INTRODUCTION AND REVIEW OF LITERATURE
INVASIVE FUNGAL INFECTIONS:

Fungi have been recognized as etiologic agents of diseases in man and animals since time immemorial. The prevalence is recorded during the vedic periods (Vyas, et al., 1993). Since 1950's there has been a dramatic increase in the number of cases of human diseases attributable to pathogenic fungi (Malcolm and Hugh, 1977). This is due to a) increase in number of reported mycosis because of increased awareness on the part of clinicians b) improved diagnostic practices c) prolonged life span of patients in whom opportunistic fungal infections are becoming increasingly frequent. Normally in-group of patients with naturally induced immuno suppression (as in the case of malignant diseases and diabetes) as well as those individuals with artificially induced immuno suppression (caused by transplantation, chemotherapy) fungal infections become life threatening situation (Riddel and Stewart, 1958). Fungal infections in man can be classed as follows:

a) SUPERFICIAL MYCOSIS: - They involve skin, hair and nails. They may not be of serious consequences except being of temporary nuisance. The causative agents include dermatophytes like Epidermophyton, Microsporum, Trichophyton, and hair infections by Trichosporon cutaneum and skin infections by Cladosporium worneckii.
b) **SUBCUTANEOUS MYCOSIS:** - agents of sporotrichosis, chromomycosis, phaeomycotic cysts etc cause it.

c) **SYSTEMIC MYCOSIS:** - They are deep mycosis involving internal organs. The deep mycosis might get widely disseminated. In the various organs and on occasions, can even prove fatal. The fungi and their spores are also capable of causing allergic conditions in man. The systemic infections can be caused by

(i) **Primary Pathogens:** - e.g. *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis* etc.

(ii) **Opportunistic Pathogens:** - e.g. *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans* and *Mucor* species. These opportunistic pathogens initiate infection only when host's resistance is impaired (Goodman and Gilman, 1980).

**INFECTION WITH CANDIDA SPECIES:**

The frequency of invasive candidiasis has increased tremendously to become the fourth most common blood culture isolate (Saral, 1991). The predominantly encountered species of *Candida* in immunocompromised host is *candida albicans*. It can occur as a superficial, invasive or systemic infection involving the respiratory and gastrointestinal tract (Luna and Tortoledo, 1985; Bodey and Fainstein, 1988, 1993). The skin and vagina are also sometimes affected due to this infection. Skin infections are caused due to the breakdown of
the mucosal membrane for e.g. in burn injuries. Mucosal surfaces are the primarily affected parts leading to colonization in the mouth and gut. The predisposing factors for *C. albicans* infection in leukemia patients are prolonged immunosuppressive therapy and impaired cell mediated immune response (Dutta, et al., 1989). The most common form is oral candidiasis and bronchopulmonary candidiasis occurs as a secondary infection due to impaired drainage of secretions from pulmonary tissues. Granulocytopenic patients are the most susceptible to develop disseminated candidiasis has recently been recognized as a syndrome and has been reported to occur exclusively in granulocytopenic individuals. (Thaler, et al., 1988).

**INFECTION WITH ASPERGILLUS SPECIES:-**

The term ‘Aspergillosis’ was first used by Frescenius in 1850 while working on a fungal infection of the air sac of a bird—bustard. He named the isolate ‘*Aspergillus fumigatus*’. There are around 600 species of genus *Aspergillus*, out of which only about 8 have been reported to be pathogenic. *Aspergillus* is ubiquitous in nature being commonly found in soil and decaying vegetation and the most common species involved in infections are *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. niger*, *A. terreus*, *A. ochraceus* and *A. clavatus* (Rippon, 1982; Pennington, 1986).
The pathophysiology of allergic disorders associated with *Aspergillus* species is best illustrated in allergic bronchopulmonary aspergillosis (ABPA). An allergic response to aspergillar hyphae is set in motion without invasion of the tissues of the host. It appears that the bronchospasm is mediated by IgE (type I reaction, immediate hypersensitivity), whereas bronchial & peribronchial inflammation are induced by the formation of immune complexes (type III reaction). The size of the conidia of *A. fumigatus* varies between 2.5-3.0 μm in diameter and is reported to be thermotolerant (active even at 50°c (Peterson and Schmpff, 1989; Talbot, et al., 1991). The conidia due to their small size are easily inhaled and can reach the respiratory tract where they colonize in prehealed pulmonary cavities causing invasive pulmonary aspergillosis. Factors apart from environmental exposure are also important for development of the disease (Gustafson, et al., 1983; Kusne, et al., 1992; Kramer, et al., 1993). Studies have demonstrated that there exists a direct correlation between the duration of granulocytopenia and the onset of infection. Patient with prolonged treatment induced granulocytopenia were found to be made susceptible to developing aspergillosis (Saral, 1991).

* A. fumigatus invades the lung tissue in immunocompromised host which leads to the necrosis of the tissues as a result of the production of toxins by the mold (Kemper, et al., 1993; Lortholary, et al., 1993). Dissemination of infection
occurs through hematogenous spread. The hyphae grows through the walls of
the blood vessels may causing thrombosis and ischemic necrosis (Young, et al.,
1970). The propensity of *Aspergillus* species to invade blood vessels might lead
to extensive hemorrhagic infraction and hemoptysis in thrombocytopenic patients.

Albeit, the pathogenicity of *Aspergillus* species depends primarily on
immunosuppression, there are several innate aspergillal virulent factors. Under
certain conditions *in-vitro* some strains synthesize proteases such as elastase, an
enzyme implicated in invasion of lung tissue (Denning, et al., 1990). An inhibitor
of complement produced by certain strains of *A. fumigatus* and *A. flavus* might
enhance pathogenicity by subverting the generation of opsonins and chemotactic
that has immunosuppressant and antiphagocytic activity *in-vitro* (Moser, et al.,
1992). The extend to which of this and other toxins are active in the
pathogenesis of human infection is still unknown.

Invasive pulmonary aspergillosis is best understood from the perspectives
of impaired pulmonary host defenses. On the basis of the studies done by
Schaffer, et al., (1982) two lines of defense were delineated against *Aspergillus*,
both of which need to overcome before infection can be established. The first
line of defence is directed against spores. The mononuclear phagocytic system
forms an efficient defence barrier by destroying the fungus in its conidial stage
(Bennett, 1988; Kan, et. al., 1988). Studies have clearly demonstrated that A. flavus spores are present in the phagolysosomes of alveolar macrophages and the spores of A. fumigatus were found to be efficiently taken up by the organs rich in the reticuloendothelial system (RES) after intravenous administration. Cortisone inhibits the first line of defense by impairing the ability of macrophages to prevent germination of conidia in-vitro and in-vivo, which results in impaired clearance to the fungus in vivo. When the first line of defense is overcome normal mice can still resist infection but neutropenic mice are susceptible to infection. Neutrophils thus form the second line of defense, which destroy the mycelia. Natural immunity is generally overcome when both the defense barrier i.e. RES and neutrophils are impaired. When a large dose of cortisone is administered it results in destruction of spores by macrophages and the recruitment of neutrophils around the fungus. Invasive aspergillosis remains a very important cause of fungal diseases in immunosuppressed hosts especially liver and bone marrow transplant cases with mortality approaching 100% (McWhinney, et. al., 1993). Documented reports indicate that invasive aspergillosis causes high mortality rate surpassing 60% even with antifungal therapy (Rinaldi, 1983)
As there is an alarming rise in the number of cases, several problems related to cure of fungal infections have been identified/recognized which are discussed below.

Increasing resistance – Long term treatment of fungal infections in immunocompromised patients has begun to show species resistant to older antifungal drugs. Several other species of fungi have also begun to exhibit resistance (Hay, 1991).

Emergence of new pathogens – Species of fungi that once posed no threat to humans are now being detected as a cause of disease in immune-deficient people. Even low virulence baker's yeast, found in the human mouth, and has been found to cause infection in susceptible burn patients (Stemberg, et. al., 1994)

Lagging research – Because pathogenic fungi are difficult to culture, and because many of them do not reproduce sexually, microbiologically and genetic research into the disease causing organisms has lagged far behind as compared to other organisms.

A wide array of antifungal drugs have been designed and investigated (Denning, 1991, Warnock, et. al., 1998). They are broadly classified as under:

Azole derivatives:
The azoles act primarily on ergosterol biosynthesis at the C-14 demethylation step, a cytochrome P-450 dependent reaction (Figure 2) (Hay, 1994). The resulting ergosterol depletion and accumulation of methylated sterols interferes with the bulk functions of ergosterol. The structure of the plasma membrane is altered, making it more vulnerable to further damage and altering the activity of several membrane-bound enzymes, such as those for nutrient transport and chitin synthesis. Severe depletion of ergosterol may also interfere with the hormone like functions of ergosterol and affect cell growth and proliferation (Parks, et al., 1992). The overall effect is fungistatic rather than fungicidal, thereby limiting and utility of these drugs (Figure 1) (Walsh and Pizzo, 1988). Some azoles can also interact with and damage the cell membrane directly. They are consequently fungicidal and toxic and therefore used only topically (Galgiani, 1993). Though the other azoles do not produce serious toxicity, they have shown to influence the endocrine system by inhibiting mammalian cytochrome P-450-dependent enzymes, which are known to synthesize steroid hormones.

**Allylamines/thiocarbamate**

There are two Allylamines antifungal in clinical use, naftifine (topical) and terbinafine (oral), and one thiocarbamate, tolnaftate (topical) (Ryder, et al 1988). All three inhibit squalene expoxidase which, together with oxidosqualene cyclase,
cyclizes squalene to lanosterol (Figure 2). The resulting ergosterol depletion and squalene accumulation affect membrane structure and functions such as nutrient uptake (Figure 1). Squalene epoxidase is not a cytochrome P-450 enzyme, and thus this class of compounds is less toxic than the azoles. Because of their poor pharmacokinetics, these compounds work only against dermatophytes although squalene epoxidase is ubiquitous and allylamines have broad antifungal activity in vitro. Moreover, they have almost similar minimum inhibitory concentration and minimum fungicidal concentration (Shadomy, et. al., 1985).

**Fluorophyrimidine 5-fluorocytosine (5-FC)**

It is mainly used in combination with amphotericin B in cryptococcal meningitis. It is deaminated to 5-fluorouracil (5-FU), converted to the nucleoside triphosphate and incorporated into RNA where it causes miscoding (Figure 1). In addition, 5-FU is converted to deoxynucleoside, which inhibits thymidylate synthase and thereby DNA biosyntheses. However, it has a limited spectrum of activity and its administration is accompanied by toxic effects such as bone marrow suppression and hepatotoxicity (Georgopapadakou and Walsh, 1994)

**Cell Wall synthesis inhibitors**

The fungal cell wall, a structure essential to fungi that has functions similar to its bacterial counterpart and that is lacking in mammalian cells, would seem to be an ideal target for antifungal agents (Figure 1). Inhibitors of the biosynthesis
of two important cell wall components, glucan and chitin, already exist. Polyoxins and the structurally related nikkomycins (both consist of pyrimidine nucleoside linked to a peptide moiety) are known to inhibit chitin synthase competitively, presumably acting as analogs of the substrate uridine diphosphate (UDP)-N-acetylglucosamine (chitin is an N-acetylglucosamine homopolymer) causing inhibition of separation and osmotic lysis (Cabib, 1987). Unfortunately, the target of Polyoxins and nikkomycins is in the inner leaflet of the plasma membrane, they are taken up by a dipeptide permease, and thus peptides in body fluids antagonize their transport. Appropriate polyoxin derivatives that bypass peptide transport have been synthesized. Another promising development is the observed synergy between chitin synthase inhibitors and glucan or ergosterol synthesis inhibitors, although its clinical significance is yet to be established (Baloch and Mercer, 1987).

Other Antifungal Agents

Less commonly used drugs include the morpholines (Figure 1). For example amorolfine, which is used in the topical treatment of nail infections also, acts on the ergosterol pathway (Figure 2) (Baloch and Mercer, 1987) Morpholines inhibit two steps in the ergosterol pathway, Δ14 reductase and Δ7 - Δ8 isomerases. Other targets in the ergosterol pathway are oxidosqualene cyclase
Figure 1. Site of action of some antifungal compounds used clinically are italicized.

(Adapted from Georgopapadakou and Walsh, 1994)
and Δ^{24} methyl-transferase, the latter being unique to ergosterol synthesis and thus a particularly attractive target (Oehlschlager and Czyzewska, 1992).

Topoisomerase I and II control the topological state of DNA so that it can undergo replication, transcription, repair and chromosomal segregation. The success of topoisomerase inhibitors in antibacterial and anticancer chemotherapy has made fungal topoisomerase particularly attentive drug targets (Liu, 1989). However it is not yet known whether fungal topoisomerase exploitable differences relative to their mammalian counterparts.

Elongation factor 3 (EF-3), a 125-kDa protein, participates in fungal but not mammalian protein synthesis is another promising target (Kamath and Chakraburty, 1989; ). It is present in most fungi and is essential for viability and disruption of its gene is lethal. It is specifically required by the yeast 40S ribosomal subunit, although its exact function in the elongation cycle is unclear. A major drawback for rational drug design is the absence of known EF-3 inhibitors.

Toxicity consideration would preclude DNA itself as being an antifungal target, although it is the likely target of the antipneumocytis drug pentamidine (Jones, 1990). After the discovery of cispentacin which inhibits homoserine dehydrogenase, the possibility of interfering with amino acid synthesis emerged, although available information may not yet be sufficient to launch a synthetic,
rational drug design program (Konishi, 1989, Yamaki, 1990). Ornithine
decarboxylase (ODC), the rate limiting enzyme in polyamine synthesis and a
favorite target for growth inhibition in cancer chemotherapy, may be another
antifungal target (Mc Cann and Pegg, 1992). Microtubule aggregation – the
target for griseiofulvin, the agricultural fungicide, benomyl and the anticancer
drugs vincristine, vinblastin and taxol – also merits reexamination as a possible
target for clinical antifungal. More speculative means to combat fungi include
inhibiting protease activity, decreasing adhesion through alternate ligands and
preventing phase transition to the more invasive form, or preventing virulence in
general, through effects on signal transduction.

POLYENE ANTIBIOTICS

Polyene macrolides are produced mostly by soil actinomycetes of the
genus Streptomyces. They exert antifungal activity by forming transmembrane
pores, which leads to the leakage of vital metabolites resulting in cell death
(Figure 1). They exhibit their activity against dermatophytes, fungi and protozoa
(Mallams, 1988). Nystatin was the first antibiotic discovered in this class of
compounds, since then over 200 polyenes have been reported and over 40 have
been elucidated for their structures (Hazen and Brown, 1950). Polyenes are
characterized by large 20-40 membered lactone ring containing 3-8 conjugated
double bonds (Medoff and Kobayashi, 1980). These molecules are amphipathic
Figure 2. Site of action of some clinical useful ergosterol synthesis inhibitors.

(Adapted from Georgopapadakau and Walsh, 1994).
in nature due to the presence of double bonds and hydroxyl groups on opposite sides of macrolide ring. Macrolide antibiotics have been classified according to the number of conjugated double bonds as trienes (aureofungin), tetraenes (etruscomycin), pentaenes (filipin), hexaene (dermostatin) and heptaenes (hamycin, amphotericin B). The heptaene group of macrolides is subclassified into aromatic and non-aromatic polyenes. The best known example of non-aromatic heptaene polyene is amphotericin B whereas hamycin belongs to the aromatic subgroup.

Polyene macrolides exhibit characteristic ultraviolet absorption spectra ranging between 200-405 nm due to the presence of conjugated double bond system (Oroshnik and Mebane, 1963). Polyenes exhibit poor solubility in water, alcohols and esters but they dissolve readily in dimethyl sulfoxide.

Kinsky (1961) has demonstrated experimentally that polyene antibiotics enhance the cell membrane permeability of a number of organisms, which results in leakage of important cellular constituents resulting in cell death. Fungal cells in general are susceptible to the polyene action. Work done on yeast's such as *Saccharomyces cerevesiae* and various species of *Candida* have clearly established that polyenes induce leakage of ions and small molecules such as K, Na, inorganic phosphates, sorbose, xylose, urea, nucleotides and phosphate esters (Marini, et al., 1961, Medoff and Kobayashi, 1980; Aggett, et al., 1982).
Bacteria do not have sterols in their membranes and are not affected by polyene antibiotics suggesting that the mode of action of polyenes be by interacting with membrane sterols, which are present in eukaryotic membranes. De Kruijff, et al. (1974) has shown that a 3β OH group, a planar ring system and hydrophobic side chain at C-17 position in the sterol molecule are essential for interaction with polyene antibiotics.

**AMPHOTERICIN B**

Amphotericin B is a heptaene polyene macrolide antibiotic isolated from Streptomyces nodosus (Ryley, et al. 1981). The molecule (MW 924.1) is about 24 Å long and consists of a free carboxyl group, a macrolide ring of carbon atoms (closed by the formation of an internal ester or lactone) and the glycosidic side chain containing the amino sugar mycosamine (Figure 3a-b). The presence of the carboxyl group at C-16 and amino group renders the molecule amphipathic character (Figure 4). Amphotericin B is a yellow amorphous powder, which is insoluble in water and shows its maximum antifungal activity between pH 4.2 and 6.5. It is the first choice of drug for the treatment of most of the systemic fungal infections and its marketed as a topical cream lotion as well as an ointment (Hoeprich, 1978; Medoff, et al. 1983; Walsh and Pizzo, 1988). Amphotericin B is commercially available in the powder form, which is known as fungizone. Fungizone consists of 50 mg of amphotericin B with 41 mg of sodium.
Figure 3(a) Structure of few antifungal used in current day therapy.

(Adapted from Andreoli, 1974)
Figure 3(b) Structure of few antifungal used in current day therapy.

(Adapted from Andreoli, 1974)
deoxycholate and 25.2 mg of sodium phosphate as buffer. Amphotericin B shows fungicidal activities against most species of Candida, Aspergillus, Mucoraceae, Blastomyces, Cryptococcus, Histoplasma and Coccidioides. The antifungal activity of amphotericin B lies in its preferential binding to ergosterol resulting in the formation of transmembrane pores of about 10 Å in diameter. The formation of the pore results in the leakage of the vital metabolites leading to fungal cell lysis. Studies have shown that the anticellular effects of amphotericin B are more inhibited by extracellular ergosterol than cholesterol and lower concentrations of amphotericin B were required to induce K leakage from ergosterol containing lipid vesicles as compared to that containing cholesterol (Kotler-Brajtburg, et. al., 1974).

Based on the experimental data for Amphotericin B – cholesterol complex, Andreoli (1974) proposed a model for the pore (Figure 5). The model suggested that C-1-C-13 and C-20-C-33 segments are visualized as a rod like array of 20-29 Å in length. The C-15 hydroxyl group, C-16 carboxyl group and C-19 mycosamine group are present on one side of the rod located at the water bilayer interphase. The rod is embedded in the interior parallel to the hydrophobic chains of phospholipid as well as to planar cyclopentanophenanthrene skeleton and C-17 acyl residues of cholesterol. According to this model two kinds of hydrogen bonds are involved between polar group of Amphotericin B and 3-OH
Figure 4. Corey – Pauling space filling model of amphotericin B .
Amphotericin B after interacting with lipid bilayer, assumes this orientation with hydrophobic c-20 - c-33 haptaene segment is parallel to c-1 - c-13 hydroxyl – substituted segment; carboxyl groups are at extreme right.
(Adapted from Andreoli, 1974)
Figure 5. Structure of amphotericin B – cholesterol pore. The dotted lines between the hydrocarbon chains of phospholipids represent short range Van der walls forces. The dashed lines represent hydrogen bonds.

(Adapted from Andreoli, 1974)
Of cholesterol. The first involves a 3OH proton and carboxyl oxygen of C-16 carboxyl group whereas the second is between 3-OH oxygen and C-17 hydroxyl proton of amphotericin B.

De Kruijff and Demel (1974) have also studied polyene-sterol interactions and have proposed mechanisms for induction of membrane permeability changes by various polyenes. They conceived that amphotericin B cholesterol complex as a circular arrangement of 8 amphotericin B molecules interdigitated with 8 cholesterol molecules. The outside portion is hydrophobic whereas the inside portion is hydrophilic due the presence of the hydroxyl groups of amphotericin B. Two such complexes i.e. half pores generate a full pore which traverses the membrane. The pore size of such complexes is about 8Å°(Kasumov, et. al., 1979).

TOXICITY ASSOCIATED WITH AMPHOTERICIN B THERAPY

Like ergosterol, amphotericin B can also interact with cholesterol found in membranes of mammalian cells (especially red blood cells) leading to hemolysis (Figure 6). This makes it highly toxic and thus has severely restricted the maximal exploitation of amphotericin B as a therapeutic agent in clinical medicine. Amphotericin B also accumulates in the cells of kidneys and produces nephrotoxicity which causes an unavoidable side effect leading to a decline in
Figure 6. Structure of cholesterol (a) and ergosterol (b). For binding of amphotericin B, number of features on the sterol nucleus are essential, namely a 3-β hydroxyl group and a Δ - 22 double bond together with a cholestane skeleton.
glomerular filtration rate, renal tubular acidosis, decreased serum potassium and diminished renal concentrating ability (Butler, et al., 1964; Finn, et al., 1977).

The decrease in glomerular filtration rate appears to be related decreased renal blood flow leading to cortical ischemia (Burgess ad Birchall, 1972). Glomerular lesions and tubular damage have also been describing (Douglas, et al., 1969). Although it is thought that glomerular filtration rate generally returns to normal shortly after therapy is discontinued, irreversible alterations in renal function have been reported (Takaes, et al., 1963).

Amphotericin-induced renal tubular acidosis is characterized hypokalemia, an inability to excrete an acid load, and occasion nephrocalcinosis, generally without induction of systemic acidosis (Finn, et al., 1977). Although the mechanism remains unclear it is though that this toxicity occurs primarily in the distal nephron (Mc Candy, et al., 1968). The defects distal nephron functions, such as impaired concentrating ability and distal renal tubular acidosis, have been attributed to the effects of the drug on tubular membranes. Amphotericin B interacts with membrane bound sterols to increase passive permeability to sodium, potassium, hydrogen ion, water and molecular weight solutes such as urea. Such effects could explain potassium wasting and the inability to maintain an acid and osmotic gradient in the distal nephron (Steinmetz, et al., 1970, Bullock, et al., 1976). Hypokalemia commonly occurs in association with
amphotericin induced renal tubular acidosis and is the most widely recognized complication of amphotericin B therapy. In addition, serum magnesium levels may also decreased during amphotericin-B therapy. Many generalized reactions are also described during amphotericin B therapy. Fever, chills, nausea, vomiting, anorexia, headache and myalgias occur with the early infusions in most patients (Goodman and Gilman, 1975; Kucers and Bennett, 1975). It has been observed that these reactions become less intense as therapy progresses although this is not always the case (Utz, et. al., 1964). The majority of patients receiving amphotericin B experience an 18-35% fall in hemoglobin concentration (Miller, et. al., 1969; Koeffler and Golde, 1977).

In an attempt to circumvent the problems associated with administration of amphotericin B, various chemical modifications have been tried. A methyl ester of amphotericin B was prepared which was reported to have greater water solubility and reduced toxicity. However the clinical testing of the salts of this compound were halted because of reports of potential neurotoxicity (Ellis, et. al., 1982, Huston and Hoeprich, 1978). Kucers and Bennett (1987) reviewed data and suggested a slight reduction of activity of this compound compared with amphotericin B against pathogenic fungi. Other esterified form of amphotericin B (such as N-d-omithyl methyl ester derivative) has also been tested (Galgiani and van Wyck, 1984).
In the recent years, lipid formulations have emerged as important delivery vehicles for bioactive molecules (Heimenz and Walsh, 1996). Amphotericin B, due to its amphipathic nature and ability to integrate into biological lipid membranes, is an ideal candidate for incorporation into lipid formulations such as liposomes (Graybill et al., 1982). In an attempt to reduce the toxicity of amphotericin B, many lipid formulations have been developed recently and these will be discussed below (de Marie et al., 1994).

LIPOSOMES:

Liposomes are lipid vesicles, which form spontaneously when the lipids (Figure 7) are dispersed in an aqueous media (Figure 8). The size of these vesicles can range from 10 nm – 10 μm in diameter depending on the method of preparation of liposomes. There are various methods for the preparation of liposomes; the common methods include vesicles formed by sonication, by detergent dialysis, reverse phase evaporation, extrusion through French press, ethanol injection and rehydration dehydration method (New, 1990; Woodle and Paphadipoulous, 1989).

Liposomes have proved to be the most promising biodegradable drug delivery system in view of their ability to incorporate both hydrophilic as well as lipophilic drugs (Figure 9). They were originally developed by Bangham et al (1965) as a model to study cell membranes. These were termed as “Liposomes”
of "Banghosomes" by Sessa and Weissmann (1968). Gregory Gregoriadis and Brenda Ryman (1972) were the first to inject liposomes to animals and study their ability to deliver the contents (albumin) into the liver. They also put forth a suggestion to use liposomes entrapped enzyme for the therapy of metabolic disorders. The utility of specific drug delivery at therapeutic sites circumventing the toxic sites has been a coveted goal in clinical therapeutics. Liposomes offer an advantage as drug carriers because they protect the drug form exerting its action before reaching the desired site and enhance the accessibility, recognition and selective interaction with target tissues (Pozhansky and Juliano, 1984; Torchilin, 1985). Targeting of liposomes can be passive or active. The natural distribution of liposome in-vivo, composed of phospholipids alone or in combination with cholesterol is referred to as passive targeting. Active targeting employs the use of specific ligands on the liposomal surface, which alters the natural tissue distribution of liposomes. Specific ligands used for active targeting include antibodies, glycolipids, asialoglycoproteins and lectins, which recognize and bind to specific molecular determinants on the target cell (Machy and Leserman, 1987).

**Long circulatory liposomes:**

One of the major limitations in employing liposomes as a delivery vehicle in-vivo is the discerning fact that these vesicles are mainly scavenged by the cells
Figure 7. Structure of phospholipid. The most well-known in this group are the glycerine phosphatides, which (linked to the basic structure of the glycerine) exhibit two nonpolar fatty acid chains and hydrophillic head group phosphorylized alcohol. The substances functioning as alcohols of biological system are serine, choline, glicerine or inosite.
of the RES upon intravenous administration. This prevents their prolonged circulation in the blood and subsequent delivery to the desired site other than the RES, despite their resemblance to the cell membranes. Although this property has been exploited for the treatment of various diseases associated with the organs (Coune, 1988), its application appeared limited in targeting drugs to these sites alone. Further, repeated administration of liposomes may also lead to RES toxicity thereby impairing the important defence mechanism of the body. For the successful exploitation of liposomes as a drug delivery vehicle, it is imperative to device ways of bypassing the RES in order to optimize their localization in other tissues. Therefore, attempts have been made by a number of investigators to alter the liposomal biodistribution by various means such as coating the liposomes with plasma proteins (Torchillin, et. al., 1980, 1983), blocking the reticuloendothelial system (Profitt, et. al., 1983; Illum, et. al., 1986), change in lipid composition (de Gier, et. al., 1969; Inoue, 1974, Gregoriadis and Davis, 1979), coating the surface with hydrophilic moieties like polysaccharides (Pain, et. al., 1984), glycolipids (Allen and Chonn, 1987) and polyethylene glycol (Savoca, et. al., 1979, Illum and Davis, 1984) and preparation of vesicles of small i.e. 70-200nm which have been reported to have long circulatory life (Liu, et al. 1991); Huang, et. al., 1992; Litzinger, et. al., 1994). Development of long
Figure 8. Self aggregation of polar lipid molecules. Depending on the temperature, the molecular shape of the lipids and the conditions in the lipid – water mixture, lipid molecule may self assemble into different colloidal particles. At higher concentrations, these particles can organize into ordered liquid – crystalline phases. Several different types of liposome are shown in the centre of the figure.

(Adapted from Lasic, 1998)
circulatory liposomes has led to several important medical applications such as cancer chemotherapy (Allen, et. al., 1992) gene delivery (Wang and Huang, 1987) and treatment of fungal infections which will be described later.

LIPID FORMULATIONS OF AMPHOTERICIN B:

1. LIPOSOMES:

   The first investigators to use liposomes as drug carriers for amphotericin B were New, et. al., (1981) who developed egg and soya lecithin liposomes with or without cholesterol incorporating amphotericin B. They employed it for treatment of leishmaniasis and observed significant reduction in toxicity and enhancement of therapeutic efficacy. They further reported that toxicity and therapeutic efficacy of the formulation was significantly dependent on the composition of phospholipids as well as presence or absence of cholesterol. They demonstrated that inclusion of cholesterol in egg phosphatidylcholine liposomes increased the \( \text{LD}_{50} \) of the preparation from 3.2 to 14 mg/kg and achieved a much larger parasite clearance.

   In 1982, first time amphotericin B incorporated into liposomes was used for treatment of fungal infections such as cryptococcosis and histoplasmosis (Graybill, et. al., 1982; Taylor, et. al., 1982). These workers reported that there was a significant reduction in toxicity (9-fold) and enhancement of therapeutic
efficacy of amphotericin B when delivered through positively charged multilamellar vesicles (MLVs) as evidenced from improved survival and reduced fungal load in treated mice. Biodistribution studies revealed that liposomal amphotericin B is rapidly cleared from circulation and accumulated in liver and lungs. They postulated that elimination from circulation and accumulation in infected organs like liver and lungs to be the reason for reduction in toxicity and enhancement of therapeutic efficacy.

However, the major breakthrough in the treatment of fungal infections with liposomal amphotericin B came with Lopez-Berestein’s work (1983, 1987). They developed multilamellar liposomes (0.5-6.0 μm) comprising of dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol in a 7:3 molar ratio with 5 mol % amphotericin B and studied therapeutic efficacy of this formulation in the treatment of candidiasis in neutropenic mice (Lopez-Berestein, et al., 1984). They reported more than 15-fold reduction in toxicity of amphotericin B with their formulation. Moreover no abnormalities in blood chemistry or histology were observed in the animals injected with encapsulated amphotericin B, while the administration of free amphotericin B was associated with nephrocalcinosis and renal parenchymal edema. Infected mice treated with single doses of 3 mg/kg liposomal amphotericin B had an increased survival time compared with those
Figure 9. Diagram of fat and water-soluble agents encapsulated liposome.

(Adapted from Hiemenz and Walsh 1996)
injected with either single dose (0.8 mg/kg) or multiple doses (0.8 mg/kg for 5 days) of free amphotericin B. That is, the encapsulated drug was almost as effective as the free drug when used in similar concentrations, while the animals treated with higher concentrations of liposomal amphotericin B had significantly improved survival (Lopez Berestein, et. al., 1984). These findings suggest that in severely infected hosts higher doses of liposomal amphotericin B (at levels not achievable with free amphotericin B) may prove to be an effective treatment approach. This was demonstrated when treatment was delayed where neither single nor multiple doses of free amphotericin B resulted in increased survival, whereas treatment with high doses of liposomal amphotericin B (4-11.2 mg/kg) showed improved therapeutic efficacy. Subsequently, this formulation was taken up for human clinical trials to treat systemic fungal infections in patients with hematologic malignancies at M.D. Anderson Cancer Centre, Houston (Lopez-Berestein, et.al., 1985). All these patients had biopsy findings or culture evidence of progression of their fungal infection while being treated with conventional amphotericin B (Weber and Lopez Berestein, 1987). It was observed that when these patients were treated with liposomal amphotericin B, the invasive disease could be controlled in more than 50% of cases. Toxicity was restricted to mild fever and hypokalemia (Lopez Berestein, et. al., 1989) and none of the patients developed substantial renal toxicity. Many of these patients who had impaired
renal function due to prior. AmB doc use showed a decrease in serum creatinine during liposomal therapy.

Another liposomal formulation called amphopilosomes was developed at the Jules Bordet Institute in Brussels, Belgium. This consisted of SUV’s most of which had a diameter of only 60nm (Sculier, et. al., 1988). These small sonicated liposomes comprising of egg phosphotidylcholine, cholesterol and stearylamine in a molar ratio 4:3:1 largely escape uptake by the mononuclear phagocytic system, and thus give high and sustained blood concentration plasma levels being 5 to 10 fold higher than after AmB doc. When they were employed for treatment of cancer patients with fungal infections more than 50% patients showed improvement. The tolerance of the formulation was very good and no renal function impairment occurred. In another study, Sculier, et. al., (1989) reported successful treatment of two patients with fungaemia due to Candida albicans and Candida tropicalis after unsuccessful therapy with AmB doc.

Though there have been many reports of successful treatment of fungal infections with these formulations, many complications during their administration have also been reported. In a particular case hypoaxemia and depressed cardiac output during administration of liposomal amphotericin B have been observed (Levine, et. al., 1991) which was thought to be related to release of thromboxane A2 caused by the large liposomes. Thus liposomal formulations
developed by Lopez-Berestein and Sculier were not taken for commercial production (Heymans, et. al., 1990)

However, these studies led to further development of other lipid formulations of amphotericin B (Brajtburj, et. al., 1990). Three major products that were subsequently developed were amphotericin B lipid complex (ABLC; Abeleet, Liposome Company, Princeton, NJ), amphotericin B colloidal dispersion (ABCD; Amphocil, Sequus Pharmaceuticals, Menlo Park, CA) and AmBisome (Nexstar, San Dimas, CA) apart from lesser known formulations like intralipid emulsion (Kirsh, et. al., 1988; Chavanet, et. al., 1992; Caillot, et. al., 1993). Many of these formulations have undergone extensive experimental and clinical evaluation.

Subsequently, many other reports appeared during this period about liposomal amphotericin B and its mechanistic basis of treatment of fungal infections. Szoka, et. al., (1987) studied the effect of encapsulation of amphotericin B in liposomes on its toxicity and antifungal activity. They reported that cytotoxicity and lethality of the liposomal preparation were a function of their lipid composition and size. They observed that sterol containing liposomes were least toxic followed by solid liposomes and fluid liposomes had highest toxicity. They also reported that small sterols containing vesicles were least lethal than large vesicles of the same composition. These researchers suggested that
reduction in toxicity of liposomal amphotericin B in SUVs could be due to faster rate of transfer of amphotericin B from small unilamellar vesicles to fungal cells as compared to MLVs (Szoka, et. al., 1987). At the same time the increase accessibility of amphotericin B in turn could account for improved fungicidal activity of sterol containing SUVs as compared to MLVs.

Physicochemical studies carried out by Jullien, et. al., (1990) have shown that in liposomes, free amphotericin B exits in equilibrium with the bound form. It has been surmised that free drug may be responsible for the killing process. Once the unbound amphotericin B attaches to the sterol of fungus, it shifts drug equilibrium from the bound form to the free form, thereby enhancing the concentration of amphotericin B attached to the fungus, which may be facilitating the killing process (Figure 10) Janoff, et. al., (1988a) observed that at high amphotericin B to lipid mole ratios, ribbon like structures of amphotericin B-liquid aggregate are formed which significantly reduce the toxicity to mammalian cell but retains effectively towards fungal cells. Hopfer, et. al., (1987) also observed that use of liposomal amphotericin B decreased the rate of transfer of the drug to the mammalian cell membrane. Mehta, et. al., (1984) reported that both free and liposomal drug kill fungal cells. These results suggest that the markedly improved therapeutic index of liposomal amphotericin B be largely due to fundamental alteration in the ability of the drug to interact with mammalian cell membranes.
Figure 10. Theory for why liposomal amphotericin B is less toxic than conventional amphotericin B. Bound amphotericin B (AmB – complex donor) is administered intravenously as compared with conventional amphotericin B. Only a small portion of amphotericin B is in aqueous phase as amphotericin B. The latter is more toxic to fungal membranes (mainly ergosterol) than to mammalian membranes (mainly cholesterol).

(Adapted from Schmitt, 1993)
rather than to alterations in pharmacokinetics or drug distribution. The reduced toxicity can also be explained in terms of reduced amount of free amphotericin B associated with the liposomal form which is not enough to lyse RBCs but can kill the fungus. It is also explainable by the fact that the uptake of liposomal amphotericin B by macrophage minimizes in interaction with RBCs (Mehta, et. al., 1994). The degree of saturation of the phospholipids also plays a key role in reducing the toxicity of liposomal amphotericin B. Reduction in toxicity of liposomal amphotericin B in the presence of sterol and saturated phospholipids may be due to the increased degree and stability of liposomes in circulation, thereby reducing the exchange of phospholipid molecules across the membrane (Juliano, et. al., 1986).

Many reasons have been attributed to the improvement in therapeutic efficacy of liposomal amphotericin B in the treatment of fungal infections. Altered tissue distribution of liposomal amphotericin B has been reported by many groups in infected state as compared to normal (Lopez-Berestein, et. al., 1984). Enhanced localization of drug at the infected site has been cited as an important reason for enhancement of therapeutic efficacy. Mehta, et. al., (1989) have shown that at low concentration, the fungizone completely inhibits macrophage differentiation and production of superoxide anion by macrophages, whereas encapsulation of amphotericin B in liposomes abolishes theses adverse effects.
These results also suggest that liposomal encapsulation of amphotericin B reduces the immunosuppressive effects exerted by free amphotericin B. Mehta, et al., (1994) also attempted to show that circulating phagocytic cells in blood play an important role in transport and accumulation of liposomal amphotericin B at the inflammatory site in vivo. The presence of liposomal amphotericin B in inflammatory peritoneal cells after intravenous administration of fluorescence labeled liposomal amphotericin B also confirmed that macrophages play an important role in the transport of intravenously administered liposomal amphotericin B to the inflammatory sites.

Ahmad, et. al., (1989, 1990, 1991) have developed various lipid formulations with a view to reduce the toxicity and enhance the therapeutic efficacy of amphotericin B. They observed that the toxicity of amphotericin B is significantly reduced after intercalation in liposomes which is composed of egg phosphotidylcholine, cholesterol and phosphatidic acid with a concomitant enhancement of therapeutic efficacy (Ahmad, et. al., 1989). They also observed that composition as well as charge plays an important role in reduction of toxicity (Ahmad, et. al., 1990).

Subsequently they also studied the effect of grafting of ligands like mannose and tufts in on liposomes on the therapeutic efficacy of amphotericin B in aspergillosis (Ahmad, et. al., 1991; Owais, et al., 1993). These authors have
reported that encapsulation of amphotericin B in tuftsin bearing liposomes increased the therapeutic efficacy, which is attributed to macrophage activation by to tuftsin. They also observed that inclusion of cholesterol in mannose-grafted amphotericin B liposomes decreased the toxicity as well as increased the therapeutic efficacy. Further tissue distribution studies indicated that this is due to the increased localization of liposomes at the infected sites, which is proposed to enhanced uptake of mannosylated liposomes by macrophages.

2. LONG CIRCULATORY AMPHOTERICIN B LIPOSOMES:

In an attempt to understand whether longer blood residence time is of importance for the improved efficacy of amphotericin B liposomes, many different studies have been carried out (Otsubo, et. al., 1998). The rationale behind use of long circulatory liposomes was that it may lead to increased accumulation of liposomal amphotericin B at sites of fungal infection other than RES organs such as kidney and lungs (Figure 11).

Figure 11. Schematic representation of four major liposome types. Conventional liposomes are either neutral or negatively charged. Sterically stabilized ("stealth") liposomes carry polymer coating to obtain prolonged circulation times. Immunoliposomes ("antibody targetted") may be either conventional or sterically stabilized. For cationic liposomes, several ways to impose a positive charge are shown (mono-, di-, or multivalent interactions).

(Adapted from Lasic, 1998).
found that the in-vitro antifungal activity of polyethylene glycol liposomes, was more than non-polyethylene glycol liposomes and AmBisome was maximum. Polyethylene glycol amphotericin B liposomes were more effective in clearing fungus at lower doses (5 mg/kg.) whereas AmBisome could achieve complete clearance at higher doses (29 mg/kg.). These workers concluded that enhanced therapeutic efficacy could be achieved at lower doses by prolonged residence time of liposomes in the blood compartment.

In another study, these workers observed that a single dose treatment with 5 mg/kg. Polyethylene glycol amphotericin B liposomes resulted in zero mortality and significant reduction of fungal load whereas 70% mortality occurred in mice treated with 5 daily doses of 5 mg/kg AmBisome (van Etten, et. al., 1998a). These workers also attempted to find out whether low uptake of polyethylene glycol amphotericin B liposomes by macrophages is the sole reason for low intracellular activity against C. albicans. With there experimental findings, they proposed that there is reduced uptake of polyethylene glycol amphotericin B liposomes by macrophages, but due to high direct antifungal activity of amphotericin B in polyethylene glycol amphotericin B liposomes, the resulting level of action against intracellular C. albicans is similar to AmBisome (van Etten, et. al., 1998b).
Otsubo, et. al., (1998) developed long circulatory immunoliposomal amphotericin B by using monoclonal antibodies (against surface glycoprotein of pulmonary capillary vessel walls) and evaluated its therapeutic efficacy against invasive pulmonary aspergillosis in mice. They observed that the survival rates for mice treated with an intravenous dose of 2 mg/kg. wt AmBisome, polyethylene glycol liposomal amphotericin B and monoclonal antibody tagged polyethylene glycol liposomal amphotericin B were 16.7, 83.3 and 100% respectively. Furthermore treatment with monoclonal antibody tagged polyethylene glycol liposomal amphotericin B produced a marked reduction in fungal load in lungs. They observed, enhanced localization of monoclonal antibody tagged polyethylene glycol liposomal amphotericin B in lungs and concluded that this is a promising formulation of amphotericin B against invasive pulmonary aspergillosis.

3. AMPHOTERICIN B LIPID COMPLEX:

Amphotericin B lipid complex (ABLC) was developed by Janoff, et. al., (1988) and it comprises of dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol in a 7.3 molar ratio with 33-mol % amphotericin B. It is derived from the original Juliano/Lopez-Berestein formulation, which also contained dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol, but a lower concentration of amphotericin B (Lopez-Berestein, et. al., 1983, 1984, 1985).
1987) ABLC is a fraction of the original MLV-liposomes, which shows a higher therapeutic index than the original formulation (Janoff, 1988a). These workers performed a series of experiments combining the lipid formulation of dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol with varying concentrations of amphotericin B. The studies suggested that the hemolytic activity of this lipid formulation at low concentrations (more than 3 mole % of amphotericin B to lipid) was similar to that of conventional amphotericin B, but as the mole ratio of amphotericin B increased and the ribbon structures began to form, there was a marked reduction of overall toxicity. As amphotericin B is increasingly complexed with the lipid, the amount of free amphotericin B in the solution is reduced, thereby decreasing the toxicity.

Freeze tech electron microscopy of the formulations made with 0, 5, 25 and 50-mole % of amphotericin B showed significant differences in the structures formed. The formulation made up of 5-mol % amphotericin B was very similar to the liposome prepared by Lopez-Berestein, et. al., (1983, 1987). As the concentration of amphotericin B was increased, the liposomal vesicles disappeared and were replaced by tightly packed ribbon structures. The ribbon like structures formed by complexing dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol with Amphotericin B were called amphotericin B
Figure 12. Putative structure of Abelect. Amphotericin B and lipid are arranged in a 1:1 interdigitated complex. One possible arrangement would require drug – lipid pairs to be arranged in cylinders. The hydrophobic polyene region of the drug would be aligned with the lipid hydrocarbon chains, and the polar hydroxyl groups would face towards the center of the cylinders pore. The cylinders would aligned side by side, thus creating a ribbon-like appearances.

(Adapted from Janoff, 1993)
lipid complex. It is apparent that the non-liposomal structures in the formulation that were like 'ribbon' were responsible for the reduction in toxicity (Figure 12).

ABLC has been compared with conventional amphotericin B in a number of animal models including mice, rats, rabbits and dogs. Clark, et al., 1991 reported that LD_{50} of amphotericin B increased from around 3.0 mg/kg. wt. to over 40 mg/kg. wt when delivered as ABLC. However, Perfect and Wright (1994) reported only 4-5-fold increase in tolerated dose of amphotericin B when delivered as ABLC in rabbits. Concentrations of amphotericin B in the liver, spleen and lungs of mice and rats appear to be much higher after administration as compared to AmB_{dec}. Higher uptake of ABLC in lungs is expected due to its large size (1.6 – 6 μM) due to which there would be physical entrapment in pulmonary capillaries (Taylor, et. al., 1982). Plasma levels of amphotericin B were consistently lower after injection of ABLC than were the levels obtained after injection of AmB_{dec}. When increasing the dose of lipid complex, the levels of drug in the liver, spleen, and lung tissue rise dramatically. However, this rise is associated with little change in the level of drug in the kidney and essentially no rise in plasma levels (Clark, et. al., 1991; Olsen, et. al., 1991; Janoff, et. al., 1993).

The efficacy of ABLC has been evaluated in a number of animal models of fungal infection. Clark, et al. (1991) observed 100% survival in aspergillosis
infected mice treated with high doses of ABLC (12 mg/kg wt) whereas treatment with AmB<sub>doc</sub> resulted in 100% mortality. Perfect and Wright (1994) used ABLC for treatment of cryptococcosis in rabbits. Though, fungal load was least with 10 mg/kg wt ABLC treatment, at equivalent doses AmB<sub>doc</sub> treatment had higher antifungal activity. Similarly Allendoerfer, et. al. (1997) also reported dose dependent efficacy of ABLC in coccidioidomycosis infected mice and observed improved survival with high dose ABLC treatment in comparison to AmB<sub>doc</sub>. Therefore like other groups they also reported enhanced therapeutic efficacy of ABLC at higher doses.

Apart from reduction in toxicity as the major reason, the efficacy of ABLC has also been attributed to the ability of lipase's either fungi or inflammatory cells at the site of infection to release the complexed amphotericin B from the lipid formulation. However, the improvement in the therapeutic index may also be related to binding to high-density lipoproteins as well as to selective interaction with fungal cell membranes (Wasan and Lopez-Berestein, 1994).

4. AMPHOTERICIN-B COLLOIDAL DISPERSION:

Amphotericin-B colloidal dispersion (ABCD, Amphocil, Seques Pharmaceuticals, Melno Park. CA) is a stable complex of amphotericin B and Cholesteryl sulfate in a 1:1 molar ratio (Figure 13). The particles form a disk like
Figure 13. Structure features of Amphocil. Interaction of amphotericin B with cholesteryl sulfate forms rigid and tightly packed lipid layers that are unable to bend sufficiently to form closed vesicular structures. Because of the presence of a polar hydroxyl region, amphotericin B probably forms a shield at the disc edges and is oriented so that the seven hydroxyl groups along its side are in contact with the polar aqueous environment.

(Adapted from Hiemenz and Walsh, 1996)
structure with a diameter of 122 nm and a thickness of only 4 nm leaving no free
drugs (Guo, et. al., 1991). ABCD has also been compared with AmB_{DOC} in a
number of animal models. Clemons and Stevens (1991) and many other groups
have reported that there was at least a 4-5 fold increase in the MTD of amphotericin
B when delivered as cholesterol-3-sulfate colloidal dispersion in comparison to
deoxycholate suspension (Patterson, et. al., 1989; Feilding, et. al., 1992; Hostler,
et. al., 1992).

Fielding, et. al., (1991, 1992) have also extensively studied the
pharmacokinetics of ABCD and compared it with AmB_{DOC} in rats and dogs. The
observed that ABCD was cleared faster from blood and peak serum levels were
significantly lower in comparison to ABCD. However, the half-life of ABCD is
much longer and it is stable retained in most tissues. Concentration of
amphotericin B in liver tissue is 2-3 times higher after injection of ABCD than after
injection AmB_{DOC}. Reduced amphotericin B distribution to kidneys and lower
levels in plasma were cited as the major reasons for reduction in toxicity and
enhancement of therapeutic efficacy.

Therapeutic efficacy of ABCD has been extensively evaluated in the
treatment of various experiment fungal infections such as coccidiodomycosis,
cryptococcosis and aspergillosis (Patterson, et. al., 1989; Hostler, et. al., 1992).
Clemmons and Stevens (1991) used ABCD for treatment of coccidiodomycosis
infected CD1 mice. They observed that AmB_{doc} has higher antifungal activity at similar doses in comparison to ABCD. Important studies of toxicity and therapeutic efficacy of ABCD have been carried out by Allende, et. al., (1992) while employing ABCD for treatment of aspergillosis in rabbits they observed that maximum survival was achieved with 5 mg/kg ABCD treatment even though 10 mg/kg. ABCD treatment rabbits had lower fungal load. They attributed this to high toxicity of ABCD at 10 mg/kg. Dose and demonstrated it by rise in serum creatinine levels. Similarly other workers have also demonstrated the enhanced therapeutic index of ABCD at higher dose in treatment of aspergillosis and cryptococcosis (Patterson, et. al., 1989; Clemons and Stevens, 1991; Hostler, et. al., 1992; Allende, et. al., 1993).

5. AmBisome:

AmBisome (Nexstar, San Dimas, CA) is a true liposomal formulation of amphotericin B with average diameter of 60.7 nm (Figure 14). It comprises of soya phosphatidylcholine and distearoyl phosphatidylglycerol stabilized by cholesterol in combination with amphotericin B in a 2.0:8.1:0.4 molar ratio (Alder-Moore and Proffitt, 1991). Many studies have been carried out which demonstrate the reduction in toxicity of amphotericin B when delivered as AmBisome (Alder-Moore, et. al., 1991). Some reports have suggested on the
Figure 14. Schematic structure of amphotericin B small unilamellar vesicle (AmBisome).

(Adapted from Hiemenz and Walsh, 1996)
basis of LD_{50} values that this liposome form of amphotericin B may be at least 15 times less toxic than conventional amphotericin B (Proffitt, et. al., 1991). Lee, et. al., (1994) reported that AmBisome could be safely delivered till 10-mg/kg wt in rabbits when 1.5 mg/kg wt AmB_{doc} was causing acute toxicity. The pharmacokinetics of AmBisome has been studied extensively (Proffitt, et. al., 1991; Lee, et. al., 1994). Due to its small size, this formulation has a long circulatory life and rate of uptake by the RES appears to be much slower than that by ABLC or ABCD. It is well known that layer lipid complexes and dispersion are readily phagocytozed by the macrophages of the RES than small unilamellar vesicles. However, it is still preferentially concentrated in liver and spleen, though a slower rate. Consequently, peak levels were much higher following AmBisome administration than those after AmB_{doc}.

Like other lipid formulations, the therapeutic efficacy of AmBisome has also been extensively evaluated in various infections and experiment animals. Gondal, et. al., (1989) employed AmBisome for treatment of disseminated candidiasis in mice and found a significant improvement in survival as compared to AmB_{doc}. Pahls and Schaffner (1994) observed dose dependent antifungal activity and therapeutic efficacy of AmBisome in treatment of candidiasis in mice. They reported that equivalent doses of AmBisome had 4-8 folds less antifungal activity as compared to AmB_{doc}. At higher dose of AmBisome (5 mg/kg wt),
100% survival was observed whereas AmBdoe could achieve only 30% survival. Alder Moore, et. al., (1991) also used AmBisome for treatment of murine candidiasis and cryptococcosis and reported improved therapeutic index.

Although the above review describes the success obtained with the use of these formulations of amphotericin B, many complications in animal and human trials continues to be reported. Moreover, contrasting results about the therapeutic efficacy of these formulations have been reported from different laboratories. Therefore, a need has remained for a further improved antifungal drug, which does not suffer from the shortcomings associated with these formulations.

The present study was in that direction, where attempts were made to prepare a formulation that has low toxicity and high therapeutic efficacy like other commercial formulation.

**IN-SITU PREPARATION OF LIPOSOMES:**

Approaches quite different from these concepts are either to form new liposome by hydration of lipid in-situ or to load existing empty liposomes just before administration.
Payne and coworkers (1986) developed the so-called proliposome concept for the in-situ preparation of liposome. The lipids were cast, finely divided, from an organic solvent on a fine powder such as Sodium Chloride and Sorbitol. A certain degree of control over the liposome particle size upon hydration of the lipid films can be obtained by the carrier, the nature of the lipid and the lipid film thickness.

A second in-situ method for loading liposomes is based on the establishment and maintenance of a pH gradient between the internal (within the liposome) and external water phase (Nichols, et. al., 1990 & Mayer, et. al., 1990).

Recently, a new method for the preparation of multilamellar liposomes was developed (Perrette, et. al., 1991), which avoids the usage of pharmaceutically non-accepted solvents. The method involves preparation of an initial proliposome mixture containing lipid, ethanol and water, which is converted in-situ into a liposome dispersion by a simple dilution step. This method was adopted in the present research work for the preparation of liposomes derived from proliposome technique with minor modifications.

INCORPORATION OF β - CYCLODEXTRINS:

The recent patent application (McCormack & Gregoradis, 1995) describes a liposomal delivery system, which involves cyclodextrin (CD) inclusion
complexes. Generally, hydrophobic substances tend to leak out of liposomes which can easily be prevented by complexing them with CDs.

Cyclodextrin, a family of Cyclic oligosaccharides composed of α - 1, 4 - linked 6,7 or D-glucose units (Figure 15) are known to accommodate water insoluble molecules ('guest') in the hydrophobic milieu of the cavity present in the hydrophobic milieu of the cavity present in there structure to yield water soluble inclusion complexes (Duchene, et. al., 1993 & Uckarma, et. al., 1987). This property of cyclodextrin has been used to improve drug formulations, for instance to increase drug solubility and stability (Szejtli, et. al., 1983; Gal-Fuzy, et.al.,1985 and Standler-Szoke, et. al., 1985), and also pharmacological action (Bootsma, et.al. 1989, Hasan, et. al. 1990 and Puglisi, et. al. 1991). It has been proposed recently (McCormack, et. al., 1996) that drug leakage problem out of liposomes could be circumvented by entrapping inclusion complexes into the aqueous phase of liposomes. These are expected (Gregoriadis, 1995) to protect complexes from drug displacement, prevent their premature excretion and, when appropriately tailored, direct complexes to target tissues.

In recent years, a number of chemically modified cyclodextrin have been developed to overcome the solubility and degradation problems associated with natural cyclodextrin. Masson, M. and Co-workers (1998) have reported an
<table>
<thead>
<tr>
<th>TYPE</th>
<th>R group</th>
<th>Degree of substitution</th>
</tr>
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<tbody>
<tr>
<td>CM-CD</td>
<td>-CH₂COO⁻</td>
<td>0.5</td>
</tr>
<tr>
<td>SB-CD</td>
<td>-(CH₃)₄SO₃⁻</td>
<td>0.9</td>
</tr>
<tr>
<td>HP-CD</td>
<td>-CH₃CH(OH)CH₃</td>
<td>0.6</td>
</tr>
<tr>
<td>A-CD</td>
<td>-COCH₃</td>
<td>1.0</td>
</tr>
<tr>
<td>M-CD</td>
<td>-CH₃</td>
<td>0.6</td>
</tr>
<tr>
<td>TMA-CD</td>
<td>-(CH₃)₂N⁺(CH₃)₃</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Figure 15. Structure of β – cyclodextrin derivatives. Their abbreviated name, functional group and degree of substitution.

(Adapted from Masson, 1998)
investigation into the effects of this ionic cyclodextrin on the chemical stability of various ionic drug compounds.

A number of safety evaluations demonstrate that three natural CDs (α-, β-, γ-CDs) and some chemically modified CDs (HP-β-, DM-β-, SBE-β-, S-β-, G2-β-CDs) may be useful in oral formulations Among them, γ-, HP-β-, SBE-β-, and G2-β-CDs seem to be suitable for use in Parenteral as well as oral formulations. It is probable that in the future, high quality and safe CDs will provide advanced drug formulations that are superior to current formulation, with CDs being substituted for surfactants and organic co-solvents in the parenteral formulation. The advantages of the potential uses of CDs, the availability of CDs and economic reasons play a decisive role in the growing interest in the CDs shown by pharmaceutical industry. This interest is reflected in the increasing numbers of pharmaceutical products being placed on the market as CD-based formulations. The legal status of CDs has been recently reviewed (Loftsson & Brewster, 1996).

Most recent studies on the use of cyclodextrin to allow I.V. administration have utilized Hydroxy propyl β - CDs (SBE-β-CDs), since safety concern with other cyclodextrin preclude their parenteral use (Rajewski, et. al., 1996 & Irie, et. al., 1997).

FORMATION OF INCLUSION COMPLEX:
The rationale design of formulations which take advantage of cyclodextrin inclusion complexation requires an understanding of the relationship between intrinsic drug solubility, the magnitude of the binding constant for the inclusion complex, and dilution effects. Most pharmaceutical agents form 1:1 complexes with cyclodextrin as described by scheme 1 (Figure 16). On the basis of structure and properties of drug as well as the cyclodextrin, higher order complexes are also possible by scheme 2 of figure 16 (Rajewski and Stella, 1996).

Method of preparing drug–cyclodextrin complexes have been reviewed (Hirayama, et al., 1987). In the solution phase, the procedure is generally as follows: an excess amount of drug is added to an aqueous cyclodextrin solution, and the suspension is agitated for up to 1 week at the desired temperature. The suspension is then filtered or centrifuged to form a clear drug–cyclodextrin complex solution. For preparation of solid formulations of the drug–cyclodextrin complex, the water is removed from the aqueous drug-cyclodextrin complex solution by evaporation, or sublimation. It is sometimes possible to shorten this process by formation of supersaturated solution through sonication followed by precipitation at the desired temperature. In some cases, the efficiency of complexation is not very high, and therefore, relatively large amounts of cyclodextrin must be used to complex small amount of drug. To add to this difficulty, vehicle additives, osmolality modifiers, and pH adjustments commonly
Figure 16. Schematic illustration of mechanism of drug – cyclodextrin inclusion complexation.

(Adapted from Rajewski and Stella, 1996)
used in drug formulations such as sodium chloride, buffer salts, surfactants, preservatives and organic solvents, very often reduce the efficiency. For example, in aqueous solutions ethanol and propylene glycol at low concentrations have been shown to reduce the cyclodextrin complexation of testosterone and ibuprofen by acting as competing guest molecules while at higher concentrations they can reduce complexation through a manipulation of solvent dielectric constant. Likewise, non-ionic surfactants have been shown to reduce cyclodextrin complexation of diazepam and preservatives to reduce the cyclodextrin complexation of various steroids. On the other hand, additives such as ethanol can promote complex formation in solid or semisolid state. Un-ionsed drugs usually form a more stable cyclodextrin complex than their ionic counterparts; thus the complexation efficiency of basic drugs can be enhanced by addition of ammonia to the aqueous complexation media (Loftsson and Brewster, 1996).

Inclusion complexation of various drugs with \( \beta \)-Cyclodextrin have been reported (Tasic, et al., 1996) i.e. kneading, co-precipitation, freeze drying or spray drying techniques are employed for the same. Although freeze-drying and spray drying methods superior show dissolution properties (Adhage, et al., 1998), they are not economical. Our endeavor was to prepare the complexes by a grinding or roll compacting method, which could be commercially exploited.
PHARMACOKINETICS OF LIPOSOme ENTRAPPED INCLUSION COMPLEX
(AMPHOTERICIN/ β - CDs) AND LIPID INTERACTIONS:

Intravenous administered CDs disappear rapidly from the systemic circulation and are excreted mainly through the kidney. Although α- and β- CDs are excreted almost completely in an intact form into the urine, γ - CD is degraded to a considerable extent in rats (Uekama, et.al., 1991) The steady-state volume of distribution (Vdss) for β-CD and 2-hydroxy propyl-β – CD (HPβCD) in rats, (Monbaliu, et.al., 1990) rabbits(Yamamoto, et.al.,1990) dogs (Monbaliu,et.al.,1990) and humans (Mesens,et.al.,1991) corresponds well with the extracellular fluid volume of each species, suggesting that no deep compartments or storage in pools are involved . The total plasma clearance (CLt) for HP-β-CD in all species tested (Monbaliu,et.al.,1990)are similar to that of inulin,a polysaccharide known to be rapidly distributed in extracellular fluid and then excreted at a rate of glomerular filtration . Furthermore, the renal clearance of HP-β- CD in humans (110 – 130 ml /min) is independent of dose administered and nearly equivalent to the reported glomerular filtration rate (125mL / min). The elimination of CDs is strongly dependent on renal function, so nonlinear pharmacokinetics for β- CD at higher doses (> 200mg / kg) in rats (Irie and Uekama,1997) can be explained by the untoward effects of β –CD on the kidney.
A recent study has shown that the sulfoalkyl ether derivatives of $\beta$-CD, when administered intravenously to mice, are eliminated in an intact form into the urine in a manner similar to that of HP-$\beta$-CD and inulin (Rajewski, et al., 1996).

Moreau and Co-workers (1992) have shown that when amphotericin B (AmB) was mixed with intralipid in neutropenic patients, renal toxicity was reduced without altering the total sodium intake, however, Joly, et. al., (1993) have demonstrated that deoxycholate AmB associated with intralipid in the treatment of Cryptococcal meningitis patients with AIDS, showed no differences in nephrotoxicity between AmB and AmB infused with intralipid. Kirsh and Coworkers (1988) further reported that the formulation of an emulsion of AmB and 20% intralipid reduced acute AmB toxicity in the treatment of systemic mucormycosis candidiasis, without altering its antifungal activity. Since AmB associates predominantly with HDL in serum following 1h of incubation and the continuous infusion of intralipid increases total serum cholesterol and HDL cholesterol, it is possible that AmB's interactions with HDL increase in the presence of a continuous intralipid infusion. Considering varying volume of distribution with respect to the varying lipid concentration in blood, the present research studies examined the influence of a concurrent lipid administration on the clearance from
the blood stream and distribution of AmB and liposomal AmB entrapped inclusion complex in tissue in rats after a single I.V. dose.