CHAPTER I

INTRODUCTION

Most drugs administered by an extravascular route are absorbed from the site of administration into the systemic circulation. The drugs need to diffuse and transport to the sites of action across barriers such as the epithelium of the gastrointestinal tract, the endothelium of blood vessels, and the membrane of the target cell. Cell membranes are composed of the lipid bilayer, which is a semi-permeable structure. Lipid-soluble drugs can easily penetrate into and across biological membranes to reach their sites of action. Water and some selected small molecules such as urea and monovalent ions can pass through the hypothetical aqueous pore of cell membrane while permeability of larger water-soluble molecules across cell membrane is limited. These water-soluble molecules pass through water-filled channels between the cells (tight junctions) by paracellular pathway. Tight junction pores have diameters of approximately 4-8 Å, and the intercellular spaces occupy only 0.01% of the total surface area of epithelium (Hidalgo, 2001). Tight junctions are thus the main barrier to passive paracellular diffusion for these large hydrophilic molecules. Molecular size and charge often limit transport of large hydrophilic drugs across the epithelium, and absorption problems occur. Palmgrén et al. (2004) reported that permeability of atenolol, a hydrophilic molecule, through Caco-2 monolayer was only 0.14×10^{-6} cm/sec. Mannitol, another hydrophilic molecule, has gastrointestinal permeability of 0.38×10^{-6} cm/sec in rat and an in vitro flux of 0.72 ± 0.05 percent/cm^2/hr across Caco-2 monolayer (Cogburn, Donovan, and Schasteen, 1991; Liang, Chessic, and Yazdanian, 2000). On the contrary, the in vitro permeability of testosterone, which is a lipophilic molecule, is as high as 31.2×10^{-6} cm/sec (Versantvoort et al., 2002). Similarly, diazepam has a high flux of 39.49 ± 1.72 percent/cm^2/hr across Caco-2 monolayer (Cogburn, Donovan, and Schasteen, 1991). Hence, relatively higher doses are required for large hydrophilic drugs to be sufficiently absorbed to exert their pharmacologic actions.

One approach to increase absorption of hydrophilic drugs is to convert them into more lipophilic prodrugs. For example, enalaprilat was structurally modified into

enalapril, which has increased intestinal absorption and can be administered orally (Ward, Tippin, and Thakker, 2000). However, this approach requires much time and resources for drug development because prodrugs must be re-evaluated as new chemical entities. Another approach for increasing the absorption is to open the tight junction using enhancers such as octylglucoside, sodium caprate, sodium taurocholate, and chitosan (Tirumalasetty and Eley, 2006; Ward, Tippin, and Thakker, 2000). The bioavailability of cefoxitin in rats was reported to increase from less than 5% to as much as 70% with palmitoyl carnitine (Sutton et al., 1993). However, the use of enhancers has been associated with lipid bilayer damages, and absorption of toxic or unwanted substances may be concomitantly increased (Ward, Tippin, and Thakker, 2000).

Apart from limited transport and absorption of hydrophilic molecules, an active process called the efflux system may limit transport and absorption of some lipophilic drugs as well. Generally, cell membranes are composed of protein carriers, which mediate transfer of drug and other xenobiotic molecules to the alternate side of the membrane. Carrier-mediated systems (transporters) can be divided into influx transporters and efflux transporters. Influx transporters bind and translocate drug molecules into the cytoplasm, but efflux transporters have the opposite effect. Efflux transporters consist of the P-glycoprotein (P-gp) or multidrug-resistance protein (MDR1) and multidrug-ressistance-associated proteins (MRP1-7). Efflux transporters are located on the apical membrane. These transporters bind with drug molecules and excrete the drugs out of the cells into the lumen of capillaries, bile, urine, and gastrointestinal tract. P-gp transporters are also expressed in normal tissues as well as in tumor tissues. High levels of P-gp expression have been observed in the endothelial cells of brain capillaries, adrenal glands, kidneys, lungs, liver, and in the epithelium cells of the intestinal tract (Jodoin, Demeule, and Béliveau, 2002). P-gp transporters have been reported to decrease efficacy of absorption of many drugs including doxorubicin, digoxin, propranolol, and vinblastine (Versantvoort et al., 2002; Troutman and Thakker, 2003; D'Emanuele et al., 2004), which has led to therapeutic failure. P-gp substrates are usually lipophilic agents with high membrane permeability. Thus, the efflux system imposes absorption and transport problems to lipophilic molecules that are P-gp substrates.

Much effort has been put towards attempts to increase transport and absorption of P-gp substrates across the epithelium and the plasma membrane. One approach is inhibition of efflux transporters using surfactants. Hugger and co-worker (2002) reported that polyethylene glycol 300 (20% v/v), Cremophor EL (0.1% w/v), and Tween 80 (0.05% w/v) could inhibit efflux transporters and increase accumulation of paclitaxel into the cells. However, these inhibitors also changed membrane fluidity, and they were toxic to the cells. Nevertheless, the increased bioavailability of a cyclosporine product, Neoral®, has come partly from inhibition of P-gp by the surfactant present in the product (Van Mourik et al., 1998).

Nanotechnology has been applied in the field of drug delivery as well as in drug discovery and pharmaceutical manufacturing (Jain, 2005). Nanoparticles are widely applied as drug delivery systems. Nanoparticulate structures include polymeric nanoparticles, ceramic nanoparticles, polymeric micelles, dendrimers, and liposomes (Sahoo and Labhasetwar, 2003). These nanoparticles may interact with cells via several mechanisms. They can fuse with cell membrane and release entrapped agents into the cytoplasm directly. Poste and Papahadjopoulos (1976) reported that negatively charged liposomes composed of phosphatidylserine (PS)/phosphatidylcholine (PC) were taken up by nonendocytotic pathway or fusion with BALB/c mouse 3T3 cells. In addition, nanoparticles may be taken up by the cells via endocytosis, and transcytosis may follow. Most cells usually take nanoparticles into endosomes. Endosomes later fuse with lysosomes to form secondary lysosomes, and lysosomal enzymes break nanoparticles. During the process of breakdown of nanoparticles, the contents of secondary lysosomes are released into the cytoplasm or exocytosis may occur, depending on the cell type and the nature of the contents of the lysosomes. If the drug in nanoparticles is lipophilic and can permeate the membrane of endosomes or lysosomes, direct delivery of the drug into the cytoplasm takes place. On the other hand, if the drug is hydrophilic and cannot permeate the membrane, it can be expelled from the cell by exocytosis. Drugs can also be transported through the cell, rather than into the cell, by another similar process called transcytosis. In transcytosis, however, endocytosis and exocytosis occur without the internalized material being changed. Transcytosis occurs mainly in polarized epithelial cells. Thus, it is possible that nanoparticles can increase permeability and/or cellular

uptake of hydrophilic drugs by these mechanisms. For example, phospholipid-based liposomes containing horseradish peroxide are internalized by endocytosis into isolated Kupffer cells (Dijkstra et al., 1984). In addition, di-2,4-octadecadiene phosphatidylcholine liposomes enhance uptake of calcein and bovine serum albumin in mouse gastrointestinal tract (Chen, Torchilin, and Langer, 1996) and polymeric calcitonin nanoparticles enhance uptake of calcitonin by Caco-2 cells (Yoo and Park, 2004).

Since nanoparticles contain the drug inside their structures, it is likely that the drug will be protected from interaction with P-gp on the apical membrane. Several lines of scientific evidence indicate that these nanoparticles enhance delivery of lipophilic drugs that are substrates of efflux transporters. For example, propranolol-G3 dendrimers enhance propranolol transport and polymeric dendrimers reduce the effect of P-gp on drugs absorption in Caco-2 cells (D'Emanuale et al., 2004). Cetyl alcohol/polysorbate nanoparticles enhance uptake of paclitaxel in rat brain perfusion model (Koziare et al., 2004). Besides, epirubicin encapsulated in dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine liposomes has enhanced absorption across Caco-2 cells (Lo, 2000). Therefore, liposomes and other nanoparticulate structures may be good candidates for drug delivery systems to enhance cellular uptake and transendothelial/transepithelial transport of both highly water-soluble drugs and lipophilic drugs that are substrates of P-gp efflux transporters.

The model systems that are currently used to determine intracellular delivery and permeability characteristics of various compounds are in vitro cell culture models. To be useful in evaluating whether a carrier system can enhance intracellular delivery and transendothelial/transepithelial transport of interested compounds, the model should have the following properties: (1) The model must behave as a sufficient barrier to hydrophilic substances. (2) The model should be permeable to lipophilic substances and (3) the model should express transport proteins of interest. Various cell culture models have been utilized for cellular uptake and transport studies such as Caco-2, IEC-18 and MDCK (Steensma, Noteborn, and Kuiper, 2004; Troutman and Thakker, 2003). Caco-2 cells have been derived from human colon adenocarcinoma. The cell line was first characterized as a permeability model in 1989 and has been extensively used as a model for uptake and transport studies (Hidalgo, 2001). When Caco-2 cells form monolayer,

intercellular tight junctions completely develop, which restrict paracellular transport. Caco-2 cells also express numerous energy-dependent efflux pumps such as P-gp and MRP (Ferrec et al., 2001). Calcein represents a hydrophilic substance marker, which has a very low tendency to associate with phospholipid membranes. It does not penetrate cellular membrane, and thus does not use the transcellular pathway (Fujita et al., 1997). Rhodamine 123 is a lipophilic substance that is a known substrate of P-gp (Litman et al., 2001). The fluorescent dye has been used to study efflux transporters in a number of studies (Troutman and Thakker, 2003; D'Emanuele et al., 2004).

Liposomes are vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules, usually phospholipids. Phospholipids are biodegradable and major structural components of biological membrane. Liposomes entrap water-soluble molecules into aqueous compartments and lipid-soluble molecules are incorporated within the membrane. Liposomes have been used in the clinical practice for treatment of systemic fungal infections and to reduce adverse effects of chemotherapeutic drugs (Grant et al., 1989; Gabizon et al., 1994). Cationic liposomes are capable of safely transferring gene to pulmonary epithelium in gene therapy for cystic fibrosis (Alton and Geddes, 1995). Therefore, liposomes are relatively safe and effective carriers for drug delivery. The composition of lipid bilayers and charge on liposomal surface are important factors influencing the mechanism and extent of liposome-cell interaction. Negatively charged liposomes enhance uptake of carboxyfluorescein by trophoblast cells (Bajoria, Sooranna, and Contractor, 1997). HeLa cells take up positively charged liposomes by endocytosis largely than either neutral or negatively charged liposomes (Miller et al., 1998). Thus, results of enhanced drug delivery using liposomes vary and are dependent on cell types and nature of delivered molecules.

Liposomes are of interest to many research groups due to their safety and proven success in clinical uses. Several lines of evidence indicate that liposomes can enhance drug delivery into the cells as well as drug transport across the epithelium (Kozubek et al., 2000). Drugs that have been studies include a wide range of characteristics, from hydrophilic to lipophilic in nature. Most studies, however, focus on a particular drug of interest. There is no study to investigate the overall feasibility of using liposomes to enhance delivery of hydrophilic substances and P-gp substrates, which are major classes

of drug with absorption problems. In this study, the feasibility of using liposomes to overcome absorption problems was studied in a systematic way. The mechanism of liposomes in increasing cellular uptake of hydrophilic compounds was also elucidated. In addition, the effects of liposomal composition on enhanced delivery of hydrophilic substances and P-gp substrates into and through epithelial cells were evaluated. Calcein was used as a model hydrophilic substance, and rhodamine 123 as a model for P-gp substrates. Caco-2 cells were selected as the model cell culture system because the cell line met the above criteria and both intracellular delivery and transepithelial transport could be evaluated.

Objectives

The specific objectives of this study were to study:

1. The feasibility of using liposomes to enhance delivery of hydrophilic substances and P- glycoprotein substrates into and through epithelial cells
2. The effect of liposomal charge on delivery of hydrophilic substances and P-glycoprotein substrates into and through epithelial cells