CHAPTER II

LITERATURE REVIEW

When a drug is administered by an extravascular route of administration, the drug must first be absorbed into the systemic circulation and then diffuse or be transported to the site of action. The drug must cross cellular membranes, which are barrier to drug absorption. Along the way to the target site, the drug must also cross various epithelia by going either through or between epithelial cells. The permeability of a drug at the absorption site into the systemic circulation is related to the physiochemical properties of the drug, the nature of drug product, and the anatomy and physiology of cell membranes.

Cell membrane (Brown and Naglieria, 2005)

Cell membranes are generally thin, approximately 70 to 100 Å in thickness. Cell membranes are composed of phospholipids in the form of bilayer interdispersed with carbohydrates and proteins. Cell membranes are semipermeable partitions that act as selective barriers to the passage of molecules. Water, some selected small molecules, and lipid-soluble molecules can directly pass through such membranes, whereas highly charged molecules and large molecules, such as proteins and protein-bound drugs, can not. The schematic structure of cell membrane is shown in Figure 1.

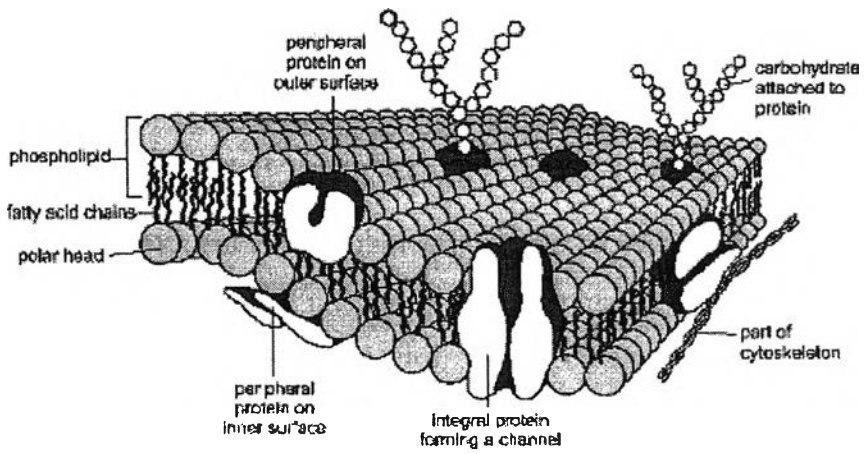

Figure 1: Structure of cell membrane (Source: www. Biologymad.com)

Barrier properties of epithelium (Hidalgo, 2001; Pollard and Earnshaw, 2002)

The epithelium consists of epithelial cell monolayer. The driving forces for drug transport across the monolayer are the concentration gradient, electrical potential difference, and hydrostatic pressure gradients. The permeability resistance of cell monolayer consists of both physical and biochemical barriers.

The physical barrier is composed of two parallel barriers: the tight junction and the lipid character of cell membrane. The tight junction constitutes the main barrier to paracellular diffusion. The diameters of tight junction pores are approximately 4-8 Å. Tight junctions are more permeable to cations than to anions and generally restrict the diffusion of all solutes larger than about 1.8 nm in diameter. Because of the narrow diameter of tight junction and the small surface area, which accounts for only 0.01% of the total surface area, this pathway is of little importance for most drugs.

The biochemical barrier consists of drug metabolizing enzymes and efflux transporters. The enzymes expressed by enterocytes include aminopeptidases, dipeptidyl peptidase IV, isoenzymes of the cytochrome P450 (CYP) superfamily such as 1A1, 1A2, 2D6, 3A4, 2C9, and 2C19, esterases, phenol sulfotransferase, and UDP glucoronyltransferase. The efflux transporters, which pump drug out of the enterocytes, include P-glycoprotein (P-gp or MDR1) and multidrug-resistance-associated proteins (MRP 1-7).

Passage of drugs across cell membranes

Drug absorption across a membrane can be divided into three main pathways:

1. Passive diffusion (Brown and Naglieria, 2005; Hidalgo, 2001; Petri, 2005)

Passive diffusion is the process by which molecules spontaneously diffuse from a region of higher concentration to a region of lower concentration or by an electrochemical gradient. This process requires no external energy. Drug molecules move

forward and backward across a membrane. The overall direction of movement is directed by the concentration gradient.

1.1 Passive paracellular transport

Passive paracellular pathway is an aqueous, extracellular route across the epithelium (Figure 2). This paracellular pathway can be divided into convective ("solvent drag") and diffusion components. The convective component is described by the rate at which a compound is carried across cell membrane by water flux. The diffusion component is the process by which water-soluble compounds move through the water-filled space between cells. The driving forces for passive paracellular diffusion are the electrochemical potential gradients derived from differences in concentration, the electrical potential, and the hydrostatic pressure. The available surface area for paracellular transport has been estimated to be 0.01% of the total surface area. The rate-limiting step in paracellular transport is the negatively charged tight junctions in the apical intercellular space of the cells. Paracellular transport appears to be insignificant for absorption of drugs with a molecular weight exceeding 200 Da. For example, many hydrophilic compounds, which use primarily the paracellular route, such as mannitol, acebutolol, sulfasalazine, and acyclovir have low permeability across tight junctions of the epithelium (Liang, Chessic, and Yazdanian, 2000). For many compounds, low epithelial permeability leads to low oral bioavailability. The hydrophilic broad-spectrum antibiotic cefoxitin has an oral bioavailability of < 5% in animals owing to poor intestinal permeability (Sutton, 1993).

1.2 Passive transcellular transport

Passive transcellular pathway is the process that involves the movement of solute molecules across the apical membrane, through the cell cytoplasm, and across the basolateral membrane (Figure 2). This is the main route of permeation for hydrophobic compounds with low molecular weights. Drug molecules move forward and backward across a membrane. If the two sides have the same drug concentration, forward-moving

drug molecules are balanced by molecules moving back, resulting in no net transfer of drug. When one side is higher in drug concentration, at any given time, the number of forward-moving drug molecules will be higher than the number of backward-moving molecules; net result will be transfer of molecules to the alternate side. Fick's first law of diffusion can describe the transport rate mathematically.

$$J = P \cdot (C1 - C2)$$

where J is the flux or the mass transport over time per surface area, P is the permeability coefficient, and (C1 - C2) is the concentration gradient over the membrane.

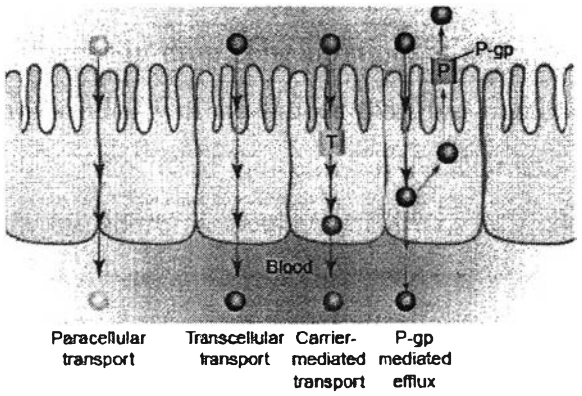

Figure 2: Passage of drugs across cell membranes (Source: Albert, 2001)

2. Carrier-mediated transport (Brown and Naglieria, 2005; Hidalgo, 2001; Petri, 2005)

Carrier-mediated transport systems are present for the absorption of ions and nutrients for the body. Carrier-mediated transport is a specialized process, requiring carrier that binds the drug to form a carrier-drug complex that shuttles the drug across the membrane and then dissociates the drug on the other side of the membrane.

2.1 Active transport

Active transport is a carrier-mediated process that plays an important role in the gastrointestinal absorption and in renal and biliary secretion of many drugs and

metabolites. Active transport is characterized by the ability to transport the drug against a concentration gradient. This process is an energy-consuming system.

2.2 Facilitated diffusion

Facilitated diffusion is also a carrier-mediated transport system, differing from active transport in that drug moves along a concentration gradient (moves from high drug concentration to low drug concentration). Therefore, this system does not require energy other than the thermal energy.

3. Vesicular transport (Brown and Naglieria, 2005; Hidalgo, 2001; Philippot and Schuber, 1994; Lodish et al., 2004; Karp, 2005)

Vesicular transport is the process of engulfing particles or dissolved materials by the cell. Vesicular transport can be classified by size of particles and type of materials. Pinocytosis is a nonspecific uptake process by which solute molecules dissolved in the luminal fluid are incorporated by bulk transport into the fluid-phase of endocytotic vesicles. On the other hand, phagocytosis refers to the engulfment of larger particles or macromolecules with diameter of more than 0.1 μm into cell. A few cell types (e.g., macrophages) can take up whole bacteria and other large particles by this process. Moreover, all eukaryotic cells continually engage in endocytosis, a process that takes up particles to form a membrane-limited vesicle of about 0.05-0.1 μm in diameter. When the cell membrane pinches off to form vesicles, the endocytic vesicles (the early endosome). The early endosome conveys its contents to the late endosome. The late endosome later fuses with the lysosome and molecules were broken down to their constituent parts by enzymes in the lysosome. Either the materials will remain sequestered in the lysosomes until exocytosis (Figure 3), or they will slowly leak out of the lysosomes into the cells.

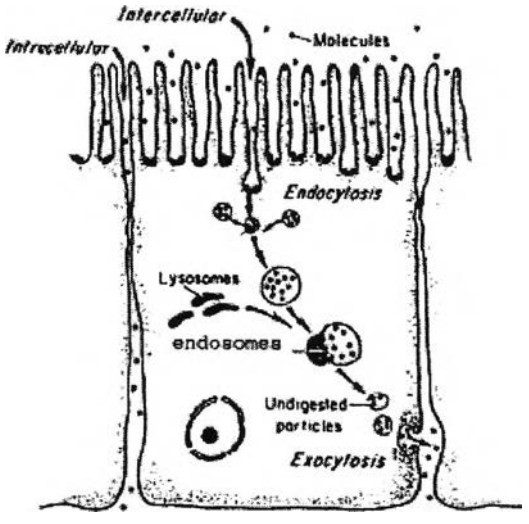

Figure 3: Diagrams intracellular transport of vesicles in enterocytes (Source: Ho et.al., 1990)

In addition, specific extracellular macromolecules can bind to receptors on the external surface of the cell membrane and are taken up into the cell. This process is referred to as receptor-mediated endocytosis. There are two different types of receptors. One group of receptors is the transferrin and low-density lipoprotein receptors, which mediate the delivery iron and cholesterol to cells, respectively. The second groups of receptors carry messages that change the activities of the cell. These ligands include hormones (such as insulin) and growth factors (such as epidermal growth factor).

Transcytosis is the vesicular transport of macromolecules from one side of the cell to the other within a membrane-bounded carrier. It is a strategy used by multicellular organisms to move materials selectively between two different environments without altering the unique compositions of those environments. Transcytosis is a widespread transport process; a variety of cell types uses it. The most familiar cell type that expresses transcytosis is the epithelial tissue. Transcytosis occurs mainly in polarized epithelial cells. In the polarized cell, net movement of material can be in either direction, apical to basolateral or the reverse, depending on the cargo and particular cellular contents of the process. However, transcytosis is not restricted to only epithelial cells, osteoclasts and neurons also carry vesicular cargo between two environments by transcytosis. In normal enterocytes, the transcytotic capacity is low; most of the endocytosed material is

localized to and degraded in the lysosomes. Intact macromolecules are believed to be transported more effectively by specialized cells called the M cells. The number of M cells is small compared to normal enterocytes.

4. Efflux transport

In contrast to the role of absorption transporters, which can enhance drug absorption, other transporters may have the opposite effect. Efflux transporters mediate the extrusion of compounds from the cell cytoplasm to the lumen through a process known as apical efflux. Two families of efflux transporters are the multidrug resistance (MDR) and multidrug-resistance-associated protein (MRP) family. P-glycoprotein (P-gp or MDR1) is the most extensively studied efflux protein.

P-glycoprotein (Hidalgo, 2001; Nielsen, 2004; Hugger et al., 2002)

P-glycoprotein (P-gp), the most studied member of apical efflux transporters, is the product of the MDR1 gene. P-gp has a molecular weight of approximately 170 kDa. The structure of P-gp is given in Figure 4. P-gp comprises 1280 amino acids and consists of two subunits with six transmembrane domains and two ATP binding sites. P-gp acts as an efflux pump that excretes toxic compounds into the bile, urine, and gastrointestinal tract. In cancer cells, P-gp extrudes chemotherapeutic agents out of the cells, thus decreasing the intracellular concentration of the antitumor drugs. P-gp is also expressed in normal tissues. High levels of P-gp expression have been observed in the epithelia of the small intestinal, colon, bile duct, and kidneys, as well as in the capillary endothelial cells of the blood brain barrier (Jodoin, Demeule, and Béliveau, 2002). The P-gp family is divided into two different classes. Class I consists of the drug-transporting P-gp, which includes the human MDR1 and the mouse mdr1a (mdr3) and mdr1b (mdr1) gene products. Class II includes the non-drug transporting P-gp such as the human MDR2 (MDR3) and the mouse mdr2 gene products (Nielsen, 2004).

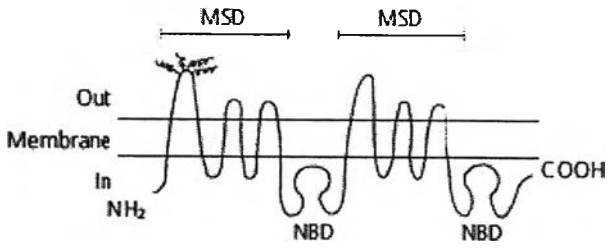

Figure 4: Schematic model of P-glycoprotein. The transmembrane segments, adenosine triphosphate-binding sequences, and location of carbohydrate chains (ع) are indicated. NBD: nucleotide-binding domain; MSD: membrane-spanning domain. (Source: Nielsen, 2004)

Substrates of P-gp (Litman et al., 2001; Wiese, and Pajeva, 2001)

The substrates most often involved in P-gp efflux include anthracyclines, anthracenes, vinca alkaloids, camptothecin derivatives, epipodophyllotoxins, tubulin polymerizing drugs, chromopeptide antibiotics, alkylating agents, and HIV-1 protease inhibitors (Table 1.) Thus, expression of the P-gp may have a role in preventing or diminishing absorption of toxic compounds and may act as biochemical barrier to drug molecules that are absorbed.

Table1: P-glycoprotein substrates

Class	Representative
Anthracyclines	daunorubicin, doxorubicin, epirubicin
Anthracenes	bisantrene, mitoxantrone
Vinca alkaloids	vinblastine, vincristine, vinorelbine, vindesine
Camptothecin derivatives	CPT-11, topotecan
Epipodophyllotoxins	etoposide, teniposide
Tubulin polymerizing drugs	colchicine, paclitaxel, docetaxel
Chromopeptide antibiotics	actinomycin D
Alkylating agents	mitomycin C
HIV-1 protease inhibitors	ritonavir, saquinavir, indinavir

Mechanisms of P-glycoprotein

The type and the number of the binding sites at P-gp are still a question of debate. One of the most popular models is "hydrophobic vacuum-cleaner" in Figure 5. According to this model, both halves of P-gp form a single transport channel through which the drug can be expelled from the cytosol not only in charged form but also in neutral form directly from the plasma membrane when entering the cell during its diffusion through the phospholipid bilayer. The drugs can be recognized and extruded out of the cell even before they reach the cytosol.

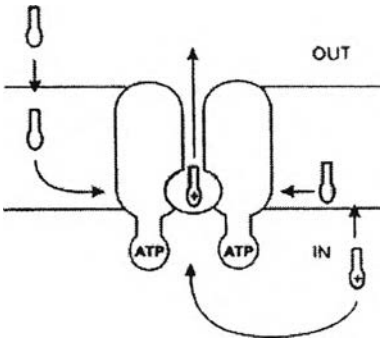

Figure 5: Hydrophobic vacuum-cleaner model of P-glycoprotein functioning (Source: Wiese, and Pajeva, 2001)

Another model of P-gp functioning proposed by Higgins (1994) presumes that P-gp acts as a flippase: the drug first intercalates into the inner leaflet of the bilayer and only then interacts with the transport protein in the membrane; the transporter then flips the drug from the inner to the outer leaflet as seen in Figure 6.

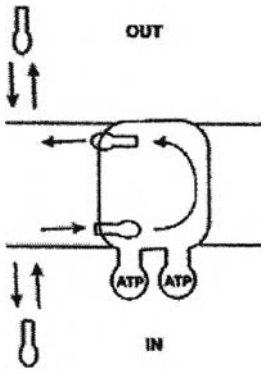

Figure 6: Flippase model of P-glycoprotein functioning (Source: Wiese, and Pajeva, 2001)

More recently, the multidrug-resistance-associated protein (MRP) transporter similar to P-gp was discovered. It is present in small quantities in almost all mammalian cells and is localized in the plasma membrane and the endoplasmic reticulum. MRP1, MRP2/cMOAT, and MRP3 have been found in human duodenum, whereas MRP1, MRP3, and MRP5 are found in human colon (Hidalgo, 2001). MRP expression leads to resistance to antineoplastics similar to that of P-gp with some exceptions (paclitaxel, mitoxantrone, colchicines and gramicidine D) (Preiss, 1998). Thus, the efflux system imposes absorption and transport problems to lipophilic molecules that are P-gp substrates.

Liposomes (New, 1997; Kozubek et al., 2000)

Liposomes are simple vesicles in which an aqueous volume is entirely enclosed by a membrane composed of amphiphilic lipid molecules (usually phospholipids). They form spontaneously when these lipids are dispersed in aqueous solution, giving rise to a population of vesicles, which may range in size form 10 nm-10 μm in diameter. Liposomes are classified into three classes based on number of bilayers and their size.

- Multilamellar vesicles (MLV) usually consist of several (up to 14) lipid layers (in an onion-like arrangement) separated from one another by a layer of aqueous solution. These vesicles are over several 100 nm in diameter.
- Small unilamellar vesicles (SUV) are surrounded by a single lipid layer and are 25-50 nm in diameter. This limit varies slightly according to the ionic strength of the aqueous solution and the lipid composition of the membrane.
- Large unilamellar vesicles (LUV) are a heterogeneous group of vesicles similar to SUVs and are surrounded by a single lipid layer. The diameter of these liposomes is very broad, from 100 nm up to cell size.

Interactions between liposomes and cells (New, 1997)

Liposomes interact with the cells by several mechanisms. The first interaction is intermembrane transfer. Intermembrane transfer of lipid component can take place upon close approach of the phospholipid bilayers without disruption of the liposomes. Lipophilic materials located in the liposomal membrane can insert themselves into other membranes via this process (Figure 7a). The second interaction is contact release. Contact release can occur when the membranes of the cell and the liposome undergo perturbations. These perturbations cause an increase in permeability of the liposome membrane. This leads to release of water-soluble solutes in high concentration in the close vicinity of the cell membrane, through which these solutes may pass (Figure 7b). Whether these processes take place to a significant extent or not depend upon membrane composition as well as the nature of the compounds themselves. Cell-induced leakage of solutes has been observed to be grater in liposome membranes with cholesterol concentration above 30 mol%.

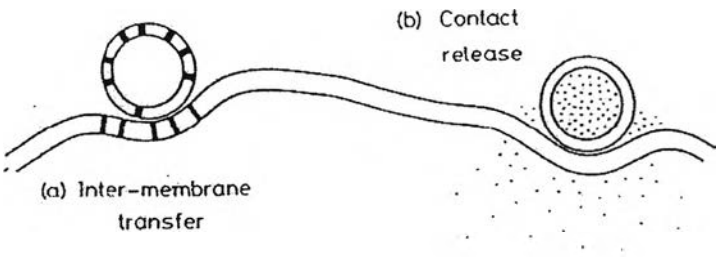

Figure 7: Contact-mediated transfer mechanisms (a) Intermembrane transfer (b) Contact release (Source: New, 1997)

The third interaction is liposome binding to cells without uptake. The liposomes may remain passively adsorbed on the cell surface with complete retention of aqueous and lipid contents within the vesicles, separate from the cell (Figure 8a). Adsorption of liposomes to the cell surface can often occur with little or no internalization of either aqueous or lipid components. It may take place by either physical attractive force or binding of specific receptors on the cell membrane to ligands on the vesicle membrane. Cellular uptake may occur after liposome adsorption. Uptake usually increases at or

below the phase transition temperature of the liposomal membrane. Moreover, after the initial adsorption stage, fusion may take place. Fusion can occur when liposomes come into close approach with cell membrane. Fusion results in complete mixing of liposomal lipids with those of the plasma membrane of the cell and the release of liposomal contents into the cytoplasm (Figure 8b).

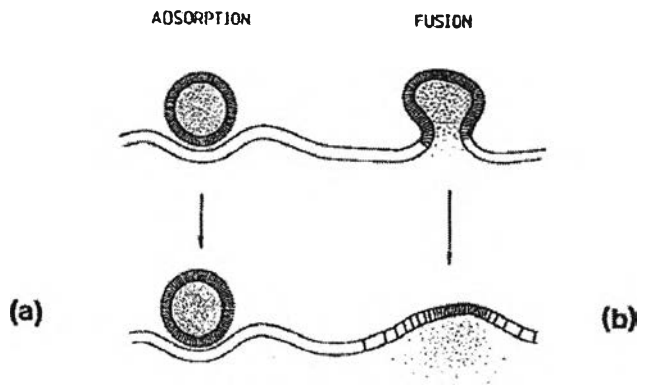

Figure 8: Liposome binding to cells with and without uptake (a) Adsorption (b) Fusion (Source: New, 1997)

Furthermore, liposomes may be taken up into the cell by phagocytosis or endocytosis. This vesicular pathway is the main route for cellular uptake of nutrients and exogenous macromolecules. After binding of liposomes to the cell surface, cells take liposomes into endosomes, which have a pH of 5 to 5.5. The early endosomes fuse with the late endosome. Then the late endosome fuses with the lysosome to form secondary lysosomes where cellular digestion occurs at approximately pH 4.5. Lysosomal enzymes break open the liposomes and hydrolyze the phospholipids to fatty acids, which can be recycled and reincorporated into host phospholipids. During liposome breakdown in lysosomes, the contents of the aqueous compartment are released. These contents will either remain sequestered in the lysosomes until exocytosis occurs (particularly if they are highly charged at low pH), or they will slowly leak out of the lysosomes into the cell. In addition to endocytosis, liposomes may also be taken up by receptor-mediated endocytosis. Liposomes coated with low-density lipoprotein or transferrin bind to the cells via surface receptors and are internalized via coated pits.

Factors influencing the uptake of liposomes into cell

Several factors affect uptake of liposomes by cells. These factors are summarized as following.

1. Liposome composition

Cellular uptake of liposomes is mediated by adsorption of liposome onto the cell surface and subsequent fusion or endocytosis. The composition of lipid bilayer and charge on liposomal surface are important parameters influencing the mechanism and extent of liposome-cell interaction. The effect of lipids used, cholesterol, and the presence of charge on the liposome bilayer on the cellular uptake has been investigated. Neutral phosphatidylcholine (PC) vesicles are incorporated into cells primarily by endocytosis, whereas negatively charged vesicles composed of phosphatidylserine (PS)/ PC are taken up by nonendocytotic pathway or fusion (Papahadjopoulos, Poste, and Schaeffer, 1973; Poste and Papahadjopoulos, 1976). Moreover, inclusion of 9% PS, phosphatidylglycerol (PG), or phosphatidic acid (PA) in liposomal membrane significantly increases uptake of 1- hydroxypyrene-3, 6, 8-trisulfonate by CV1 cells (Lee, Hong, and Papahadjopoulos, 1992). Furthermore, epithelial adenocarcinoma HeLa cells and lymphocytic leukemia Jurkat T cells with externalized PS are more susceptible to fusion with 1,2-Dioleoyl-3-trimethylammoniumpropane (DOTAP) liposomes than control cells (Stebelska, Wyrozumska, and Sikorski, 2006).

2. Bilayer fluidity

The physical state of liposomes is of considerable importance in determining pathway by which lipid vesicles are incorporated into culture cells. For MLV as well as SUV, lipid fluidity determines whether the predominant pathway for incorporation into cells is by endocytosis or fusion. Solid charged vesicles (PS/DPPC/DSPC 1:4.5:4.5) and fluid neutral vesicles (PC) are incorporated into 3T3 cells almost exclusively by endocytosis. In contrast, the major incorporation pathway for fluid charged vesicles

(PS/PC, 1:9) is via a nonendocytotic mechanism, which involves fusion of lipid vesicles with the plasma membrane (Poste and Papahadjopoulos, 1976).

3. Size

Uptake of liposomes also depends on physical parameters such as their size. Heath, Lopez, and Papahadjopoulos (1985) found that sonicated methotrexate-γ-aspartate liposomes were less taken up than liposomes extruded to 0.1 μm or 0.2 μm, but more than unextruded vesicles. The size of liposomes with greatest accumulation in tumors is around 0.1 μm in diameter (Uchiyama et al., 1995).

4. Cell type

Liposome uptake by different cells has a varying dependence on the liposomal composition. In human neutrophils, liposomes containing cholesterol is more efficient in enhancing the production and release of reactive oxygen species than liposomes containing only PC (Atrouse, 2002). With monocytes/macrophages, there is no apparent difference in uptake with varying amounts of cholesterol in liposomal formulations (Katragadda, Bridgman, and Betageri, 2000). Moreover, negatively charged liposomes containing PS and DCP were capable of delivering greater amount of stavudine into the macrophages than either neutral or positive liposomes (Katragadda, Bridgman, and Betageri, 2000). Futhermore, trophoblast cells take up negatively charaged liposomes more efficiently than neutral and positively charged liposomes (Bajoria, Sooranna, and Contractor, 1997). In addition, liposomes with negatively charged phospholipid membrane have an enhanced uptake by CV1, L929, CL18, and AKR/J SL2 cells when compared to neutral liposomes (Lee, Hong, and Papahadjopoulos, 1992; Heath, Lopez, and Papahadjopoulos, 1985). In contrast, Miller et al. (1998) found that HeLa cells took up positively charged liposomes by endocytosis better than either neutral or negatively charged liposomes. The murine derived mononuclear macrophage cell line (J774) endocytoses both HPTS cationic and anionic liposomes better than neutral liposomes

(Miller et al., 1998). These findings suggest that different cells have different uptake mechanisms and different liposome-binding sites on their surfaces.

Cell culture models (Braun et al., 2000; Artursson and Borchardt, 1997)

In drug design and drug development, adequate model systems must be introduced early to avoid loss of promising compounds at an advanced stage due to insufficient absorption into and distribution throughout the body. Besides toxicity, the pharmacokinetic characteristics were a major reason for failures of compound in studies. Several models have been established as tools for prediction of in vivo barriers. Cell culture models have been used for high throughput screening of drug absorption/metabolism, to screen for potential absorption problems in drug discovery, and in development of drug delivery systems. Studies using cell cultures are easy to perform in large numbers. It requires only small sample volumes. It is high throughput with low variability between replicates. The advantages of cell cultures have been summarized as follows: (1) rapid evaluation of the permeability and metabolism of the drug; (2) the opportunity to study mechanisms of drug transport under controlled conditions; and (3) the opportunity to perform studies on human cells (Audus et al., 1990). Various cell types of epithelial and endothelial origins have been used for transport studies in search of in vitro models for in vivo barriers such as epithelia of GI tract, nasal mucosa, skin, and the blood-brain barrier. In these models, epithelial or endothelial mono/multilayers are cultivated in permeable cell inserts. Cells are allowed to grow and, in some cases, to differentiate for a few days to a few weeks. At this time, the cells have formed confluent layers with appropriate barrier properties for drug transport.

Successful use of cell culture models is closely related to standardization of the cell culture used. Standardization of cell culture models is important because cell cultures obtained from different laboratories are widely different. Differences in culture conditions (seeding density, cell feeding routine, and composition of the media) and age of the cells are factors that are known to produce dramatic inconsistencies among different laboratories. Thus, standardization of cell cultures should be performed before any uptake or transport studies.

Standardization of cell cultures (Braun et al., 2000)

Standardization is essential for successful use of cell culture models. The origin of cells and passage numbers should be controlled. Growth medium should not be changed, and if changes are made, the growth characteristics of cells have to be checked under the new conditions. The support membrane is another important component influencing cell growth and differentiation of cells. Thus, it is important to keep standard supports for cells. Furthermore, confluence of cells should not be the only criterion when performing transport studies, because significant differences exist between confluent cells in the exponential growth phase and confluent cells in the stationary growth phase. Qualitative and quantitative differences are encountered in protein expression. Besides protein expression, the tight junction formation, which is usually expressed in terms of transepithelial electrical resistance (TEER), can also change in dependence of the growth phase. Thus, the exact time of study needs to be specified in terms of number of days after seeding or after the cell culture has reached confluence. For characterization of cell culture, the confocal laser-scanning microscope is of great value for optical scans through the cell layers with subsequent image restoration. To characterize tight junction formation, TEER values, which can change according to the growth phase, can be monitored. The complete regular network of tight junction can also be monitored with the permeability value of water-soluble substances such as mannitol or PEG 4000 (Versantvoort et al. 1998). In addition, transporters such as P-gp can be localized by fluorescence immunocytochemistry with specific antibodies. For functional assessment of P-gp, transport studies can be used by comparison of the permeability values of known P-gp substrates between the two transport directions. P-gp function is verified when the efflux ratio is more than two (Faassen et al., 2003). The efflux ratio is defined as the permeability in the basolateral to apical (B-to-A) direction divided by the permeability in the apical to basolateral (A-to-B) direction. Rhodamine 123, vinblastine, and propranolol are examples of substrates of efflux transporters that have been used for such purposes (Troutman and Thakker, 2003; D'Emanuele et al., 2004). Inhibition of the carrier-mediated component by specific P-gp inhibitors such as verapamil or cyclosporine A is also helpful (Braun et al., 2000; Litman et al., 2001).

Cell Proliferation (Freshney, 2000)

Measurement of cell proliferation rates is often used to determine the response of cells. Quantitative analysis of culture growth is also important in routine maintenance, as it is a crucial element for monitoring the consistency of the culture and determining the best time to subculture, the optimum dilution, and the estimated planting efficiency at different cell densities. Changes of test medium, serum, supplement, and culture vessels require quantitative reassessment of cell growth.

The growth state of a culture is a kinetic process. It is critical in the design of cell culture experiments. Cultures vary significantly in many of their properties between the lag phase, the period of exponential growth (log phase), and the stationary phase (plateau). The log and plateau phases give a vital piece of information about the cell line, the population doubling time (PDT) during exponential growth, and the maximum cell density achieved in the plateau phase (Figure 9). Therefore, it is important to take account of the status of the culture both at the initiation of an experiment and at the time of sampling, in order to determine whether it is proliferating or not and, if it is, the duration of the PDT and cell cycle time. The measurement of the PDT is used to quantify the response of the cells to different inhibitory or stimulatory culture conditions such as variations in nutrient concentration, hormonal effect, or toxic drugs. It is also a good monitor of the culture during serial passage and enables the calculation of cell yields and the dilution factor required at subculture. Cells that have entered the plateau phase have a greatly reduced growth fraction and a different morphology. They may be more differentiated and may become polarized. Therefore, cell cultures are most consistent and uniform in the log phase and sampling at the end of the log phase gives the highest yield and greatest reproducibility.

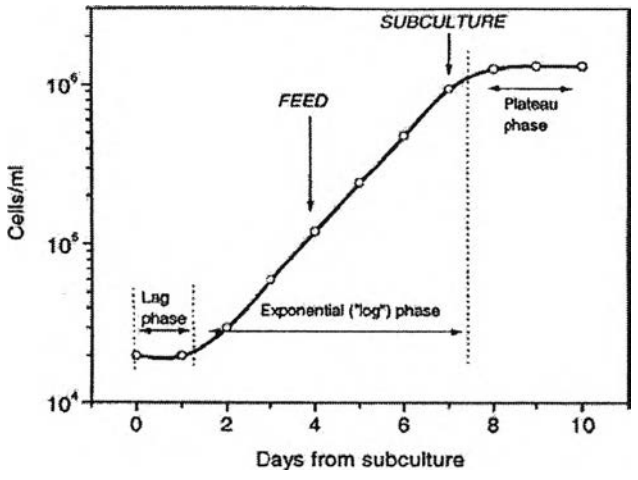

Figure 9: Semilog plot of cell concentration versus time from subculture, showing the lag phase, the exponential phase, and a plateau, and indicating times at which subculture and feeding should be performed. (Source: Freshney, 2000)

Caco-2 (Ferrec et al., 1999; Gan and Thakker, 1997)

Caco-2, an epithelial human colon adenocarcinoma cell line, has been the most popular cellular model used to predict absorption of drug molecules. In culture, Caco-2 differentiates spontaneously to enterocytes under conventional condition upon reaching confluence, after 14-21 days of seeding. When cultured on a microporous filter, Caco-2 cells form monolayer of highly differentiated epithelial cells. The cells in the monolayer possess brush border on the apical surface, tight junctions between adjacent cells, and a variety of proteins such as hydrolases and microvillar transporters. Several active transport systems are expressed in Caco-2 cells (for example, those for sugars, amino acids, dipeptides, bile acids, vitamins, and hormones). Several enzymes are also found in the brush border membranes (e.g., amimopeptidase, alkaline phosphatase, sucrase, dipeptidyl aminopeptidase, and γ- glutamyl transpeptidase). The existence of phase I metabolizing enzyme (CYP1A1) and phase II metabolizing enzymes (glutathione s-transferase, glucoronidase, and sulfotransferase) in this cell line has been reported. However, CYP3A, which is present in almost all intestinal cells, is very weakly expressed in Caco-2 cells. In addition, Caco-2 cells have expression of energy-dependent drug efflux pumps such as P-glycoprotein, multidrug-resistant-associated proteins, and

lung cancer-associated resistance protein. Thus, Caco-2 cells seem to be suitable in drug absorption studies with well physicochemical barrier properties. Caco-2 cells were used to study various drug delivery systems, examples of which are displayed in Table 2.

Table 2: Examples of studies on drug delivery systems using Caco-2 cells as a model

Dosage form	Drug/marker	Reference
Biodegradable nanoparticles	salmon calcitonin	Yoo and Park, 2004
Dendrimers	propranolol	D'Emanuele et al., 2004
Poly (amidoamine) dendrimers	paclitaxel	El-Sayed et al., 2003
Micelles	cholesterol	Ramaswamy et al., 2002
Liposomes	epirubicin	Lo, 2000
Folic acid-PEO-labeled liposomes	Texas Red®-dextran 3000	Anderson et al., 1999

Culture medium (Freshney, 2000; Ferrec et al., 1999)

Tissue culture media have been developed with conditions that simulate the situation in vivo. In particular, the environment of cell culture is regulated with regard to temperature, osmotic pressure, pH, essential compounds (such as carbohydrates, amino acids, vitamins, proteins, and peptides), inorganic ions, hormones, and extracellular matrix. Culture medium component is dependent on the requirements of cells. In addition, cell culture requires serum, which is the most significant for cell growth, and serum at a concentration of 2-20 % is mostly added to the medium for optimal cell growth. Culture medium for passage of Caco-2 cells is mostly composed of DMEM with fetal bovine serum (FBS) (10-20%), nonessential amino acid (1%), sodium pyruvate (1%), L-glutamine (1%), and penicillin-streptomycin (1%). Culture conditions for Caco-2 include a culture medium at pH 7.4, with an osmolality of 260-320 mOsm/l, temperature of 37 °C, with 5-10% CO_2 in the atmosphere, and the relative humidity of 90-95%. However, Invitrogen Corporation is producing a variety of new nutrient medium formulations and supplements that minimize or eliminate many of problems associated with the use of animal sera as a supplement. Advanced media produced by the company are basically the conventional media with additional amino acids and sodium pyruvate,

ethanolamine, glutathione, ascorbic acid phosphate, insulin, transferrin, Albumax®, and trace elements. This new media enable researchers to reduce FBS supplementation by 50- 90 % with no change in cell growth, morphology, or function of cells in several cell lines including MDBK, Hep-2, Vero, WI-38, MDCK, A549, COS-7, and MRC-5. It can also save the time and labor usually spent in qualifying new lots of animal sera. Additionally, since there are fewer lot-to-lot changes, there are few chances of experimental variability. There is also reduced risk of interference or variability from undefined protein or other serum constituents. Before Caco-2 model is used to study the uptake and transport of interested substances, standardization of the cell culture model using standard markers are important. For transcellular pathway, lipophilic marker compounds known to be transported through the cell such as alprenolol, clonidine and diazepam have been used (Cogburn, Donavan, and Schasteen, 1991). On the other hand, for paracellular pathway, hydrophilic markers such as mannitol, PEG-4000, phenol red, and Lucifer yellow have been used to verify integrity of the tight junctions (Liang, Chessic, and Yazdanian, 2000; Cogburn, Donovan, and Schasteen, 1991). For screening of P-gp expression, known substrates of P-gp are tested for the efflux ratio by comparison of A-to-B and B-to-A transport in a two-chamber diffusion system. Specificity to P-gp is confirmed by inhibition of the carrier-mediated component with specific P-gp inhibitors such as, verapamil or cyclosporine A (Braun et al., 2000).

Uptake studies (Jaroszeski and Heller, 1998)

In uptake studies, drug is added to the apical side of cells and the accumulation of the drug is measured. The extent of drug accumulation is quantitatively analyzed by digesting the cell with a detergent. If a fluorescent marker is used, the amount of accumulated marker is determined by spectrofluorometric method. When flow cytometry is used, the fluorescence can be detected without cell disruption. Flow cytometry is a laser-based technology that is used to measure characteristics of biological particles. Flow cytometers scan single particles or cells as they flow in a liquid medium past an excitation light source. The underlying principle of flow cytometry is that light is scattered and fluorescence is emitted as light from the excitation source strikes the

moving particles. Light scattering and fluorescence is measured directly for each particle that passes the excitation source.

Transport studies (Ferrec et al., 2001; Braun et al., 2000)

Transport studies are conveniently done in a two-chamber diffusion cell. A commonly used commercial product is the Transwell® system (Corning, USA) (see picture in Appendix I). In transport studies, drug is added to the apical side of cells and the appearance of the drug on the basolateral side is followed with time. Conditions are chosen such that sink conditions are maintained throughout, which means that the kinetics of transfer is followed up to a maximum in the receiver chamber of about 10% of the total amount of drug applied. The system should be adequately stirred so that the effects of the aqueous boundary layer can be reduced. A drug absorption rate coefficient is determined from the initial linear appearance curve in the receiving (basolateral) chamber. A linear relationship between the A-to-B flux at several concentration levels is an indication of a passive diffusion. A tight correlation between the flux or apparent permeability coefficient (P_{app}) and TEER is significant in implying the paracellular route of drug transport. When drug transport in two directions are compared as the efflux ratio ($P_{app, BA}/P_{app, AB}$), the ratio of more than 2 generally indicates that the drug is a substrate for the efflux transporter(s) (Faassen et al., 2003). To use Caco-2 monolayers to study drug absorption, Caco-2 cells are allowed to grow as a monolayer and differentiate on a semi-permeable membrane with a defined pore size. Caco-2 monolayer generally has good integrity with complete morphological and functional differentiation as well as sufficient expression of P-gp after 21 days of culture. Drugs having complete absorption in humans often have a high permeability coefficient ($P_{app} \geq 1 \times 10^{-6}$ cm/s), whereas poorly absorbed drugs have a low permeability coefficient ($P_{app} < 1 \times 10^{-7}$ cm/s) in Caco-2 cells (Artursson and Karlsson, 1991).

Calculation in transport studies with cell culture models (Petri, 2005; Braun et al., 2000)

The apparent permeability coefficient (P_{app}) is used to quantify transport in a two-chamber diffusion system. Drug transfer from the donor to the receiver chamber through the cell layer is measured as increases in drug concentration in the receiver chamber over time, and the differential increase of drug is calculated. P_{app} (cm/s) values are determined as follows:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{C_0 A}$$

where dQ/dt (mol/s) is the increase in the amount of drug in the receiver chamber per over the time interval, A (cm^2) is the growth area of the cell culture insert, and C_0 (mol/ml) is the initial drug concentration in the donor chamber.