CHAPTER I

Introduction
And
Review of literature
1. INTRODUCTION

1.1. Drug delivery systems

1.1.1. Need for Drug Delivery Systems

Human beings have utilized drugs to improve their health since early civilizations; beginning with the use of primary herbs, through the emergence of synthetic chemistry in the 20th century, and into the modern era of nanotechnology and combinatorial chemistry. Multiple natural and synthetic chemicals have been utilized as effective therapeutical drugs throughout history [Drews 2000]. Because of low water solubility, poor membrane penetration and short in vivo half-life, a significant number of drugs molecules have been identified as poor therapeutic candidates [Weiss et al. 1993; Lipinski et al. 2001; Langer 1983]. With the potential to impact patients, a significant effort is currently made to overcome these problems.

1.1.2. Criteria for Drug Delivery Systems

Drug delivery systems are formulations or devices that improve the therapeutic efficiency of pharmaceuticals. The delivery system aims to control drug bio-distribution profiles as well as drug concentration, release and targeting [Pillai et al. 2001]. The design for desirable drug delivery systems is summarized below:

First, the drug delivery system should improve the drug's physiochemical properties, such as solubility and stability in vivo.
encapsulation of poorly water-soluble drugs with a reservoir, in which the
delivery system solubilizes the drugs and concurrently protects from
macrophages and proteins, is the most common delivery approach [Kwon et
al. 1994; Langer 1990].

Second, a drug delivery system should transport the drug to the
desired sites. Drug delivery systems may transport the therapeutic agents
through the circulatory system or directly to a specific target such as an
organ, tissue or cell. The delivery systems should permeate physiological
barriers such as the endothelial [Deen et al. 1983] and reticulo-endothelial
systems [Arshady 1996]. Thus, membrane permeability, delivery size and
surface properties must be considered. Targeting strategies can be further
utilized to direct the encapsulated drugs to a specific biological target.

Third, a drug delivery system should extend the drug’s bioavailability
[Kaparissides et al. 2006]. For many treatments, the drug level in the
circulatory system must be maintained within an effective concentration
range. Often, delivery systems should provide sustained drug release within
the optimum therapeutic concentration range for an extended period of time.

Last, a drug delivery system should be biocompatible [Charman et al.
1999]. The delivery system itself should not provoke undesired
physiological effects such as toxicity.
1.2. Drug Carriers

A delivery system mainly comprise of a drug carrier that acts as a vehicle in the delivery of the drug. Several carrier systems and targeting agents have been considered as means to deliver drugs to specific cells or tissues. Among the carriers one can name: Colloidal drug carriers (such as liposomes, emulsions, nanoparticles and Polymeric micelles) and protein microspheres that are extensively being studied and used.

1.2.1. Liposomes

Lipid molecules with polar heads and non-polar tails can self-assemble into liposomes, which are structurally composed of a bilayer structure and an aqueous core. Classical liposomes were composed of phospholipids and cholesterol, which were first studied by Bangham et al. as early as 1965 [Bangham et al. 1965]. Early studies of liposomes as drug delivery vehicles were hindered by their rapid clearance from the bloodstream, due to adsorption of plasma proteins (opsonins) to the phospholipid membrane, thereby triggering recognition and uptake of the liposomes by the mononuclear phagocytic system (MPS), also referred to as the reticulo-endothelial system [Lasic et al. 1995; Gregoriadis 1995; Lasic 1997; Woodle et al. 1995]. Research on liposomes as drug delivery systems was rejuvenated by the inclusion of polyethylene glycol (PEG) conjugated lipid molecules in the lipid bilayer to help evade MPS recognition [Woodle et al. 1995].
1995; Lasic 1997; Woodle et al. 1992; Woodle et al. 1995; Allen et al. 1989]. PEG molecules in the lipid bilayer create a steric barrier, which inhibits the interactions of liposomes with plasma proteins (such as opsonins and lipoproteins) and cell receptors [Sapra et al. 2003]. The inclusion of pegylated lipid extends the half-life of liposomes from less than a few minutes (classical liposomes) to several hours (sterically stabilized liposomes, also called Stealth liposomes) [Sapra et al. 2003]. In contrast to classical liposomes, which have dose-dependent and saturable pharmacokinetics at higher lipid concentrations, pegylated liposomes (Stealth liposomes) show dose-independent kinetics of blood clearance [Lasic 1995; Woodle et al. 1995]. Ligands and antibodies or antibody fragments can also be readily attached to the PEG corona of Stealth liposomes, which result in a targeted drug delivery system. To date, targeted Stealth liposomes are used most exclusively for applications involving active targeting [Allen et al. 2004].

1.2.2. Emulsions

Emulsion is a heterogeneous mixture of two immiscible liquids with emulsifier that stabilizes the dispersed droplets. They can be utilized as carriers for hydrophilic or hydrophobic drugs for different therapeutic applications. For instance, oil-in-water emulsions have served as carriers for lipophilic drugs and many of them are available commercially [Rossi et al. 2007]. These emulsions can alter the biodistribution of the incorporated
drugs and enhance their accumulation in target tissues. For example, Constantinides et al. [2000] have formulated a submicron emulsion of paclitaxel using vitamin E (tocopherol) as the internal phase (Tocosol®-paclitaxel). When the emulsion was injected into melanoma-bearing mice, it was found to be less toxic and had greater antitumor activity than Taxol®. However, Tocosol®-paclitaxel, unexpectedly, failed in a phase III study conducted in breast cancer patients. The response rate was 37 % for Tocosol®-paclitaxel versus 45 % for Taxol®, and was coupled with more side effects than seen in vitro [Bulitta et al. 2009]. Although there is no clear explanation for this failure, the weak interactions between tocopherol and paclitaxel might explain the lower efficacy of the formulation.

1.2.3. Nanoparticles

Nanoparticles are colloidal particles with a rigid core. They are either made from i) a polymeric or lipidic matrix in which a drug is dissolved or dispersed or ii) from drug nanocrystals stabilized by a polymer [Hawkins et al. 2008; Gaucher et al. 2007; Gaucher et al. 2009]. They are usually larger than micelles (100-200 nm) and are generally more poly-disperse. Amphiphilic copolymers have been exploited as emulsifiers for the preparation of nanoparticles. They form stable films, where the hydrophobic block is oriented toward the core while the hydrophilic block creates a hydrated corona and provides steric stabilization for the nanoparticles. Many copolymers have been employed in the preparation of nanoparticles for the
delivery of anticancer drugs. Examples of these polymers include PEG-b-PDLLA and PEG-b-poly(lactide-co-glycolide) [Gaucher et al. 2007; Gaucher et al. 2009; Esmaeili et al. 2008]. Alternatively, albumin nanoparticles (nab-paclitaxel; Abraxane®) have been used for paclitaxel delivery [Hawkins et al. 2008]. These protein nanoparticles consist of albumin-bound paclitaxel with a mean particle size of 130 nm. They facilitate drug transport into tumors through albumin receptors and caveolae-mediated transport across endothelial cells, which increase the intra-tumoral accumulation of paclitaxel [Hawkins et al. 2008]. Preliminary evidence suggests that this process may be facilitated through binding of albumin to SPARC (secreted protein acid and rich in cysteine), an extracellular matrix glycoprotein that is overexpressed and associated with poor prognosis in a variety of cancers. In a recent in vivo study, intratumoral paclitaxel accumulation was found to be 33 % higher for nab-paclitaxel compared to Cremophor® EL-paclitaxel when each formulation was administered using equal doses of paclitaxel [Desai et al. 2006]. These nanoparticles have been recently approved for use in patients with metastatic breast cancer.

1.2.4. Polymeric micelles

Polymeric micelles (PM) are formed via, the self-assembly of amphiphilic copolymer chains in aqueous milieu. They present a core/shell architecture wherein the hydrophobic core serves as a microenvironment for
the solubilization of poorly water-soluble drugs while the hydrophilic corona acts as a stabilizing interface between the core and the external medium. In water, hydrophobic interactions are generally the main driving force behind the micellization process. PM usually have fairly narrow size distributions with diameters ranging from 10 to 100 nm. Incorporation of the drug inside the micelles often decreases the toxicity of the entrapped drug, allowing for higher doses to be administered and greater efficacy. For example, Dufresne et al. [2004] demonstrated that paclitaxel incorporated in poly(N-vinylpyrrolidone)-b poly(D,L-lactide) (PVP-b-PDLLA) did not reach the maximum tolerated dose (MTD) even at 100 mg/kg and showed greater anti-tumor activity than Cremophor® EL micelles, whose MTD was established at 20 mg/kg. Because of the higher MTD, paclitaxel could be injected at higher doses (60 mg/kg) where it induced three- and two fold increase in the plasma and tumor area under the concentration-time curves respectively, versus Cremophor® EL (20 mg/kg). Indeed, paclitaxel formulated in PEG b-PDLLA micelles are now in phase II clinical trials, either alone or in combination with cisplatin [Lee et al. 2008; Kim et al. 2007].

1.2.5. Protein Microspheres

Microspheres may be defined as homogeneous particles or monolithic microcapsules ranging in size between 0.1 μm to 1000 μm. Some authors have further sub-classified microspheres with diameters less than 1 μm as
"nanospheres". Consideration of albumin microspheres as carriers for targeting tissues and organs was introduced in the early 1960's. Radio-labelled albumin microspheres were used to study the phagocytic capacity of the reticulo-endothelial system (RES) [Wagner et al. 1963] and the peripheral circulation in humans [Wagner et al. 1965]. Albumin microspheres were suggested for use in drug delivery to the RES because of their chemical and physical stability and their rapid clearance by phagocytic cells [Kramer 1974]. The synthesis and use of albumin microspheres to improve the efficacy of therapeutic drugs has been the topic of many reviews [Kreuter 1983; Gupta et al. 1989; Arshady 1990].

1.3. Current modalities for developing an ideal drug carrier

Various attempts to solve the problem of solubilization of insoluble drugs are associated with loading poorly soluble drugs (usually, hydrophilic molecules) into various nanosized pharmaceutical carrier, such as liposomes (drugs are loaded into the hydrophobic membrane of the liposome), micelles (drugs are loaded into the hydrophobic core of the micelle, and oil-in-water emulsions (Jones et al. 1999; Torchilin 2007). Although, many experimental data are available showing a dramatic increase in the drug efficacy after loading into nanocarriers, the general problems associated with these approached include: relatively low loading capacity of the drug into the nanocarriers (between 0.5 and 25 % by weight, usually below 10 % wt); the impossibility of using the same protocol for making solubilized forms of
different drugs, since each drug required its own specific conditions for solubilization; difficulties with the scaling up the technology; difficulties with controlling surface properties or surface composition of such nanosystems; insufficient storage stability of such systems and their instability in the body (Torchilin 2007).

Currently, the most popular approach to solubilize poorly soluble drugs and prepare their dosage forms with sufficiently high bioavailability is the use of micellar drug carriers made of various materials (Lasic 1992; Masuda et al. 1986; Muranishi 1990]. Such encapsulations not only increase the bioavailability of the drug, but also protect them from destructive factors upon parenteral administration and beneficially modify their pharmacokinetics and biodistribution [Maeda et al. 2001; Maeda et al. 2000].

However, there exists a number of problems associated with micellar carriers, which include low loading efficacy of the drug into the micelles (usually well below 5% wt); the difficulty of using a single protocol for solubilizing different drugs. Also there are problems associated with controlling the rate of release of the drug from micelles. These problems are further compounded by instability of the micelles and it is not easy to scale-up the technology.
1.4. Amphotericin B

Amphotericin B is a product of *Streptomyces nodosus*, an actinobacteria isolated from a Venezuelans oil sample, and its use as an antifungal was first described in 1956 [Gold et al. 1956]. AmB's macrocyclic structure as shown in Figure 1.1 was determined in 1970 [Mechlinski et al. 1970]. The structure has rigid hydrophobic all-trans heptaene fused to a hydrophilic polyol chain. AmB is thus an amphiphilic molecule; its amphiphilic behavior dominates our understanding of its interaction with cell membranes. Additionally, AmB is characterized by a polar head group of a carboxylic acid and an amine on the sugar group. Together, these groups make AmB zwitterionic in neutral aqueous solution and give the molecule the amphoteric property of its name. The pKa of the carboxylic acid proton is 5.6 while that of the amine is 10; AmB's isoelectric point is then 7.8. The molecular weight of AmB is 924.1 and its length (21 Å) is approximately equal to the length of a phospholipid, and thus similar to half a lipid-bilayer thickness [Holz 1974]. AmB is poorly soluble in most solvents due to its amphiphilic nature. Pure AmB in aqueous solution has a critical micelle concentration (CMC) of 6 X 10^{-7} M [Tancrede et al. 1990] but is more soluble (mM range) in the polar aprotic solvents dimethylsulfoxide (DMSO) and dimethylformamide (DMF). Below the CMC, AmB exists as monomers, while above the CMC AmB forms aggregates. The mean number of AmB monomers per aggregate is unknown.
and is thought to correspond to a wide size distribution [Gaboriau et al. 1997]. AmB absorbs blue light strongly, with an extinction coefficient $>10^5$ M$^{-1}$ cm$^{-1}$ at 410 nm. The absorption spectrum of AmB depends on the drug's aggregation state; peak absorption for the monomer is 410 nm, aggregated solutions show a blue-shift to $\sim$340 nm. AmB was recently determined to be fluorescent, with the monomer fluorescing between 500 nm and 650 nm, and aggregates identified as dimers fluorescing between 400 nm and 550 nm [Gruszecki et al. 2003].

Figure 1.1. Chemical Structure of AmB. AmB is characterized by an all-trans heptaene on one side of the macrolide ring and numerous hydroxyl groups on the other side.

1.4.1. Use of Amphotericin B

AmB's clinical use is for the treatment of systemic fungal infections, most commonly of *Candida*, *Cryptococcus* or *Aspergillus* species. AmB has additionally been used clinically in the treatment of many other fungal infections. Such fungi may form part of the natural flora found in the...
body (e.g. in the gut or respiratory tract) that are normally kept in check by the body's immune system. As such, patients suffering systemic fungal infections are typically already immuno-compromised. Large increases in the number of recorded fungal infections accompanied the rapid spread of AIDS in the 1980's and 1990's, although organ-transplant anti-rejection drugs, cancer chemotherapy treatment, and malnutrition are also important causes of immunodeficiency.

Intravenous administration of AmB is the backbone of treatment for severe and otherwise fatal fungal infections because of AmB's broad spectrum, potency, and rapidity of action. Additionally, the conventional formulation of AmB is relatively affordable compared to other anti-fungal drugs. Illustrating the magnitude of AmB's importance is that the American national demand for conventionally-formulated AmB is approximately one million 50 mg vials per year [Petraitis et al. 2005]. AmB's use as an antifungal is particularly important as it is fungicidal whereas many other treatments only limit fungal growth (fungistatic effect). Despite its use as a front-line antifungal for more than 50 years, resistant strains are rare [Bryskier 2005]. AmB is also the treatment of choice for the parasite-caused disease Leishmaniasis. Approximately 12 million people globally are thought to be currently infected with Leishmaniasis, and it kills 500,000 annually.

Approved indications for the use of AmB therapy vary according to the formulation. The conventional form, Fungizone ® (FZ - Bristol Myers
Squibb, New York, USA), has been approved by the American FDA for treatment of aspergillosis (due to *Aspergillus fumigatus*), torulosis (*Cryptococcus neoformans*), systemic candidiasis (*Candida spp.*), North American blastomycosis, coccidioidomycosis, certain susceptible forms of Zygomycetes, sporotrichosis (*Sporothrix schenckii*), and infections due to susceptible forms of Conidiobolus and Basidiobolus. FZ is also indicated for American mucocutaneous leishmaniasis.

In addition to its approved indications, AmB has been found to act as a potentiator of existing anti-cancer drugs [Tsujino et al. 2006; Kleinberg 2006], as a mediator of prion disease progress (transmissible spongiform encephalopathies such as the bovine form: mad cow disease) [Adjou et al. 1997; Pocchiari et al. 1991], as a possible treatment for Hodgkin's Lymphoma in combination with acyclovir [Richard 2006], to have potent activity against malaria-causing parasite *Plasmodium falciparum* [Hatabu et al. 2005], as a first alternative to benzimidazoles for the treatment of Alveolar echinococcosis [Reuter et al. 2003] and to be a stimulant of the immune system [Yamaguchi et al. 1993]. Additionally, a 2006 editorial in Clinical Infectious Diseases hailed reports of promising antifungal activity on administration of fungal heat shock protein with Amphotericin B as possibly marking the arrival of a 'third age' of antimicrobial therapy [Casadevall 2006]. This approach represents combination therapy where an antifungal is administered along with an immuno-modulator (the fungal heat shock protein in this case) to boost the host immune system; ‘reviews’ of the outlook have been published [Pirofski et al. 2006].
1.4.2. Amphotericin B Mechanism of Action

Immediately following intravenous administration of FZ, some 95% of AmB is released from the deoxycholate and is taken up by lipoproteins (both LDL and HDL) within the bloodstream, particularly those with high cholesterol content [Brajtburg et al. 1984]. The remaining 5% is carried unbound in the blood. The bloodstream disperses the AmB throughout the body. AmB is cleared from the serum quickly, depositing in the cholesterol-rich cell membranes in the spleen, liver, kidney and lung. Cholesterol-bound AmB in HDL is taken up via reverse cholesterol transport mechanisms while cholesterol-bound AmB in LDL is taken up via the cell’s LDL receptors [Wasan et al. 1994; Barwicz et al. 1997]. The LDL-bound portion is associated with acute nephrotoxicity; HDL-bound component is associated with decreased toxicity [Wasan et al. 1994]. Complexation of AmB with lipid promotes its association with HDL, hence it is a part of the reason why the lipidic AmB formulations show reduced toxicity. Clearance of AmB occurs via macrophages in the liver and through radical-initiated auto-oxidation [Lamy-Freund et al. 1985].

Despite being used clinically for many years and the subject of many studies, AmB’s mechanism of action is still poorly understood. Part of what is known has been determined by analogy to the related polyene antibiotics nystatin and filipin. Filipin is a cyclic pentaene and shares the general hydrophilic polyol chain and conjugated polyene structure of AmB, but
lacks the sugar moiety. Nystatin is a closer analogue of AmB, differing in the placement of two hydroxyl groups but with the primary difference being the heptaene of AmB is replaced by a diene-tetraene moiety in nystatin. All three compounds are thought to form membrane pores by binding to sterols making the cellular constituents to leach out.

It was recognized early on that AmB is active against cell types whose membranes include sterols (e.g. eukaryotic cells such as mammalian and fungal) but not active against species with sterol-free cell membranes (e.g. bacteria). Interaction with cell membrane sterols was thus postulated to be an integral part of AmB's mechanism [Lampen 1969]. At the cell membrane level, AmB was found to induce significant changes in water, mono- and divalent cation, and small non-electrolyte molecule permeability across sterol-containing lipid bilayers. Both liposome and cellular (Acholeplasma Laidlawii) models were studied [Bolard 1986; Bard et al. 1980] and the loss of these intracellular species leads to cell death [Bolard 1986; Brajtburg et al. 1984; Lampen 1969]. These results led to the hypothesis that specific AmB interaction with fungal cell membrane sterol (ergosterol) was the basis of its action and that interaction with cholesterol in mammalian cell membranes was the basis of its toxicity. This premise has become known as the “Sterol hypothesis”. Three broad fields of evidence exist for this sterol hypothesis: 1) Fungal and mammalian cell membranes contain sterol and are known to be sensitive to AmB. 2) Free sterols were
found to antagonize AmB-induced $K^+$ leakage of *Candida* and *Mycoplasma*
cells [Archer et al. 1975]. 3) Physico-chemical measurements show that AmB has favorable interaction with sterols.

Both studies of AmB binding to sterol-containing phospholipid vesicles and solution-based binding experiments confirmed more specific binding to ergosterol than cholesterol [Vertut-Croquin et al. 1983; Bolard 1986]. The binding constants for AmB with cholesterol and ergosterol were found to be $2.1 \times 10^5 \ M^{-1}$ and $1.66 \times 10^6 \ M^{-1}$, respectively [Witzke et al. 1984]. This resemblance in binding constants explains the specificity of AmB to mammalian and fungal membranes. Ergosterol and cholesterol share similar chemical structure as shown in Figure 1.2. Ergosterol has two additional double bonds, one within the ring structure and the other on the side chain, as well as an additional methyl substituent on the side chain. There is conflicting experimental evidence on the mode of AmB-sterol binding. The heptaene of AmB may interact hydrophobically with various portions of the sterol. Computer modeling as well as experimental studies have suggested both the nature of the sterol side chain and of the sterol ring structure to be the dominating governors of binding efficiency [Baginski et al. 1997; Gaboriau et al. 1997; Pirofski et al. 2006].
The sterol specificity of AmB has been suggested to be in part due to differences in cell transmembrane potential [Brutyan et al. 1996]. Additionally, these differences are proposed to be the cause of AmB's non-uniform tissue distribution [Szoka et al. 1993]. There is a potential-dependence to the opening and closing of AmB channel, but the mechanism of this is not certain. The mechanism might be related to an increase in antibiotic intra-membrane or surface concentration. A third possible mechanism is a potential-sensitive lipid conformation which favors insertion of pre-formed AmB aggregates ("pre-pores") into the membrane from a surface-bound position [Brutyan et al. 1996].

Gruda et al. [1988] were the first to recognize the relation between AmB aggregation state and its therapeutic effectiveness. Using absorbance
spectroscopy of AmB binding to solvent-dissolved ergosterol, they noted monomeric AmB showed little binding to ergosterol, but that the presence of small aggregates (possibly dimers) resulted in near immediate binding. Further investigations confirmed aggregation state dependence of AmB activity, with AmB monomer active against ergosterol-containing membranes only and AmB aggregates active against both cholesterol and ergosterol containing membranes [Barwicz et al. 1992; Gruda et al. 1988; Bolard et al. 1991]. Since these studies, considerable effort has been made to develop AmB formulations that deliver the more selective monomer. This is a challenging drug-delivery task; due to AmB’s low CMC, direct administration of a normal 1 mg/kg/day dose would require about 120 liters of AmB solution [Barwicz et al. 1992]. The trick then is to deliver the AmB in a relatively concentrated solution but to allow only monomeric AmB release from the formulation.

1.4.3. Toxicity of Amphotericin B

Although widespread, the use of AmB is limited by its dose-dependent toxicity. Toxicity in the context of this thesis means the adverse effects of the drug which are detrimental to human health. Conventional AmB, Fungizone (FZ), is the de facto standard of AmB toxicity against which other AmB formulations are compared. Therapeutic use of FZ provokes both acute intravenous infusion toxicities which are usually reversible, and chronic kidney toxicity which may be permanent. Its administration is associated with a long list of side effects (Table 1.1), but
the most important toxic effect is FZ's kidney toxicity. Acute renal failure has been reported for between 49% and 65% of patients receiving AmB treatment [Deray 2002]. Accordingly, the patient's renal function and history is taken into account when determining if FZ therapy is suitable. Those patients with renal dysfunction or who otherwise cannot tolerate FZ may be prescribed an alternative AmB formulation or a non-AmB anti-fungal.

Table 1.1. Partial List of Toxic Side Effects of Fungizone ® (FZ)

<table>
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<tr>
<th>Toxic Side Effect</th>
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<tbody>
<tr>
<td>Kidney toxicity including acute failure</td>
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<tr>
<td>Vertigo &amp; Hearing Loss</td>
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<tr>
<td>Cardiac Arrest</td>
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<tr>
<td>Anaemia</td>
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<tr>
<td>Nausea &amp; Vomiting</td>
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<tr>
<td>Acute Liver Failure</td>
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<tr>
<td>Fever, Rigors, Chills</td>
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<tr>
<td>High or Low Blood Pressure</td>
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<tr>
<td>Low Blood K+ or Mg2+ Concentration</td>
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1.4.4. Dose-Dependent Toxicity of FZ

The dose-dependent kidney toxicity of FZ is a cumulative effect resulting from chronic treatment. The renal dysfunction may be characterized by decreased renal blood flow, decreased rate of glomerular filtration (a standard measure of kidney function) and impairment of electrolyte re-absorption. Irreversible renal toxicity is associated with
cumulative doses of > 4 g AmB [Deray 2002]. At lower cumulative doses (0.5-1 g) acidosis of the renal tubules is common, but this effect generally is reversed on cessation of FZ therapy.

More generally, the local concentration of AmB is important. For a typical adult dose of 1 mg/kg/day, maximum AmB concentration in blood plasma is approximately in the range 1-3 μg/ml [Terrell et al. 1987]. Chronic treatment results in AmB build-up to higher concentrations in the kidneys, liver and lungs. At concentrations >5 μg/ml, AmB is non-specifically toxic to cells (cytotoxic). The therapeutic index of a drug is a way to measure its relative effectiveness. The therapeutic index is the efficacy-to-toxicity ratio, often defined as the maximum tolerated dose over the minimum curative dose. Clearly, AmB's therapeutic index is limited by its cytotoxicity, resulting in a low index. The aggregation state of AmB is related to its toxicity, with aggregated AmB found to induce cytotoxicity and damage to DNA (genotoxicity) [Egito et al. 2004; Bolard et al. 1986]. Monomeric AmB showed no genotoxic effect and only slight cytotoxicity related to an oxidative-damage mechanism.

1.4.5. Mechanism of AmB Toxic Side Effects

Studies have indicated that multiple mechanisms may be responsible for the long list of AmB side effects, in addition to those caused by pore formation and increased cellular permeability [Brajtburg et al. 1990; Holz 1974]. Specific cellular and molecular mechanisms producing the toxic
effects have not been elucidated, but certain themes have been implicated. It has been well established that AmB degrades via an auto-oxidation mechanism and this may result in peroxidation of cell membrane lipids [Sokol-Anderson et al. 1986; Lamy-Freund et al. 1993]. The products of lipid oxidation within both low-density lipoproteins (LDL) and high-density lipoproteins (HDL) themselves are highly toxic and may be expected to provoke a physiological response [Sevanian et al. 1995; Hurtado et al. 1996]. Tancrede’s group [1990] has shown that FZ induces structural changes in LDL and increases LDL oxidation. The inhibitory effect of AmB on various cellular enzymatic systems has also been reported earlier [Vertut-Doi et al. 1988; Capuozzo et al. 1990]. More recently, studies have linked them with increased production of cytokines involved in signaling of inflammation [Shadkchan et al. 2004].

1.4.6. Formulations of Amphotericin B

The first approved formulation of AmB was FZ, consisting of a 1:1.8 ratio of Amphotericin B and the bile salt- sodium deoxycholate buffered with sodium phosphates. Addition of the deoxycholate surfactant is required to provide sufficient AmB solubility for intravenous administration and results in a colloidal dispersion of AmB. Rinnert et al. [1977] used light-scattering techniques to find that FZ aggregates held about 2000 molecules of AmB on average. Van Etten et al. [2000] used cryo-transmission electron microscopy to find as reconstituted FZ has an average micelle size of about
4 nm, as well as thread-like structures of aggregated micelles. At the concentration used for intravenous injection, FZ consists principally of aggregated AmB.

Several other commercial formulations of AmB exist; each designed to limit its toxic side effects. Of these, Abelcet® (ABLC - Enzon Pharmaceuticals, Bridgewater, NJ, USA) and Ambisome® (Astellas Pharma, Deerfield, IL, USA) are the most commonly used. ABLC is a lipid-complexed form of AmB, consisting of drug and lipid in 1:1 molar ratio. Two lipids are used, dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) in 7:3 molar ratio. The resultant complex holds AmB in an aggregated state and forms ribbon-like structures 2-5 in size [Janoff et al. 1988]. Despite requiring administration of an AmB dose that is five-fold that of FZ, the formulation is better tolerated and has a decreased nephrotoxic effect. The higher dosages are required to maintain the same efficacy as FZ because a smaller fraction of the AmB is free in the plasma and thus available for anti-fungal activity. Acute toxicity of ABLC is similar to the acute toxicity of FZ. Ambisome is a unilamellar liposomal version of the drug. The liposomes are smaller than 100 nm diameter and consist principally of saturated phospholipids, cholesterol and AmB. Like ABLC, Ambisome is also given in higher AmB dosages than FZ but is better tolerated. Lipid-associated formulations of AmB give a different tissue distribution of AmB compared to FZ. They favor higher
concentrations in the liver, spleen and kidneys, where fungal infections are likely to reside [Lopez-Berestein et al. 1984]. This represents a desirable targeting of the drug.

Both ABLC and Ambisome have been found to retain the broad spectrum of antibiotic action of the FZ formulation. Generally speaking, they are used when FZ is unsuitable. The lipid based formulations of AmB all have improved therapeutic index compared to FZ, but have a serious cost disadvantage. Daily costs for an average adult on lipid-based AmB are $300-$1000 USD whereas with FZ this cost is about $5 USD [Kleinberg et al. 2006]. All formulations of AmB are administered intravenously and require hospitalization because close monitoring of its toxicity is required.

1.4.7. Heat-Treated Fungizone (HTFZ)

Mild heat treatment of Amphotericin B as FZ has been suggested as a possible way of reformulating AmB with the combined benefits of retaining FZ's low cost and decreasing its toxicity. As far back as 1979, Ernst et al. showed in light-scattering experiments that heating of FZ solutions leads to an increase in aggregate apparent mass by some 400-fold. Knowledge that AmB aggregation state modulates its therapeutic efficacy led Gaboriau et al. [1997] to characterize the physico-chemical properties and toxicity of heat-treated FZ in two seminal papers in the late 1990's. The structure of HTFZ aggregates from a 5 mg/ml solution was found to be pleiomorphic (varied) cobweb structures of approximately 300 nm in their largest dimension using
a combination of cryo-transmission electron microscopy and laser-light scattering [Van Etten et al. 2000]. HTFZ therapy resulted in decreased toxicity to both in vitro human and animal kidney cell lines and in vivo rats and rabbits [Van Etten et al. 2000, Gaboriau et al. 1997]. The anti-fungal and anti-parasitic activity of AmB has been found to be unchanged by the heating process; HTFZ maintains a low minimum inhibitory concentration (MIC) and minimum lethal concentrations (MLC) similar to FZ [Hatabu et al. 2005; Bartlett K et al. 2004].

1.4.8. Summary of AmB Characteristics

AmB remains a drug of choice in the treatment of systemic fungal infections, but its use is limited by its toxicity, particularly that of the FZ formulation, which is the most widely used. Approved formulations with decreased toxicity are too expensive for widespread application. An improved understanding of AmB's mechanism of action is needed to aid the development of an inexpensive, effective AmB formulation. One possible advance in this direction is HTFZ, formed by simple heating of FZ, which shows decreased toxicity in preliminary studies. Minimal work on HTFZ's mechanism of action has been carried out.

At the cell membrane level, AmB is known to increase membrane permeability, particularly when sterols are present in the membrane. AmB binding to sterols to form aqueous pores within the bilayer is the proposed rationale for the increased permeability and accordingly for AmB's
mechanism of action towards both fungal and mammalian cells. This explanation, by sterol-hypothesis, has been challenged by studies that suggest there is pore formation even in sterol-free membranes; therefore the mode of actions is still not clear.

Chemically, study of AmB is complicated by its aggregation behavior in most solvents. This is of prime importance, since the aggregation state of the drug has been correlated with its effectiveness and toxicity. All reports of AmB effectiveness within a particular biological model must be read with care to note the AmB aggregation state which produced these results. The approach used in this thesis was to examine AmB formulations with comparison to free AmB and to understand their complexation with various proteins by determining their physico-chemical and biological properties.

1.5. Curcumin

*Curcuma longa* L. is a tropical herb usually seen in Southeast Asia. Since medieval times, the herb was reported in the literature for its various therapeutic effects. The curative property was attributed to the yellow pigment constituent in the rhizome of the plant. This active principle is curcumin (diferuloyl methane) (Figure 1.3). Curcumin has a broad range of therapeutic properties. In recent times *in vitro* studies of curcumin and its various synthetic analogues show that this compound exhibits biological activity, especially anti-inflammatory (Crohn's disease, arthritis, and several disorder of the cardiovascular system) and anti-metastatic, anti-angiogenic
(anticancer potential and several other miscellaneous diseases) [Aggarwal et al. 2009]. In addition to the above, it also has anti-oxidant, anti-viral, and anti-infective activity. Curcumin with its antioxidant and anti-inflammatory activity has invaluable therapeutic advantages. Experimental studies have demonstrated that curcumin is a potent scavenger of reactive oxygen species (ROS), which include superoxide anion radical and hydroxyl radical. Various patho-physiological effects of curcumin at the cellular level include: induction of apoptosis (programmed cell death) in cancer cell lines through down-regulation of pro-angiogenic genes mediated by transcription factor NF-κB8 and IκB kinase [Aggarwal et al. 2009].

![Chemical structures of curcuminoids](Aggarwal et al. 2007).

**Curcumin I:** R1 = R2 = OCH3  
**Curcumin II:** R1 = OCH3, R2 = H  
**Curcumin III:** R1 = R2 = H

Figure 1.3: Chemical structures of curcuminoids (Aggarwal et al. 2007).

### 1.5.1. Limitations in Formulating Dosage Forms and Delivery Systems

Despite the demonstrated efficacy of curcumin, it appears that its poor systemic bioavailability after oral dosing compromises the potential for therapeutic uses. The major reasons contributing to the low bioavailability...
of curcumin include poor absorption and rapid systemic elimination [Strimpakos et al. 2008]. Oral drug administration is usually considered as a practical and easy way to administrate drugs. However, in order for a drug from solid dosage form to be absorbed, in this case through the epithelial layer of the intestine, these substances must become dissolved. Curcumin is a hydrophobic compound with very low solubility in water. The partition coefficient and solubility in water was measured to be 3.2 μg/ml and 0.6 μg/ml, respectively [Kurien et al. 2007; Patel et al. 2009]. When watersolubility is less than 1 μg/ml, which is the case for curcumin, the bioavailability from oral formulations such as conventional tablets may be unacceptable [Pouton et al. 2006]. This was demonstrated in clinical trial study to evaluate the pharmacokinetics and effective dose of curcumin in humans. In this study a number of patients were given 8000 mg of free curcumin orally per day in order to achieve detectable systemic levels. However, beyond 8 g, the bulky volume of the drug was unacceptable to the patients [Cheng et al. 2001; Bisht et al. 2007]. Furthermore, studies performed on humans and animals showed that orally administrated curcumin undergoes rapid metabolism in the liver particularly via, glucuronidation, while curcumin given intraperitoneally or systemically undergoes reduction [Aggarwal et al. 2009]. Metabolites produced from these pathways show low or no pharmacological activity [Aggarwal et al. 2007; Aggarwal et al. 2009].
1.5.2. Delivery Systems for Curcumin

It is necessary to improve the bioavailability of curcumin in order to fully utilize the potential of this agent, and therefore a growing number of research groups are working on this aim. There are studies designed to investigate new approaches that could overcome these limitations seen with free curcumin. Number of studies has evaluated the liposomal formulation \textit{in vivo} and their effectiveness. The study conducted by Li et al. [2005] investigated the effect of liposomal curcumin on pancreatic carcinoma cells and suppression of KF-kB activity. The incorporated curcumin in liposomes showed a dose related increase in apoptosis of carcinoma cells and suppression of NF-kB activity. Moreover, the liposomal curcumin was found to be as effective as or better than free curcumin. Another experiment studied the effect unilamellar liposomal curcumin after tumor implantation in mice. This study concluded that liposomal curcumin could increase the life span of the animals by up to 74% in comparison with untreated [Rubya et al.1995]. The study conducted by Kunwar et al. [2006] compared the cellular uptake of liposomal and albumin-loaded-curcumin by the spleenic lymphocytes and EL4 lymphoma cells. They reported that liposomes were able to deliver more curcumin into the cells than human serum albumin.

The absorption of a micellar formulation was evaluated using inverted rat intestinal sacs. This micellar formulation was composed of phosphatidylcholine and sodium deoxycholate. The authors reported that
after incubation for 3 hours, the percentage of free curcumin absorbed was 49 %, whereas the percentage for micellar formulation was 56 % [Suresh et al. 2007].

In another approach phospholipid complex of soya phospholipid and curcumin was tested in rats. The study showed higher plasma concentrations, and longer half-life of phospholipid complex in comparison with free curcumin. Furthermore the bioavailability was also seen to be improved significantly after oral administration. The relative bioavailability of curcumin was estimated to be around 330 % for the phospholipid complex as compared to free curcumin [Liu et al. 2006].

Another strategy of delivering curcumin is self-microemulsifying drug delivery system (SMEDDS). This system is basically composed of isotropic mixtures of oil, surfactant, cosurfactant and drug which have the ability to form oil/water microemulsion when it comes in contact with aqueous medium in gastro intestinal tract after oral intake [Borhade et al. 2008]. The curcumin-SMEDDS formulation was composed of 57.5 % surfactant, 30 % cosurfactant and 12.5 % oil. The in situ evaluation of this formulation showed that the absorption percentage of curcumin-loaded SMEDDS was 3.86 times higher than that of curcumin suspension [Cui et al. 2009].

"Nanocurcumin" is another formulation recently developed for curcumin [Bisht et al. 2007]. The principle of this formulation is that
curcumin is encapsulated in cross-linked polymeric particle with a hydrophobic core and a hydrophilic shell. The size of these particles lies in nanometre range and typically less than 100 nm. The group tested the product on pancreatic cancer cells and NF-kB and reported to be effective in inhibition of these cells and has similar activity as free curcumin on inflammatory cytokines [Bisht et al. 2007].

Loaded solid lipid nanoparticles (SLN) is another type of nanoparticle based delivery formulations. The system is usually consisting of biodegradable solid lipids. At room temperature the particles are in the solid state. Therefore, the mobility of incorporated molecules is reduced, thus it may offer possibility of modified release [Mühlen et al. 1998]. The study on SLN loaded curcumin was performed by Tiyaboonchai et al. [2007] and aimed at using this formulation in topical application. The stability and release was tested and found that properties of cream containing curcumin incorporated into SLNs was improved in comparison to free curcumin in the cream formulation.

Using pharmacological agents such as piperine (a component of black pepper) as suppressor of glucuronidation process of curcumin was also investigated. It was reported the inhibition of this process which occur primarily in the liver and in the intestine could enhance the bioavailability of curcumin [Aggarwal et al. 2007].
As presented, there are numerous studies suggesting different approaches of delivery systems in order to improve the absorption of curcumin. All of these studies have concluded that it is possible to develop formulations and methods which can improve the bioavailability and give higher plasma concentrations.

1.6. Scope of the thesis

The principle objective of this work is to explore the use of proteins as drug carriers. The use of protein appears to be promising, since the ligand-binding sites of a great number of proteins are well characterized [De Wolf et al. 2000]. In natural systems, the occurrence of high selectivity and binding affinity for a specific small ligand is invariably attributable to the presence of proteins. Among these, albumins and lectins have been studied extensively and have generated much expectation in the field of drug delivery for quite some time. In the current era lectins are gaining greater importance’s as drug delivery agents because of their high degree binding specificity to cell surface glycans [Bies et al. 2004]. Lectins have been earlier studied for its use in the delivery of drugs to oral cavity for treatment of gingivitis, ulcerations and oral carcinomas [Smart 2004]. Caseins derived from milk have also been studied for the microsphere preparation and carrying anticancer molecules such as doxorubicin [Willmott et al. 1992].

Amphotericin B a potent polyene is one among the drugs used in therapy for patients with deep seated fungal infections caused by acquired...
immunodeficiency syndrome and transplantations. Though it remains a potent antifungal, its use is hampered by its low solubility in water and toxic side effects. Some of the formulations that are currently used and their limitations have been discussed above. In the present study, proteins have been considered as a possible mode of drug delivery system in order to circumvent these limitations.

Several published reports show that AmB has a permeabilizing effect on red blood cell membrane. Brajtburg et al. [1985] have reported that the auto-oxidation of the polyene drug releasing reactive oxygen species (ROS) leads to these anti-cellular effects. Hence, we thought that the addition of a free radical scavenger or an antioxidant in the above protein-AmB complex would help to lessen the hemolytic activity of the drug.

Curcumin has been reported to possess antioxidant activity and known to exhibit various pharmacological effects including antimicrobial and anticancer properties. Coincidently, curcumin too is insoluble in aqueous medium and is poorly absorbed by the body. Various formulations and delivery systems evaluated in the past have been reviewed above. Even though there are a few reports on the amelioration of toxic effects of certain drugs by curcumin, the use of drug alone with curcumin in a protein complexed form and its beneficial biological effects have not been reported. This prompted us to evaluate the use of curcumin in combination with amphotericin B and further investigate the use of proteins for carrying these ligands.
The present study encompasses the following objectives:

1. To characterize the complex formed between amphotericin B (AmB) and carrier proteins (Sclerotium rolfsii Lectin (SRL), Albumin and casein).

2. To determine the antifungal activity of the protein-AmB complex.

3. To characterize the complex formed between curcumin and carrier proteins.

4. To evaluate the ability of AmB-protein complex, to lyse the erythrocytes (in vitro membrane damage assay).

5. To determine whether curcumin/curcumin-protein complex has any protective effect on the damage of red blood cells caused by AmB/AmB-protein complex.