomes are able to exit from the circulation and gain access to target tissues. The anatomy of microcirculation plays an important role in determining whether liposomes can gain access to extravascular tissues (78-80).

According to the architecture of the endothelium lining and the underlying subendothelial basement membrane the blood capillaries can be divided into three main categories. In the continuous capillaries, endothelium forms a continuous lining in which adjacent endothelium cells adhere via tight junctions. The subendothelial membrane is also continuous. In the case of fenestrated capillaries the endothelium is interrupted by fenestrae which are 300-800 Å in diameter. The endothelial membrane is spanned by a thin membranous diaphragm which is 4-6 nm thick. The subendothelial membrane is continuous. Discontinuous or sinusoidal capillaries are found only in the liver, spleen and bone marrow. They are thin walled and have large gaps in the endothelium which may be of several thousand Angstrom in diameter. The basement membrane is lacking in most of the species in the case of liver but an interrupted basement membrane is present in these vessels in bone marrow and spleen.
The diameter of the gaps in the endothelium would be a limiting factor for the extravasation of the liposomes. The diameter of the gaps being 100 nm, small unilamellar liposomes can pass through such openings whereas larger liposomes are retained within the sinusoid. The opening of the endothelium of the hepatic sinusoids contain gaps larger than 100 nm and hence allow the penetration of multilamellar reverse phase and large unilamellar vesicles. However the frequency of these openings is low and because of their irregular distribution the extravasation of large liposomes is less efficient (78). Continuous and fenestrated capillaries are a major mechanical barrier to the exit of liposomes from the circulation. Liposomes are unable to escape from the micro-circulation in organs lined by either of the capillaries. Thus successful targeting is limited to a few tissues lined by discontinuous capillaries i.e. spleen, liver and bone marrow. Liver is composed of two types of cells mainly parenchymal cells (Hepatocytes) and a smaller proportion of Kupffer cells which form part of the reticuloendothelial system. Some workers believe that liposomes are taken up by non parenchymal cells by their strong phagocytic activity and are responsible for most of the uptake (79). Electron microscopic and autoradiographic studies show that they are taken up by both types of cells. Studies done with liposome entrapped Horseradish peroxidase showed an initial involvement of Kupffer cells and endothelial cells followed by parenchymal cells (80).
Targetting of liposomes

Mechanistically, targetting of liposomes can be classified into

(i) Passive targetting
(ii) Active targetting

The term passive targetting designates the natural localization of liposomes prepared from phosphatidylcholine alone or in combination with cholesterol in the various tissues of the body after administration. Intravenously injected liposomes are preferentially taken up by mononuclear phagocytes of reticuloendothelial system in the liver, spleen, bone marrow and in the circulating macrophages (monocytes). The potential utility of passive targetting has been successfully employed in the treatment of visceral leishmaniasis. Leishmania is a parasitic disease mainly caused by L. donovani. The parasite predominantly resides in the macrophages of the liver and spleen. There are a number of drugs (Pentavalent antimonials, meglumine antimoniate, sodium stibagluconate and Amp-B) which are available but the use of these drugs is limited due to hepatic, cardiac and renal toxicities.

Alving et.al. (81) tested the effect of these drugs, incorporated into liposomes and found that antimonial drugs are 700 times more effective as compared to free drug in curing leishmaniasis. New et.al. (82) also demonstrated that liposomal formulation of Amp-B is more active against
visceral leishmaniasis in mice. In the case of spleen localized bacterial infection *Listeria monocytogenes* in experimental mice, liposome entrapped ampicillin was found to be 80 fold more effective in reducing the bacterial count compared to free drug.

The issue of targeting liposomes to specific tissues has attracted considerable interest but the maximum uptake by organs rich in reticuloendothelial system restricts its maximal utility in the biological system. Targeting of liposomes to specific tissues requires a recognition marker on the surface of liposomes. This has been achieved by modifying the liposomal surface with ligands like sugars, antibodies or other molecules that have specific affinity for those cells. For effective targeting, liposomes can also be designed to release their contents under controlled conditions. This includes pH sensitive (83) as well as temperature dependent liposomal systems (84).

Ryman *et al.* (1971) reported the use of liposome encapsulated enzyme for delivery into cells (72). Around the same period, Ashwell and Morell (85-87) in a series of brilliant experiments, demonstrated that a specific receptor on hepatocytes mediates clearance of B-galactose terminated glycoproteins from circulation. A specific receptor on the cell surface of the reticuloendothelial system of rats including the liver sinusoids as well as macrophages was recognized by Stahl *et al.* (88).
Encouraged by these observations Ghosh et al., demonstrated that by grafting different glycosides on the surface of liposomes it is possible to direct the latter to different cell types of rat liver (89). Galactosylated liposomes are mainly taken up by the liver hepatocytes while mannosylated liposomes are taken up mainly by non-parenchymal cells (90). This sugar specificity was completely abolished if asialofetuin was injected along with the asialo GM liposomes. Similarly there was a partial decrease in the uptake of mannosylated liposomes when mannan was used as competitive inhibitor.

**Stability of liposomes**

Liposomal stability has been the focus of much attention of many workers. Work has been going on, to increase the shelf life of liposomes. The drawback of liposomes is that they cannot be stored for a long time prior to their use as they are prone to aberrations in their structural and functional properties.

Disacharide sugars in this regard have played an important role in increasing the stability of the liposomes. It was found that, liposomes when freeze dried in the presence of trehalose, retained most of their structural and functional integrity on rehydration (91). Membrane structure is preserved by trehalose probably due to specific interaction of sugar with the phospholipid head groups (92). This causes a depression in the transition temperature and
subsequent maintenance of the liquid crystalline phase in the
dry liposomes. Trehalose and sucrose were found to be the
most effective in this regard.

Other factors which may play an important role in the
stability of liposomes are lipid oxidation and the presence
of contaminants in the lipids. Physical properties are also
attributed to play an important role. Multilamellar vesicles
prepared from palmitoyl phosphatidyl choline, cholesterol and
dicetyl phosphate showed low leakage of the solute even over
a period of one year. Small unilamellar vesicles containing
cholesterol remained stable for at least 53 days without any
leakage (93).

In vivo fate of intravenously administered liposomes is
an almost immediate release of entrapped solutes in the
circulation due to its interaction with plasma high density
lipoproteins (94). The exchange of phospholipids from the
liposomes takes place through the interaction of HDL. This
occurs as a consequence of collisions, when a sufficient ratio
of apo-HDL to phospholipid ratio is attained. The resulting
apo-HDL liposomes break down to smaller particles bearing a
portion of the liposome derived lipids and of a size similar
to that of HDL. This suggests that HDL has an ability to
remove phospholipid from vesicular membrane and causes lysis
of liposomes. This transfer of phospholipid by HDL can be
minimised by --

i) increasing the amount of cholesterol,
ii) using a phospholipid which undergoes thermal phase transition at temperatures greater than 37°C and iv) structural modifications of PC.

This obstacle can be overcome by adjusting the cholesterol content in the lipid bilayer. Permeability of various solutes from liposomes in the circulation can be minimized by the addition of cholesterol. It has been shown that the greatest stability can be achieved by using equimolar amount of cholesterol and phospholipids in the liposome preparation (97). The stabilizing effect of cholesterol in liposomes prepared from other phospholipids was similar, but this effect was not so pronounced with dihexanoyl phosphatidyl choline (98). The permeability of liposome is controlled by head group interactions (95). The permeability increases with increasing temperature, decreasing chain length and increasing unsaturation (96). However, the mechanism of cholesterol mediated stabilization of liposomes is not very clear. It is suggested that—

i) packing of liposomes induced by cholesterol prevents the interaction of apo-HDL with the phospholipid.

ii) The apo-HDL interacts with liposomes but the former is unable to remove the phospholipids due to tight packing of liposomes.

Phospholipids in biological system are mainly degraded by phospholipases. Among these, phospholipase-A selectively
hydrolyses C-2 bond of the ester group and most commonly present in liver cells. Stabilization can also be achieved by exposing negative charge on the surface of liposomes. This has been confirmed by the incorporation of gangliosides in the liposomes. A similar effect was observed with phosphatidyl serine containing liposomes (99). The effect of sialoglycolipids was more specific in preventing the binding of immuno proteins to the liposomal surface. The gross physical structure of liposomes also plays an important role in terms of stability. SUVs are cleared less rapidly than MLV while the neutral and positively charged SUVs are cleared less rapidly than the negatively charged SUVs.

Gupta et. al. (100) successfully demonstrated that liposomes can be stabilized by the structural modification of phosphatidyl choline. These workers successfully introduced an NH residue adjacent to the carbon atom of their C-2 ester group, without adversely affecting the physicochemical properties of the phospholipid molecule. Liposomes prepared from these structurally modified phospholipids lead to more ordered packing and hence decrease transfer of HDL (101). It also prevents the serum induced leakage of entrapped solutes and resists the phospholipase A-2 action in the liver. Such liposomes are stable in the blood circulation for longer time.
Limitations in the use of Liposomes as Drug Delivery System

The wider application of liposome as a carrier (in vivo) has several limitations.

1.a) Uptake of liposomes by reticuloendothelial system

A major portion of the i.v. injected liposomes are taken up by the mononuclear phagocytic cells of the reticuloendothelial system (102) and as a result, other tissues have little access to the injected liposomes.

b) The Endothelial Barrier

The use of liposomes as drug carriers for treatment of solid tumor where the vesicles have to pass out of blood stream is restricted because of the endothelial barrier. It has been demonstrated by Poste et al (103) that MLV or SUV (600 Å diameter) can not cross the pulmonary endothelia to reach the alveoli. They suggested that the liposome material in the alveoli had been carried by diapedesis of monocytes. Liposomes are able to cross the endothelium when there is a tissue damage because of certain disease. Application of liposomes through oral route or delayed release of liposomes by the use of pH sensitive or temperature sensitive liposomes does not need to cross the barrier. So where the endothelial barrier is a problem one can look for other routes of drug delivery systems using liposomes.
c) Effect of Immunoadjuvant properties of liposomes

In cancer chemotherapy liposomes containing chemotherapeutic drugs could be targeted to cancer cells by coating monoclonal antibodies (Mab) raised against tumor cell specific antigen. This therapy has not proven to be useful because of immunoadjuvant effect of liposomes. Chemotherapy requires repeated injections of the liposome encapsulated drug and this may lead to production of antibodies against Mab. So before drug is targeted to a specific cell type, liposome grafted Mab will be neutralised by anti Mab-antibodies and thus efficient drug delivery will not be achieved. Basten et al. (104) suggested an approach to overcome this difficulty. They used I labelled antigen on liposomes surface due to which immune reactive lymphocyte was damaged. This ‘Antigen suicide technique’ may be useful for drug therapy using liposome grafted monoclonal antibodies against tissue specific marker, but the approach needs proper and careful evaluation.

Advantages of using liposomes

Liposomes have distinct advantages of being nontoxic and biodegradable, as they are composed of naturally occurring lipids. Biologically active materials encapsulated within the liposomes are protected to a great extent from immediate dilution or degradation. These properties of liposomes make them promising drug carrier systems for the transport of drugs or other bioactive molecules to disease affected organs.
From the point of view of drug targeting, the relevant question is - how does the carrier device interact with the target cells? The liposome cell interactions which can take place are:

1. The peripheral liposomal membrane may be fused with the plasma membrane leading to the release of vesicular contents into the cytoplasm.

2. Exchange of lipids between liposomes and plasma membrane or adsorption of liposome to the cell surface without internalisation of liposomal contents.

3. Liposomes may be taken up by the cells through the process of endocytosis.

Fusion of the liposomes with plasma membrane provides an attractive method for the introduction of hydrophilic molecules into the cell cytoplasm or lipophilic molecules into plasma membrane. In this process cellular organelles may become accessible to the material entrapped, though the evidence is controversial. Many workers believe that a fraction of liposomes absorbed on to cells does indeed fuse with those cells (106). Generally, endocytotic process is thought to play the most prominent role in the uptake of liposomal contents leading to its sequestration in the lysosomal apparatus of the cell (107). Endocytosis has been divided into two types: phagocytosis, covering the internalization of larger particles and pinocytosis describing the uptake of smaller particles like lipoproteins, SUVs, and low molecular weight solutes.
Endocytosis of liposomes by cells may be facilitated by grafting specific ligands to the liposomal surface. Model membrane studies have identified that the density of ligand on the surface of liposome plays an important role in the ligand receptor interaction. Poste and Papahadjopoulos demonstrated that the uptake of liposomes by cell fusion or endocytosis is dependent upon the fluidity and surface charge of liposomes (108). The predominance of any of these mechanisms is controlled by the chemical and physical properties of liposomes, like vesicle size, surface charge, lipid composition, and density of ligand.

**Liposome as Antifungal drug carrier**

The polyene antibiotic treatment of most fungal infections is usually straightforward but systemic use of polyenes is limited due to their toxicity in biological system. In an attempt to reduce the toxicity of Amp-B, several derivatives have been made. The important ones which have been studied for antifungal activity and toxicity are methyl ester of Amp-B (109) and its N-d-orthonyl derivatives, N-propionyl-SH Amp-B, (110) and N 1-deoxy-D-fructose-1yl (111) derivative. Most of the derivatives show either equal or less antifungal activity without any significant reduction in drug toxicity. Furthermore the derivatisation process involves a number of cumbersome chemical steps due to which several derivatives of Amp-B have not gained acceptance as an antifungal measure and have rather remained a subject of academic interest.
In recent years, liposomes have been maximally exploited as a drug carrier for antifungal agents like Amp-B, 5-fluorouracil, grisofulvin. Our area of interest being liposomes as carriers for Amp-B, discussion will be restricted to the potential usefulness of liposomal Amp-B.

Amp-B is an ideal candidate for incorporation into liposome because of its amphipathic nature and its ability to integrate into biological lipid membranes. Administration of Amp-B intercalated into liposomes is an effective way of reducing its toxicity in biological systems. In 1981, New et al. (82) incorporated Amp-B into liposomes and demonstrated that liposomal Amp-B is less toxic as compared to deoxycholate stabilized formulation of fungizone. They also showed that presence of sterol in the liposome preparation markedly increased LD from 3.2 mg/kg for lecithin liposomes to 14 mg. This is likely to be related to the affinity of sterols for Amp-B.

It was observed that the degree of saturation of phospholipids plays an important role in reducing the toxicity of Liposomal Amp-B. Liposomes composed of phospholipids with saturated acylchains are non toxic, whereas liposome intercalated Amp-B composed of phospholipids containing unsaturated acylchains are almost as toxic as Amp-B (112). The reduction in toxicity of liposomal Amp-B in the presence of sterol and also with saturated phospholipids might be due to an increase in their order and stability and thus decreasing the flux of molecules across the membranes.
Since the last decade, much interest has been centered on the use of liposomes as a drug carrier for Amp-B in the treatment of several parasitic and systemic fungal infections. New et al. (82) have demonstrated that Amp-B intercalated into liposomes is less toxic and more effective in the treatment of leishmaniasis than Amp-B. In vitro studies by Juliano et al. (1984) suggest that liposomal Amp-B prepared from dimiristoylphosphatidylcholine and dimiristoyl phosphatidyl glycerol is toxic to fungal cells but not to red blood cells (113). In case of severe infections such as histoplasmosis (114), cryptococcosis (115), candidiasis (116) liposomal Amp-B has been shown to be less toxic than fungizone but retains similar antifungal activity. The reduction in toxicity allows the use of higher dose of Amp-B thus improving therapeutic index. There are conflicting reports regarding the therapeutic efficacy of liposomal Amp-B in the treatment of fungal infections. Taylor et al. (117) have shown that at equal doses of liposomal Amp-B with that of fungizone the liposomal Amp-B was not as effective as the commercial Amp-B in the treatment of experimental candidiasis. Juliano et al. have also checked the therapeutic efficacy of liposomal Amp-B and commercial Amp-B in the experimental model of candidiasis. They found that animal survival rates with the two formulations at equal dosage of Amp-B were similar (118). Szoka's observations (119) seem to lend support to the boosting effect of liposomal Amp-B on its therapeutic index.
The role of sterol in the liposomal formulation of Amp-B was also investigated in the treatment of experimental murine candidiasis by Juliano et al. (120). These workers have shown that there is no significant difference in the survival time of mice infected with Candida albicans treated with sterol containing liposomal formulation of Amp-B compared with those treated with sterol free liposomal Amp-B. The tissue distribution of free and liposomal Amp-B has been reported by Lopez-Berestein in normal as well as in infected animals (121). An attempt has also been made by Szoka et al. (122) to study the tissue distribution pattern of Amp-B in egg phosphatidyl choline/cholesterol/tocopherol acid succinate liposome in normal mice.

Encouraged by these findings Lopez-Berestein, (123) and Sculier et al. (124) have done clinical trials to treat Aspergillosis in cancer patients with liposomal Amp-B and found that liposome associated Amp-B offers better therapy than fungizone.
AIMS AND OBJECTIVES

Amphotericin-B in particular and polyene antibiotics in general have been widely used in clinical practice to treat various systemic fungal infections such as candidiasis, histoplasmosis Aspergillosis etc. The severe toxicity of the drug such as nephrotoxicity, azotemia, renal tubular acidosis, hypokalemia, fever, etc. impose a potential barrier in the exploitation of Amp-B as a proper therapeutic measure. Chemical derivatisation of the drug has been done to eliminate such side effects but these are far from ideal for therapy. In recent years, lot of attention has been paid in the use of liposome as drug carrier to locate the drug to specific sites which in turn could reduce the toxicity and improve therapeutic efficacy. In case of various fungal diseases such as Candidiasis, Histoplasmosis, Cryptococcosis liposomes have been used as a drug carrier for Amp-B in experimental animals. Results from various laboratories indicate that liposomal Amp-B is less toxic while being equally effective in curing fungal infection, and could permit the use of a high amount of drug, which otherwise would be toxic in free form. Encouraged by the promising results of reduced toxicity of liposomal Amp-B in candidiasis, some workers attempted to use liposomal Amp-B in the treatment of aspergillosis in cancer patients. However (123-124) no attempt has been made to evaluate the therapeutic efficacy and toxicity of liposomal Amp-B in experimental animals which deserves proper study before the
formulation enters into evaluation for clinical trial in humans. Keeping this in mind the present investigation attempted to develop a suitable animal model for aspergillosis in BALB/c mice which could provide a systemic evaluation of the therapeutic efficacy of liposomal Amp-B and free drug.

Once the suitable animal model has been developed it is necessary to select the parameters for clinical evaluation of the drug. The toxicity of the free and liposomal Amp-B has been done to find out a dose permissible for use in therapy. Several manipulations in liposomal formulations have been done by altering lipid composition in an attempt to have a suitable formulation that is less toxic and more effective.

In case of experimental candidiasis, the therapeutic efficacy of liposomal Amp-B has been checked by the survival of animals after therapy and the fungal load in disease affected organs. In the present investigation a similar approach i.e. the determination of survival and CFU have been taken as parameters to evaluate the efficacy of liposomal and free drug.

The concept regarding the role of macrophages as a secondary drug carrier for liposomal drug is becoming a major issue. In an attempt to ascertain whether macrophages play a definite role in the liposomal delivery of Amp-B, a liposomal device has been made with mannose grafted on its surface. The idea of grafting mannose, originated from the
well documented fact that macrophages have receptors for mannose. Such a device will enable one to target drug to macrophages and thereby improving drug uptake. Besides, if macrophages happen to be the carrier cells, an improvement in therapeutic efficacy would be observed. The present thesis focuses on this particular issue of the role of macrophages with special emphasis on its role in the targeting of liposomal drug.

It has been shown by Lopez-Berestein's group that liposomal encapsulation of Amp-B could alter the organ concentration of the drug and more drug gains access to the diseased site and thus improving the therapeutic efficacy (121). Whether similar explanation hold true experiments have been designed to study the tissue distribution of liposomal Amp-B by the quantitation of the drug using HPLC. It is quite reasonable to postulate an altered tissue distribution pattern in infected animals as compared to normal, because infection is always associated with damage in various tissues and capilaries and alteration in the properties and population of various cells that are involved in the defence process. The present work also focuses on this issue and tries to study the tissue distribution in normal as well as in infected animals. Search for a suitable explanation for the improved therapeutic efficacy in the light of these observations has also been attempted.