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NON-COVALENT CARRIER ENHANCED PROTEIN ABSORPTION-CELLULAR AND SUBCELLULAR MECHANSTIC STUDIES

by

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NON-COVALENT CARRIER ENHANCED PROTEIN ABSORPTION - CELLULAR AND SUBCELLULAR MECHANSTIC STUDIES

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A family of low molecular weight organic compounds (carriers) has been synthesized by Emisphere Technologies Inc. recently. These carriers, when co-administered with certain proteins (e. g. α-interferon, insulin, and human growth hormone), can markedly enhance the oral bioavailability of these proteins. Meanwhile, no obvious damage to the tissue was observed.

It has been suggested that the carriers can induce and stabilize an intermediate state of the protein, making the protein more hydrophobic and therefore more transportable. The aims of this study are: 1) to provide evidence to distinguish the mechanism of carrier enhanced protein absorption from the enhancement mechanism of classical penetration enhancers; 2) from a biological perspective to indirectly support the existence of a more hydrophobic form of protein upon interacting with the carriers.

Based on studies previously carried out in our laboratory using excised tissues and cell cultures, selected related studies were carried out on two *in vitro* cell models with primary emphasis on the subcellular level mechanistic studies. A series of cytotoxicity studies reveals that after treating cells with effective concentrations of carriers for short time periods, no substantial damage to certain subcellular organelles was detected. Long time

treatment of cells with carriers caused some damage to the cellular components. Quantitative uptake/transport measurements, coupled with visual observations, demonstrate a significant enhancement of human growth hormone (hGH) absorption in the presence of carriers. This enhancement of hGH can be observed before cytotoxicity occurs. Carrier enhanced hGH absorption is mainly demonstrated through the transcellular route. In the presence of carrier, the influx of hGH is through a simple passive process whereas the efflux of hGH is subject to a P-gp efflux pump.

These results suggests that the main mechanism of carrier enhanced protein absorption is not through the carrier effect on cells, i.e., the carrier cytotoxicity, but rather, it is the interaction between carrier and protein that makes the protein more hydrophobic and consequently more transportable.

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CHAPTER 1: INTRODUCTION

I. Oral Route for Peptide and Protein Drug Delivery

Possessing unique physico-chemical properties, peptide and protein drugs require a suitable route or a proper mode of delivery. The oral route is by far the most convenient and commonly used route for administration of therapeutic compounds. Greater than two-thirds of the dosage forms marketed are for orally administered drugs (Ranade, 1991). A large absorptive surface area, relatively high permeability, and successful patient compliance due to ease of administration make the oral route the first choice for many drugs. Yet, the oral route poses a challenge to peptide and protein drug absorption primarily due to enzymatic and cellular transport barriers of the intestinal mucosa. The large size and polar nature of peptide and protein molecules, coupled with high diffusional resistance of the gastrointestinal mucosal to these molecules, render their transport difficult. In addition, even if a small amount of peptides and proteins is taken up intact, these macromolecules will probably undergo storage or metabolism within the enterocytic cell before being transferred to the systemic circulation. Moreover, even if this barrier is overcome, the compound can still be metabolized by the liver or excreted into the bile. Therefore, the systemic availability of many orally administered peptide and protein drug is extremely low (0.1-2%) (Krishnamoorthy et al., 1995).

Orally administered peptide and protein drugs can be metabolized by a number of peptidases at one or more sites before reaching the systemic circulation. Potential sites of presystemic metabolism are the intestinal lumen, brush border, cytosol of the epithelial cells

(enterocytes) and the liver (Davis, 1986). Intraluminal metabolism can occur by pepsin secreted by gastric cells and by trypsin, chymotrypsin, and elastase secreted by pancreatic cells. Brush-border enzymes like carboxypeptidases can act on oligopeptides having 2-6 amino acid residues. The cytosolic fraction of enterocytes can convert peptides to their constituent amino acids primarily on account of peptidases present within the fraction (Krishnamoorthyet al., 1995). The liver is an important organ for protein metabolism. Being well perfused and composed of several cell types that enables recognition and internalization of protein and peptide drugs, including hepatocytes, Kupffer cells and endothelial cells, the liver is a primary site for removal of peptides and proteins following oral delivery.

Besides the enzymatic barrier, absorption of peptide and protein drugs is also hindered by resistance exerted by the intestinal membrane. The epithelium of the gastrointestinal tract, with a 200 μm thick mucus layer, directly interfaces the intestinal lumen as the primary barrier limiting and controlling the passage of substances from the lumen to the blood capillaries underneath. The major mechanisms of peptide and protein drug transport through this cellular barrier, as well as the methods utilized to potentially enhance protein drug absorption, will be discussed in depth in subsequent sections.

II. Mechanisms of Peptide and Protein Transport through Cellular Barriers

It has been proposed that cellular transport of peptides and proteins can occur paracellularly and transcellularly. The paracellular pathway refers to passive diffusion of a

compound through the aqueous space between cells. Transcellular absorption includes passive diffusion, transporter-mediated transport and endocytosis. Cellular efflux mechanisms have not been investigated as thoroughly as absorption mechanisms. Currently, the two main efflux systems that have been identified are P-glycoprotein (P-gp) and multidrug resistant associated protein (MRP) efflux systems.

A. Paracellular Pathway (Passive Diffusion)

The paracellular pathway is an aqueous, extracellular route across endothelial and epithelial cells that is followed by substances according to their size and charge (Cereijido et al., 1993). Due to its aqueous nature, it is generally used by water and small hydrophilic molecules. It is considered especially important in the absorption of amino acids and monosaccharides from the lumen of the intestine, where their concentration is sometimes high enough to drive passive transport in the desired direction. Proteins can only permeate through a few types of epithelia, for example, glomerular and choroidal epithelia (Kluge et al., 1986; Renkin et al., 1973).

The primary restriction of a solute to enter through the paracellular pathway of the epithelia is the junctional complex placed at the outermost end of the intercellular space, called the tight junction (occluding junction). Although the molecular structure of the tight junction is still uncertain, transmission electron microscopy shows that the tight junction appears as a series of connections between the outer leaflets of the two interacting plasma

membranes of adjacent cells. Freeze-fracture electron microscopy reveals that the tight junction seems to be composed of a network of strands that completely encircles the apical end of each cell in the epithelial sheet. The strands are thought to be composed of long rows of specific transmembrane proteins in each of the two interacting plasma membranes, which join directly to each other to occlude the intercellular space (Alberts et al., 1994).

Permeability of the tight junction to small molecules varies greatly in different epithelia. The proximal tubule, for example, is a very "leaky" epithelium, and junctions here are predominantly composed of a single fibril. The distal tubule and collecting ducts are relatively "tight" with approximately six anastomosing junctional fibrils. Tight junctions in the epithelium lining of the small intestine are 10,000 times more leaky to inorganic ions such as Na^+ than those in the epithelium lining of the urinary bladder. Epithelial cells can transiently alter their tight junctions in order to permit an increased flow of solutes and water through breaches in the junctional barriers.

There is a strong relationship between the structure of the tight junction and its permeability. The ability of tight junctions to restrict the passage of solutes increases logarithmically with increasing numbers of strands in the tight junction network (Alberts et al., 1994). On the other hand, disruption of tight junctions increases the permeation of certain molecules that are believed to transport through the paracellular pathway. The epithelial tight junctions have been reported to be essentially impermeable to molecules with radii $\geq$ 15 Å (Cereijido et al., 1981; Madara et al., 1985). The dimension of the paracellular

pathway is, however, reported to be equivalent to a pore radius of 25 Å (Pappenheimer et al., 1987). The tightness of the tight junction can be evaluated by the value of transepithelial electrical resistance (TEER). Usually, a higher TEER reflects tighter epithelia and thus lower permeability of solutes going through the paracellular pathway. These values fluctuate widely according to the characteristic organization of the epithelia. They may vary from as low as 6 ohms cm^2 (e. g., the proximal tubule of the kidney) (Boulpaep et al., 1971) to as high as several thousands ohms cm^2 (e. g., the epithelium of the urinary bladder) (Civan et al., 1968).

Tight junctions are also observed in endothelia. It appears that almost all endothelial cells, including those of sinusoids and lymphatic vessels, have intercellular tight junctions that serve as barriers to proteins and other large molecules. Possibly, microvessels in hemopoietic tissues lack tight junctions (Bowman et al., 1991). Like their epithelial counterparts, the endothelial cell tight junction appears in thin sections as a fusion of adjacent cell membranes and selectively restricts movement of protein tracers from the lumenal to ablumenal side. In general, morphology of the endothelial tight junctions appears similar, but not identical, to epithelial tight junctions. The tight junctional complexes of endothelial cells lining high pressure large vessels such as the aorta and muscular arteries appear relatively simple. These tight junctional complexes do not completely restrict movement of horseradish peroxidase through the paracellular route. The gap junction contained within loops of the tight junction strands is a prominent feature of this complex. In arterioles, the tight junctional complexes tend to be more complex than those in larger arteries. They

exhibit more strands and particles, but gap junctions still appear to be contained within the complexes. They are also less likely to be permeated by tracers than larger vessels. Capillaries possess the most complex occluding junctions. They exhibit complex, anastomosing tight junctional strands similar to the ones found in epithelia. It is suggested that the low permeability to proteins is correlated with complexity of the tight junctions. Microvessel beds in the kidney (Mink et al., 1984) and bronchi (Hirabayashi et al., 1984), which are fenestrated and exhibit high permeability to proteins, also possess complex tight junctions. Post-capillary venules generally exhibit simple tight junctional complexes, and gap junctions are absent. These junctions appear to be the most leaky to proteins compared to those in other areas of the vasculature. In general, the endothelium of large vessels is less complex and more leaky than that derived from small diameter vessels with the exception of postcapillary venules which are the leakiest of all the vessels. The biochemical components of endothelial tight junctions are similar to epithelial tight junctions.

Intercellular junctions of arteriolar and capillary endothelia appear to be closed and are functionally impermeable to solutes with a diameter of 20 Å, which is similar to the cutoff of epithelia tight junctions. However, 25%-30% of the junctions appear open in the endothelia of postcapillary venules (Taylor et al., 1984). These intercellular junctions allow protein molecules to go through without much restriction. In addition, transendothelial channels formed by a single vesicle or chains of two or more fused vesicles that open simultaneously on both sides of the endothelium also provide large openings for the transport

of macromolecules such as proteins. In this sense, the endothelium is considered much more "leaky" than the epithelium.

B. Transcellular Pathway (Passive Diffusion, Transporter-mediated Transport and Endocytosis)

The transcellular pathway refers to the route of a solute that traverses through one side of the lipid bilayer of the cell membrane, moves through the cytoplasm, and then travels through to the other side of the cell membrane. This process can be either simply passive diffusion or be mediated by transporter systems. For molecules that travel simply by passive diffusion along their concentration gradient, properties such as lipophilicity, size, and charge may play a dominant role in controlling their permeation ability. Commonly, small, lipophilic drugs can be rapidly incorporated into the cell membrane and be absorbed to a relatively large extent, whereas the inefficient partitioning of peptide and protein drugs into the biological membrane is one of the contributors to poor intestinal absorption of this category of drugs (Lee et al., 1990a).

Transporter-mediated transport is a process by which a transporter protein transfers a solute molecule across the lipid bilayer. This process is similar to an enzyme-substrate reaction and transporters behave like specialized membrane bound enzymes. Transporter-meidated transport can be non-energy dependent (facilitated) or energy dependent (active). For example, most animal cells take up glucose from extracellular fluid by facilitated

transport through glucose transporters, but the intestine and kidney cells take up glucose from the lumen of the intestine and kidney tubules where the concentration of sugar is lower by actively cotransporting with sodium ions. The energy that drives active transport can be obtained from ATP hydrolysis or by energy stored in ion gradients. Various nutrients, such as glucose, amino acids, di/tri-peptides, and vitamins, have been found to be transported by transporter-mediated systems. Drugs whose chemical structure mimic these nutrient structures may use the transporters of the nutrients and be transported in this way. Examples are cephalosporins (Inui et al., 1992), angiotensin-converting enzyme inhibitors (Friedman et al., 1989a; 1989b; Thwaites et al., 1995), and renin antagonists (Hidalgo et al., 1995).

Endocytosis is a process whereby cells internalize their surface to form membrane limited vesicles that entrap extracellular molecules. There are three types of endocytosis: fluid-phase endocytosis, adsorptive endocytosis and receptor-mediated endocytosis. Fluid-phase endocytosis is the most general form among these three. In fluid-phase endocytosis, molecules dissolved in the extracellular fluid enter the cell as the fluid is trapped in the invaginated and engulfed cell membrane. In adsorptive endocytosis, molecules nonspecifically bind to the cell surface and then get internalized by the cell. Receptor-mediated endocytosis is the process in which accumulation of a particular solute is mediated by a specific ligand-receptor interaction that can be highly selective and confined to the region of a particular cell type.

In the process of endocytosis, cells internalize their surface and form vesicles that contain extracellular particles. These vesicles then migrate to the perinuclear region of the cell. During migration, vesicles fuse with a series of acidic subcellular organelles, particularly endosomes and lysosomes. Sometimes, these vesicles fuse with membranes on the opposite side of the cell monolayer, and exocytose their cargo to the other side of the cell. This process is called transcytosis. Endo/transcytosis is more active in macrophages, neutrophils, neonatal intestine and most of endothelial cells. It is very limited in certain types of epithelial cells such as epithelial cells in the adult small intestine.

Most macromolecule transport is mediated by endo/transcytosis. It is considered as an important route for cellular internalization of peptides and proteins. For example, immunoglobulin G (Kraehenbuhl et al., 1969), nerve growth factor (Siminoski et al., 1986), and epidermal growth factor (Thornburg et al., 1984) are known to enter intestinal mucosal cells through endocytosis.

C. Efflux Mechanisms (P-glycoprotein and Multidrug Resistance-associated Protein)

The first direct evidence of a specific alteration of the plasma membrane in multidrug resistance came from the study on Chinese hamster cells that were resistant to the drug colchicine. Electrophoresis was used to separate components of cell plasma membranes, and the results revealed that there was a unique glycoprotein in drug-resistant cells that seemed to be absent in the drug sensitive ones. It was named P-glycoprotein (P-gp) for its association

with the apparent permeability barrier to drugs that accompanied multidrug resistance (Kartner et al., 1989).

P-gp is a relatively large plasma membrane glycoprotein that has a molecular weight of 170KDa. It functions as an energy-dependent efflux drug pump that prevents the drug from accumulating within the cell thus rendering the intracellular drug concentration subtherapeutic. P-gp can be saturated by relatively hydrophobic substrates and inhibited by modulating agents, such as verapamil, and cyclosporin A. It is energy-dependent but not dependent on co-transport of Na^+ or an electrochemical gradient.

Figure 1-1 shows the proposed structural orientation of P-gp within the plasma membrane. As can be seen, the protein has 1280 amino acids and can be regarded as two halves. It spans the plasma membrane twelve times and is believed to create a transmembrane pore. It has two ATP-binding sites on the intracellular portion of the protein. This deduced structure possesses characteristics of a membrane energy dependent efflux pump to exclude cytotoxic agents from resistant cells through a channel formed by the transmembrane segments of P-gp.

The presence of the two ATP binding sites defines P-gp as a member of the ATP binding cassette superfamily of protein. This superfamily has many members, including bacterial transporters, and eukaryotic transporters, such as analogues of the MDR1 gene in protozoans, and many other types of transporters in mammalian cells. These transporters

handle nutrients, proteins and peptides, carbohydrates, drugs, pigments, and many other substrates, both at the cell surface and in interior membranes.

P-gp is widely expressed in many organs such as the intestine, liver, kidney and blood brain barrier. The liver absorbs and processes nutrients and metabolizes endogenous compounds and xenobiotics. P-gp is expressed in the liver likely serves to transport these compounds to their correct compartments. The MDR1 gene, which encodes P-gp, is expressed at a low level in the normal liver, but is inducible by chemotherapeutic drugs and xenobiotics (Chin et al., 1990; Gant et al., 1991). The localization of P-gp is also found in the proximal tubules of kidney and is demonstrated to mediate the transport of digoxin (de Lannoy et al., 1992). In brain, P-gp has been shown to be expressed specifically in the capillary endothelial cells forming the blood-brain barrier. Cell lines such as Caco-2, T84, and HCT-8, derived from the intestine, have been found to express high amounts of P-gp on the apical surface so that substrates for this pump are directionally transported from the basolateral to the apical surface (Cavet et al., 1996; Hunter et al., 1993; Zacherl et al., 1994).

It has recently been found that P-gp is implicated in contributing to the poor penetration of certain lipophilic peptide drugs. Transport studies of Cyclosporin A (Augustijns et al., 1993) and the lipophilic peptide AcPhe $(NMePhe)_2$ NH_2 (Burton et al., 1993) in Caco-2 cells showed that for both peptides, transport was lower in the apical-to-basolateral direction than in the opposite direction and was concentration dependent in the

apical-to-basolateral direction. In addition, the incorporation of P-gp inhibitors increased the apical–to-basolateral transport rate of drug while reducing it in the opposite direction.

Another protein, named multidrug resistance–associated protein (MRP), has recently been reported (Figure 1-2). This protein has a MW of 180-195 KDa and is a membrane glycoprotein involved in a non-P-gp multidrug resistance of human tumor cells. The existence of this protein came from evidence that a number of *in vitro* selected tumor cell lines have been described to be resistant to a broad range of natural product drugs but do not overexpress the MDR1 gene (Baas et al., 1990; Kuiper et al., 1990; McGrath et al., 1989; Mirski et al., 1987; Zijlstra et al., 1987). MRP is a newly identified member of the ATP-binding cassette transmembrane transporter superfamily (Cole et al., 1992; Krishnamachary et al., 1993; Grant et al., 1994; Higgin, 1992). Recent gene transfection studies showed that MRP, like MDR1 P-gp, can actually confer resistance to a wide spectrum of natural product drugs such as vincristine and etoposide (Grant et al., 1994).

MRP is expressed in normal tissues. It has been found that organs such as lung, stomach, kidney, spleen, colon, thyroid, testis, urinary bladder, adrenal gland and muscle all have MRP expression. Besides the protective function of MRP in preventing the invasion of toxic agents to the body, it is speculated that MRP plays some other important physiological roles because of its wide distribution in various tissues. However, the nature of this role is still unclear.

There are some common features for P-gp and MRP. They both belong to the ATP-binding cassette transmembrane transporter superfamily and function as energy dependent efflux pumps to exclude toxic agents from getting into the body. They are both related to the multidrug resistance phenomena. There is considerable overlap in the resistance spectrum of these two proteins. Both proteins transport important anticancer agents such as doxorubicin, etoposide, and vincristine. P-gp and MRP can also co-exist and be overexpressed in the same cell. The apical sides of Caco-2 cells and primary cultured brain endothelial cells have been found to have both proteins overexpressed. There are also a number of features that distinguish MRP from P-gp. Possessing different molecular weights, they are structurally different. In most cases, they distribute in different tissues. More importantly, they efflux substrates with different characteristics. While P-gp has been identified as a transporter of certain hydrophobic and some positively charged compounds, the common feature for MRP substrates is believed to be anionic in nature.

III. Experimental Approaches to Improve Protein Drug Absorption

Various ways to enhance the bioavailability of non-parenterally administered peptides and proteins have been studied. They can be classified into four categories: (1) Structure modification; (2) Formulation modification; (3) Use of penetration enhancers; (4) Protein conformation modification.

A. Structure Modification

Chemically altering the drug to provide a new species with different physico-chemical properties has been used as one of the strategies to improve peptide and protein drug absorption. These modified drugs, as compared to their parent compounds, should possess properties such as greater lipophilicity, less hydrogen-bonding potential, and less accessibility to enzymatic degradation. Approaches to reduce proteolysis have included chemical modification of the peptide backbone, N- and C-terminal modification or blocking. Bioreversible N-α-hydroxyalkylation of the peptide bond against carboxypeptidases or other proteolytic enzymes is one of these approaches. Additional structural modification includes substitution of nondisulfide-bonded cysteine residues with nonsulfur-containing amino acids and chemical replacement of labile methionine residues with nonoxidizable analogues such as norleucine (Krishnamoorthy et al., 1995).

The structure of peptides and proteins has been modified for delivery purposes. Palmitoylation of insulin, to increase the lipophilicity of insulin, has been shown to facilitate the transfer of insulin across mucosal membranes of the large intestine and improve the stability of insulin against intestinal enzymatic degradation (Muranishi et al., 1992). Lipidic amino acids have been chemically conjugated to some poorly absorbed peptide drugs, and this conjugation has been shown to increase the cellular uptake of these drugs (Toth, 1994). Enkephalins are orally inactive due to their extreme susceptibility to rapid hydrolysis by intestinal peptidase, but the oral bioavailability of a chemically modified prodrug is

significantly improved (Roemer et al., 1977). The disadvantage of this approach, however, lies mainly in the undesired biological response that may be created by the introduction of new chemical entities.

B. Formulation Modification

The improvement of peptide and protein drug absorption has been attempted through a variety of formulation modifications. The focus of these modifications has been to protect proteins from a hostile environment. This is especially important for oral protein delivery in which protein drugs encounter the enzymatic barrier of the gastrointestinal tract. It has been shown that coadministration with protease inhibitors has successfully promoted the oral absorption of pentagastrin, insulin, and RNAase (Lee, 1990b). The enhancement of absorption was significant (11.5 times) for pentagastrin through the duodenum of the rat when the pancreatic duct was ligated to exclude pancreatic digestive enzymes from the small intestine (Lee, 1990b). Surfactants such as sodium dodecyl sulfate (SDS) are able to denature proteases such as α-chymotrypsin (Shao et al., 1993). Insulin absorption can be enhanced by the trypsin and chymotrypsin inhibitor aprotinin and Bowman-Birk inhibitors (Morishita et al., 1992a; 1992b). Aminopeptidase inhibitors such as bacitracin, bestatin, and sodium-glycocholate have also been shown to enhance insulin absorption (Augustijns et al., 1995; Yamamoto et al., 1994). In addition, some penetration enhancers themselves are protease inhibitors. For example, sodium glycocholate is an insulin protease inhibitor.

Enzymatic degradation of proteins may also be blocked by encapsulating these drugs into lipids or polymeric matrices such as liposomes, nanoparticles and w/o/w emulsions (Zhou, 1994). An azo polymer capable of remaining stable along the gastrointestinal tract but decomposes at the ileocecal junction to release insulin was used as a potential delivery system for insulin (Saffran et al., 1986). However, one of the difficulties of using the above methods is that the protein structure may be irreversibly destroyed when incorporated into these systems. Also, one should be concerned about the negative outcome of using these materials, such as cytotoxicity and immunogenicity. So far, none of these formulation approaches have improved the oral absorption of protein drugs sufficiently enough to be of therapeutic benefit (Wu, 1999a).

The physical properties of drugs can be modified through certain formulation modifications. These modifications includes the shielding of the ionic charge of the drug, disrupting drug aggregation, increasing drug solubility, and modifying the partitioning properties of the drug-carrier complex (Robinson et al., 1999). For example, anionic surfactants such as SDS are able to completely disassociate the insulin hexamer into monomers, thus increasing its solubility for absorption (Shao et al., 1993).

C. Use of Penetration Enhancers

Penetration enhancers are substances that facilitate the absorption of solutes across biological membranes. They are often required for absorption of peptides and proteins in

pharmacologically active amounts. Robinson (Robinson et al., 1999) has classified penetration enhancers into the following categories: (1) surfactants, (2) bile salts and derivatives, (3) fatty acids and derivatives, (4) chelating agents, (5) sulfoxides, (6) Polyols, (7) Monohydric alcohols, (8) Others. Table 1-1 listed some typical examples of penetration enhancers for each category.

Penetration enhancers usually exert their influence by compromising the integrity of biological membranes. Perturbation of the membrane can happen in the tight junctions between cells (paracellularly), on the lipid bilayer of the cell membranes (transcellularly), or on both. Calcium chelators, such as EDTA, that decrease the extracellular calcium concentration can result in an expansion in the dimension of the tight junction, and as a consequence, enhance the permeability of some substances that are usually excluded from the tight junctions under normal conditions. Actin filament disruptors such as cytochalasins prevent actin from polymerizing, thus disrupting the intercellular organization of tight junctions (Madara et al., 1986). Certain plant toxins, such as phorbol esters, are also tight junction disruption agents (Madaraet al., 1985).

Penetration enhancers may also increase the transcellular permeability of peptides and proteins by affecting membrane lipids, proteins and other membrane components. Surfactants, in general, affect membranes by solubilizing membrane components. Fatty acids and their derivatives are found to act primarily on the phospholipid components of membranes thereby creating disorder and resulting in increased permeability (Muranushi et

al., 1981; Sato et al., 1988). For example, medium chain monoglycerides are believed to enhance rectal cefoxitin absorption by extracting cholesterol out of the cell membrane (Watanabe et al., 1988). Some fatty acids and salicylates can also act on the protein components of the membranes. For oleic acid and some other salicylates, their enhancement mechanism is associated with an effect on nonprotein thiols such as glutathione in the cell membrane (Suzuka et al., 1988).

The relationship between the structure of penetration enhancers and their enhancement efficiency has been elucidated. The enhancers' hydrophobicity has been found to be an important determinant of efficacy (van Hoogdalem et al., 1988). It has been shown that medium-chain fatty acids and derivatives, i.e., chain length of C12-C18, demonstrate maximum absorption enhancement (van Hoogdalem et al., 1988; Watanabe et al., 1988; Yata et al., 1985). The rank order of effectiveness in glycerides is mono->di->triglycerides. Moreover, using both branched and unbranched fatty acids ranging in chain length from C15 to C18, Aungst (Aungst, 1989) has elucidated the structural features of fatty acids essential for their penetration enhancement efficacy. The relationships obtained indicate a parabolic dependence of the penetration enhancement activity of saturated fatty acids on their lipophilicities. This relationship applies to both cases when the unbranched and branched fatty acids are considered separately and together. The optimum Log P value for fatty acids as penetration enhancers is approximately 5.2. It is, on this basis, that has been recommended that fatty acids of chain length C10 to C12 be used as adjuvants with transdermal preparations. Similar results were obtained for other categories of penetration

enhancers. The potency of bile salts in enhancing the nasal absorption of insulin was found to correlate with increasing hydrophobicity of the bile salts' steroid nucleus (Gordon et al., 1985). For salicylates, it is indicated that both Log P and pKa play a major role in their enhancement effect (Levitan et al., 1972).

Safety is always a concern when using penetration enhancers. One way to evaluate toxicity of absorption enhancers is to use an *in vitro* model, such as Caco-2 cell, or intestinal segments of animals. These *in vitro* studies can provide rapid assessments of the toxicity of the penetration enhancers, and furthermore, elucidate the effectiveness and mechanisms of their functions. Typical toxicity assays include the evaluation of the enhancer effect on the integrity of cell paracellular tight junctions. These include transepithelial electrical resistance (TEER) measurement, and permeation measurement of well-known paracellular markers, such as mannitol, sucrose, and PEG. For cell membrane damaging effects, assays indicating protein leakage such as lactate dehydrogenase (LDH), or dye penetration such as the trypan blue exclusion test, are often used. Specific subcellular compartment damage can also be evaluated by choosing appropriate assays. Table 1-2 presents some representative *in vitro* studies describing the properties of some penetration enhancers.

D. Protein Conformation Modification

Intracellular proteins are found to translocate through membranes not in a tightly folded state, but rather, in a relatively loose conformation, termed a molten globular state. It

has been shown that denaturation of some precursor proteins with urea accelerates their translocation across membranes (Verner et al., 1988). These detailed findings have recently evoked a certain interest to pharmaceutical scientists from a protein delivery point of view. A few attempts at modifying the three dimensional conformation of proteins to potentially increase their absorption have been made recently. One study has shown that a cyclic peptide is more effective in permeation than the corresponding linear form due to its smaller molecular radius and increased lipophilicity (Pauletti et al., 1996). Another study has demonstrated that initially unfolding basic fibroblast growth factor (bFGF) to a more linear conformation of bFGF, followed by treatment with a permeation enhancer, may significantly increase the permeation of this protein across rabbit buccal mucosa (Johnston et al., 1998). The mechanism of this enhancement has been suggested to be due to a change in protein conformation. Furthermore, research has suggested that the transport activity of certain carriers synthesized by Emisphere Technologies, Inc. is correlated with their effects on protein conformation (Milstein et al., 1998). Studies in this particular area have only begun recently, however, they represent a very interesting area that needs to be further explored.

IV. Emisphere's Carrier Enhanced Protein Absorption Technology

The first generation of Emisphere's carrier was discovered in the late 1980s. Steiner and Rosen (Steiner et al., 1990) found that microspheres formed from thermally condensed α-amino acid mixtures could be used to promote oral drug absorption. These materials, initially called proteinoids, were highly complex, structurally uncharacterized mixtures

consisting mainly of di-, tri-, and tetrapeptides and certain polymer components. Subsequently, microsphere formation of structurally defined N-acylated α-amino acids and oral delivery of salmon calcitonin in rats and primates was demonstrated. These compounds were shown to promote the oral delivery of both salmon calcitonin and α-interferon in rats and primates when dosed as an aqueous solution instead of as a microsphere suspension (Leone-Bay et al., 1995a; 1995b). Recently, a series of low molecular weight N-acylated, non-α-amino acid derivatives (named carriers) were synthesized. This new generation of compounds, when co-administered with human growth hormone (Leone-Bay et al., 1996a) by oral gavage to rats and primates, can increase the oral bioavailability of this drug significantly.

The synthesis of this family of amino acid derivatives (structures of example carriers shown in Figure 5-13 denoted as E414 and E352) followed standard techniques. For example, the acylated α-amino acids (or non-α-amino acids) are synthesized by dissolution of an α-amino acid (or non-α-amino acids) in aqueous sodium hydroxide (or organic solvents, such as tetrahydrofuran) followed by the addition of a selected acid chloride. After the mixture is stirred at room temperature for about 2 hour, the product is isolated by precipitation from the acidified reaction mixture and purified by recrystallization (Leone-Bay et al., 1995b).

No clear structure-activity relationship has been established for the enhancement ability of carriers toward proteins. But it is noticed, through a study using a series of N-

acylated α-amino acids, that the aromatic amide derivatives are significantly less efficient vehicles than the alicyclic amides for salmon calcitonin delivery. The overall trend also suggested that compounds with high partition coefficient values are more effective in producing oral delivery of calcitonin (Leone-Bay et al., 1995b), a small polypeptide which lacks a tertiary structure. Meanwhile, the ability of a group of non-α-amino acids to promote the absorption of recombinant human growth hormone, a large and structurally complex protein, increases with increasing lipophilicity in the amide portion of the molecules. But in the case of α-interferon, the relationship between partition coefficient and drug delivery is less clearly defined. This weak correlation supports the contention that the partition coefficient is important but not sufficient for drug delivery. There are other factors that play critical roles in promoting absorption.

The target proteins, which the carrier delivers, are significantly dissimilar in their structure and function. Salmon calcitonin is a single-chain polypeptide hormone of 32 amino acid residues. It has molecular weight of 3500 Da and functions to regulate a number of physiological processes including calcium ion levels in the blood. Clinically, calcitonin is indicated in the treatment of symptomatic Paget's disease, hypercalcemia, and postmenopausal osteoporosis. Alpha-interferon has molecular weight of 20,000 Da. It is a lymphokine consisting of 165 amino acids with nonspecific antiviral activity. Clinically, α-interferon is indicated in the treatment of hepatitis, hairy-cell leukemia, and AIDS-related Kaposi's sarcoma. Growth hormone is a 22,000 Da protein that consists of 191 amino acids.

It is secreted by the anterior pituitary gland and primary used in the treatment of growth hormone deficiency in children.

Absorption enhancement by the carrier is not due to enzyme inhibition. It has been demonstrated that the derivatized amino acids are only weak enzyme inhibitors as compared to Bowman Burke inhibitor, a known inhibitor of trypsin, and actinonin, a known LAP inhibitor. Drug transit across the intestinal membranes is also not the result of mucosal damage. Autopsy focusing on the gastrointestinal tracts of animals including stomach, duodenum, jejunum, and ileum followed by histological examination indicates that no significant damage occurs (Brayden et al., 1997; Leone-Bay et al., 1995b).

Carrier enhancement appears to be specific toward their targets. It has been demonstrated that while absorption of salmon calcitonin and α-interferon was increased by a specific carrier, heparin absorption, with the same carrier, was not enhanced (Leone-Bay et al., 1995b). Some carriers can enhance hGH absorption in isolated rabbit intestinal tissues, but have no effect on mannitol, a paracellular marker, progesterone, a transmembrane marker and thyrotropin releasing hormone (Mlynek, 1995). In addition, same carriers can successfully enhance hGH transport significantly, yet fail to enhance lysozyme permeation (Wu, 1999a).

The precise mechanism of carrier enhanced protein absorption is not completely clear but it is suggested that these carrier molecules are able to non-covalently bind to and interact

with the protein molecules. This interaction further stabilizes a partially unfolded, intermediate conformation of the protein, which makes the protein more hydrophobic and thus more transportable (Leone-Bay, et al. 1996b). The change in protein conformation induced by carriers is reversible so that proteins, once dissociated from the carriers, may refold into their native state and resume their normal biological activity (Milstein et al., 1998). Unfortunately, due to the intrinsic properties of the carrier, many conventional spectroscopic techniques commonly used to study intermolecular interactions were found not to be applicable here because of interference from the delivery compounds at the wavelengths of interest or interaction with the specific probes designed to evaluate intermolecular phenomena.

Using techniques that may avoid carrier interference, the nature of carrier enhanced drug absorption has been gradually revealed. Differential scanning calorimetry (DSC) data have shown that upon adding carriers to certain proteins, a lowering of protein melting point, Tm, occurs (Milstein et al., 1998). Tm is the temperature at which a protein undergoes thermal denaturation. In general, the Tm of a protein is altered by noncovalent association with ligand (Connelly, 1994). This indicates that the carriers are able to induce a conformational change of the protein molecule. Furthermore, lowering of Tm by the carrier precisely reflects the transport efficacy of the carrier. Carriers that do not have enhancement activity for the protein also do not show an effect on the protein Tm (Milstein et al., 1998). Further investigation on carrier and protein interaction shows that when the carrier demonstrates maximum stabilization of partially folded intermediates of protein, it,

meanwhile, exhibits maximum enhancement of protein bioavailability (Vangala, 1998). Coincident with the preliminary calorimetry data, a capillary zone electrophoresis (CZE) studies demonstrated that co-injection of hGH with an ineffective carrier did not change the appearance of the elution time of each individual (hGH and carrier). However, the injection of a mixture of hGH and an effective carrier shortened the appearance of elution time (Leone-Bay, et al. 1996). This indicates formation of a complex between hGH and the effective carrier. In addition, using a molecular modeling technique, four compounds which represent good, fair and poor delivery carriers were chosen to study their interaction with hGH. Examination of the relative interaction energies of these four compounds indicates that the extent and site of interaction follows the order of their *in vivo* activities. It is suggested that the interaction occurs with specific residues of protein rather than specific types of residues, and the interaction happens primarily between carrier and hydrophilic residues, suggesting that hydrophilic interactions such as electrostatic and hydrogen-bonding effects may play a significant role in the specificity of the interaction (Leone-Bay et al., 1996b).

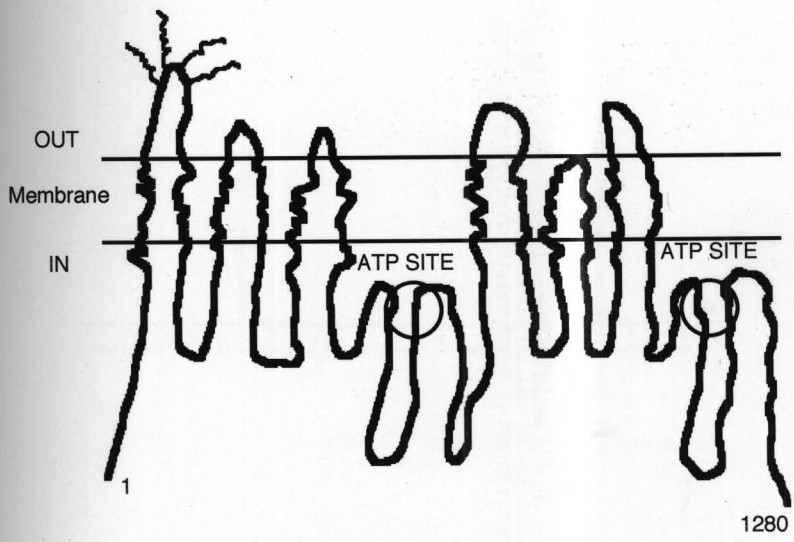


Figure 1-1: Cartoon model of the structure of P-glycoprotein. This model shows the 1280 amino acids of P-glycoprotein, the potential glycosylation sites, and the ATP binding sites (circled). Modified from reference (Gottesman et al., 1988).

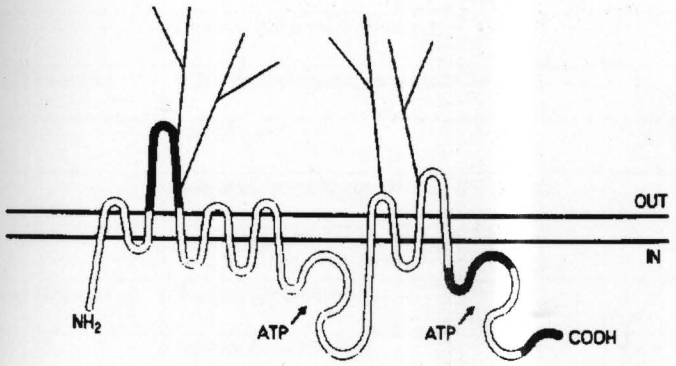


Figure 1-2: Artistic view of the secondary structure of multidrug resistant-associated protein showing the putative orientation of the transmembrane segments and the predicted glycosyl trees on the extracellular loops. Reprint from reference (Flens et al., 1994).

Surfactants (Ionic)	Sodium lauryl sulfate
	Sodium laurate
	Polyoxyethylene-20-cetyl ether
	Laureth-9
	Dioctyl sodium sulfosuccinate
Surfactants (Nonionic)	Polyoxyethylene-9-lauryl ether (PLE)
	Tween 80
	Nonylpenoxypolyoxyethylene (NP-POE)
	Polysorbates
Bile salts and derivatives	Sodium glycocholate
	Sodium deoxycholate
	Sodium taurocholate
	Sodium taurodihydrofusidate (STDHF)
	Sodium glycodihydrofusidate
Fatty acids and derivatives	Oleic acid
	Caprylic acid
	Mono(di)glycerides
	Lauric acid
	Linoleic acid
	Acylcholines
	Acylcarnitine

	Sodium caprate
Chelating agents	EDTA
	Citric acid
	Salicylates
Sulfoxides	Dimethyl sulfoxide (DMSO)
	Decylmethyl sulfoxide
Polyols	Propylene glycol
	Polyethylene glycol
	Glycerol
	Propanediol
Monohydric alcohols	Ethanol
	Isopropanol
Others	Urea and derivative
	Unsaturated cyclic urea
	Azone (1-dodecylazacycloheptan-2-one) (laurocapram)
	Cycoldextrin
	Enamine derivatives
	Terpenes
	Liposomes

Table 1-1: Classification of Penetration Enhancers. Modified from reference (Robinson et al., 1999).

Enhancer	Membrane	*In vitro* Toxicity Studies
Na lauryl sulfate	Caco-2	TEER, MTT, Morphologic changes
Dioctyl Na sulfosuccinate	Caco-2	TEER, MTT, Morphologic changes
Na caprate	Caco-2	Trypan blue exclusion, Protein release, DNA staining
MCG:PC (3:1)	Caco-2	TEER, Morphologic changes
Deoxycholate	Caco-2	Trypan blue exclusion, Protein release, DNA staining
Glycocholate	Caco-2	MTT
Taurodihydrofusidate	Caco-2	TEER, Trypan blue exclusion
Saponin, DS-1	Caco-2	TEER
Palmitoylcarnitine	Caco-2	TEER, LDH, DNA staining, Neutral red
Palmitoylcarnitine	Rat colon	TEER, Histologic examination

LDH= lactate dehydrogenase; MTT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; TEER=transepithelial electrical resistance; MCG=medium chain glyceride; PC=phosphatidylcholine

Table 1-2: Overview of representative studies describing *in vitro* effects of intestinal permeation enhancers. Modified from reference (Aungst, 2000).

CHAPTER 2: STATEMENT OF RESEARCH PROBLEM AND OVERALL PLAN OF RESEARCH

I. Statement of Research Problem

Proteins have been recognized as an important category of drugs since the beginning of this century. Recently, with the development of protein engineering and DNA technology, large quantities of highly purified protein, with desired sequences, have been synthesized for medical application. However, the rapid progress in molecular biology has not yet been matched by suitable formulation and development of protein drug delivery systems. The physicochemical and biological properties of proteins, as well as the influence of these properties on their administration routes and delivery systems, have hindered development of these drugs. The characteristics associated with protein drugs, including molecular size, short plasma half-life, requirements for specialized mechanisms for transport across biological membranes, susceptibility to breakdown in both physical and biological environments, tendency to undergo self-association, complex feedback control mechanisms, and peculiar dose-response relationships (Lee et al., 1990a), have led to the need to explore suitable routes for protein drug administration and develop new systems for delivery of protein drugs.

A variety of routes have been explored to deliver protein and peptide drugs to the systemic circulation. Among these, parenteral administration is the most common. The obvious drawback of this delivery route is patient inconvenience. Recently, some non-oral mucosal routes, such as nasal, buccal, rectal, vaginal, ocular and pulmonary, have been

examined as alternative routes to injection. Some of these have shown certain potential for peptide and protein absorption. However, most have significant limitations.

The oral route represents the most convenient and acceptable route of administration for any drug. Due to the hostile environment of the gastro-intestinal tract towards protein and peptide drugs, however, oral administration of these agents appears to give the least bioavailability as compared to other mucosal routes. A number of experimental approaches have been attempted to improve the oral absorption of peptide and protein drugs. One type is chemical modification of the protein to make it less sensitive to pre-systemic metabolism and more permeable to the gastrointestinal epithelial membrane. But creating a new chemical entity increases the possibility of additional and potentially undesirable biological effects. Other types of experimental approaches include formulation modification, the use of enzyme inhibitors and various penetration enhancers. The disadvantages of these types of systems are high variability, low bioavailability and damage to the tissue.

Emisphere's non-covalent protein drug delivery system is aimed at producing more transportable forms of proteins by modifying the three dimensional structure of the protein molecules. It has the potential to incorporate the advantage of traditional experimental approaches of protein drug delivery systems while minimizing their disadvantages. However, this technology is still at a relatively early stage. Many fundamental studies have not been completely clarified. This includes cellular and subcellular level studies on how the carrier/protein complex is absorbed and effluxed through the cell lipid bilayer and the

subcellular organelles, as well as studies on how the carrier enhancing mechanism is different than that of the traditional penetration enhancers. In addition, there is a lack of direct evidence to support the proposed mechanisms suggested by previous studies. Therefore, it is necessary to gain a more fundamental understanding of this technology, which in turn, will provide information for the design of new carriers.

II. Overall Plan of Research

1. Classical penetration enhancers function by compromising the integrity of the biological membrane and thus increase drug absorption by damaging the tissue. On the contrary, preliminary *in vivo* studies suggest that Emisphere's carriers cause no obvious damage to tissue while enhancing protein absorption. The first part of this project is an overall evaluation of carrier cytotoxicity, using a series of toxicity assays. The carrier effect on the integrity of cell membranes, subcellular organelle (lysosome), and paracellular tight junctions will be examined quantitatively. Evidence obtained from high resolution imaging will be used to assess the carrier effect on cell nucleus damage. This series of cytotoxicity studies will serve as a foundation for further mechanistic studies, to provide a basis of evidence to distinguish the mechanism of carrier enhanced protein absorption from the enhancement mechanism of classical penetration enhancers.
2. It is suggested that the carriers enhance protein absorption by interacting with the protein to produce a more transportable form of the protein. Unfortunately, due to the intrinsic properties of the carriers, many analytical methods that have been used to characterize

protein and small molecule interactions are not applicable in studying this system. Therefore, the second part of this project intends to elucidate the mechanisms of this carrier enhanced protein absorption, from biological point of view, to indirectly support the existence of a more hydrophobic form of protein upon interacting with carriers. Specifically, the enhancing efficiency of various carriers towards protein absorption will be investigated by transport studies. The carrier concentration dependency towards protein absorption and the kinetic uptake of proteins in the presence and absence of carriers will be quantified by flow cytometry measurements. Confocal laser scanning microscopy is to be utilized to follow the uptake/transport pathways and subcellular localization of proteins. These quantitative measurements, as well as direct microscopy, will be coupled with cytotoxicity results obtained from the first part of the study, to address the underlying mechanisms of carrier enhanced protein absorption.

3. The epithelial and vascular endothelial membranes represent two main barriers in the delivery of protein drugs from the site of administration to the site of action. The permeation properties of the carrier enhanced protein absorption system on these two distinct barriers has not yet been explored. The third part of this project is to quantitatively measure the uptake and efflux of human growth hormone (hGH) enhanced by carriers, using an epithelial cell model (Caco-2 cell) and an endothelial cell model (CAPE cell). By comparing the mechanisms of carrier enhanced protein absorption on these two cell lines, which possess distinct uptake/efflux features, and further relating these findings to the previous transport studies, we should obtain mechanistic insight, as

well as a clear understanding of cellular uptake and subcellular trafficking of the carrier enhanced protein absorption system.

CHAPTER 3: EXPERIMENTAL OPTIMIZATION

I. Experimental

A. Materials

Caco-2 cell line (passage 17) and CAPE cell line (passage 15) were obtained from American Tissue Culture Collection, Rockville, MD. Dulbecco's Modified Eagle's medium (DMEM) containing 4.5g/L glucose and 1% L-glutamine, heat-inactivated fetal bovine serum (FBS), non-essential amino acids, penicillin and streptomycin mixture, gentamicin, and trypsin-EDTA were obtained from Gibco, Grand Island, NY. Krebs-Ringer Buffer (KRB), Type I collagen (from rat tail) and Type A gelatin (from porcine skin) were obtained from Sigma Chemical, St. Louis, MO. Glacial acetic acid was obtained from Fischer Scientific, Pittsburgh, PA. Snapwell® plates with insert (0.4 μm pore size) were obtained from Costar, Cambridge, MA. The side-by-side diffusion chambers, gas manifold and block heater were obtained from Precision Instrument Design, Los Altos, CA. [^{125}I] human growth hormone (100-102 μCi/μg) was purchased from NEN DuPont Research Products, Boston, MA. N-[8-(2-hydroxybenzoyl)amino] caprylic acid (carrier E414, Figure 3-1) was kindly supplied by Emisphere Technologies, Inc. Tarrytown, NY.

B. Preparation of Transwell Filters

a). Coating transwell filters with collagen

Dilute acetic acid (0.1%) was prepared by diluting glacial acetic acid 1000 times with sterile water. Twenty five mg of collagen was dissolved in dilute acetic acid solution to make a 0.1 mg/ml collagen solution and stored at 2-8 °C. One day before seeding cells, 0.1ml of the above collagen solution plus 0.4 ml sterilized methanol were added to the upper chambers of the transwell inserts. The inserts were dried overnight in a hood and used within 24 hours after coating.

b). Coating transwell filters with gelatin

The transwell inserts were washed with 0.5% acetic acid solution at 50 °C for 20 minutes and rinsed twice with distilled water. The inserts were then placed in a beaker containing gelatin solution (5 mg/l) at 100 °C for 1 hour, dried overnight in a hood and heated in an oven (100°C) for 1 hour. The inserts were used within 24 hours after coating.

C. Cell Cultures

Caco-2 cells, a human colon carcinoma cell line, were maintained in DMEM containing 10% FBS, 1% non-essential amino acids, 100U/ml penicillin and 100μg/ml streptomycin. Cells of passage number 25-60 were used. The cells were maintained in tissue culture flasks at 37°C in 90% relative humidity and 5% CO_2. Trypsinization of cells was done by first washing the cells twice with phosphate-buffered saline (PBS), then treating cells with 0.5% trypsin plus 0.53 mM EDTA.

CAPE cells, a pulmonary artery endothelial cell line, were grown in the same growth medium as Caco-2 cells except the FBS concentration in the growth medium was 20% instead of 10%. Cells of passage number 16-23 were used. Cells were maintained and cultured the same way as for Caco-2 cells described above.

D. Diffusion Apparatus

The diffusion chambers used in the experiments consisted of two acrylic half cells representing a donor and a receiver chamber (Figure 3-2), each with a volume of 6 ml surrounded by an external heating/cooling block, to maintain the cells and solutions at a constant temperature. The temperature of all experiments was maintained at 37°C. The cell monolayers, grown on polycarbonate inserts, were placed into the diffusion cell openings between two O-rings. The two half cells were joined together by means of ring clamps to prevent leakage. The bathing solutions were continually gassed with 95% O_2 plus 5% CO_2 to mix the solution and aerate the cells.

E. Transport Studies

Cell suspensions were prepared at a concentration of 2×10^5 cells/ml and 0.5 ml of cell suspension was placed in the upper chamber of polycarbonate membrane Snapwell® culture plate inserts pretreated with collagen or gelatin. The lower chambers of the culture plates were filled with 2 ml of cell growth medium. The culture medium was changed every other

day to maintain cell growth. When conducting transport experiments, the inserts containing cell monolayers were placed in the diffusion chambers. Five ml of transport medium was placed in each donor and receiver compartment. Mixing and aerating in the diffusion cell was achieved by a 5% CO_2/95% O_2 airlift. Temperature was maintained at 37°C by a water-heated jacket. The top of the diffusion chambers was covered with parafilm to avoid loss of transport medium due to evaporation. One ml of sample was taken from the receiver side every 20 minutes and replaced with an equal volume of transport medium. Samples were placed in plastic liquid scintillation vials and 8 ml of scintillation cocktail was added (BioSafe II, Research Products International, Mount Prospect, IL). These samples were counted for total counts per minute (cpm) on a Beckman LSC 6000IC (Beckman Instruments, Arlington Heights, IL). In order to determine the initial donor concentration, a 1 ml sample was taken from the solution at the beginning of each experiment, and counted for total cpm. The permeability coefficient (P) was calculated using the following equation:

$$P = \frac{V}{A \cdot C_o} \cdot \frac{dC}{dT} \qquad \text{Eq. 1}$$

Where dC/dt is the flux across the monolayer, V is the volume of the chamber (5 ml), A is the surface area (1 cm^2), and C_0 is the initial concentration in the donor compartment.

F. Transepithelial Electrical Resistance (TEER) Measurement

Cells were seeded on the transwell inserts at a density of 2×10^5 cells/ml as described earlier. At different time intervals, TEER value was measured by an EVOM epithelial voltohmmeter equipped with an adjustable tip spread "chopstick" electrode set (World Precision Instruments, Sarasota, FL) as shown in Figure 3-3. The resistance of a blank filter, i.e., without cell monolayer, was measured the same way. The actual TEER of the cell monolayer was calculated by subtracting the value for the blank filter from that with cells in place at each time point.

G. Statistical Analysis

All statistical analysis was done using student's t-test, through the computer program Minitab. Only p-values less than 0.05 are considered statistically significant.

II. Results

A. Effect of Varying Cell Postseeding Times on CAPE Cell Barrier Function towards hGH Transport

Human growth hormone permeability coefficient was measured on a CAPE cell monolayer with varying cell postseeding times and the results were compared with the one on the blank filter (Figure 3-4, 3-6). It is shown that in the absence of the carrier (Figure 3-4), hGH transport through the cell monolayer shows no statistical difference compared to the

one on the blank filter when CAPE cell were postseeded for less than 9 days. Only when cell postseeding time was increased to 14 days was there a statistically significant difference between hGH permeability from the cell monolayer and the one from the blank filter.

The presence of 37.5 mg/ml carrier can enhance hGH permeability on CAPE cells that were postseeded for 14 days (Figure 3-5). Due to this enhancement effect, the barrier function of CAPE cells towards hGH transport is diminished. Therefore, in the presence of 37.5 mg/ml carrier, no significant difference was observed between hGH permeability on the filter alone and the one on the cell monolayers with varying cell postseeding times, even for 14 days (Figure 3-6).

Table 3-1 summarizes the results of hGH permeability coefficient through CAPE cells with varying postseeding times.

B. Effect of Membrane Coating Materials on CAPE Cell Barrier Function towards hGH Transport

The transwell inserts were coated with two different materials, collagen and gelatin, and CAPE cells were seeded on the transwell inserts for 9 days. Human growth hormone permeability coefficient through cell monolayers that were grown on these matrixes were determined. As shown in Figure 3-7 and Table 3-2, there is no significant difference for hGH permeation through cells that were grown on these two different supporting materials.

C. TEER Development

CAPE cell TEER development with varying cell postseeding times was measured and the results were compared with Caco-2 cells (Figure 3-8). It is shown that the TEER for Caco-2 cell monolayers can achieve ~ 500 ohms cm^2 after postseeding for one week and reach ~ 800 ohms cm^2 at week three. However, under the same experimental condition, TEER of CAPE cells was maintained at around 10 to 20 ohms cm^2 during the entire experimental period.

III. Discussion

In general, a drug needs to overcome two main barriers in order to achieve its site of action: the epithelial barrier and the vascular endothelial barrier. Earlier studies in our lab have examined the carrier enhanced protein absorption system using Caco-2 cells as an epithelial cell model. However, the barrier function of the endothelial monolayer for enhanced protein absorption has not been investigated. Because the vascular endothelium is the first barrier to the movement of macromolecules from blood to the sites of action in tissues, passage through the endothelium may be a rate-limiting step in the distribution of drugs into extravascular spaces. To achieve a more complete understanding of carrier enhanced protein absorption through the vascular wall, we utilized an endothelial cell model,

which possesses different transport mechanisms compared with an epithelial cell model, to further investigate the underlying mechanisms of carrier enhanced protein absorption.

Bovine pulmonary artery endothelial cell line (CAPE cell) was chosen as our *in vitro* endothelial cell model. This cell line has been in use since the early 1980s for various studies, most of which have focused on permeability studies. It has been characterized as endothelial in origin by the presence of angiotensin-converting enzyme activity, factor VIII-related antigen and Weibel-Palade bodies. In addition, the cells are able to produce a large amount of fibronectin in the matrix as assessed by immunocytochemical techniques (Del Vecchio et al., 1981; 1983). The cells also form tight junction complexes when grown on filters, and the cell monolayers are able to restrict albumin permeability through intercellular junctions (Siflinger-Birnboim et al., 1987).

To validate the experiments, we examined the feasibility of using CAPE cells as an endothelial transport model under the experimental condition in our lab. We first conducted a series of experiments to examine the barrier function of CAPE cells toward hGH transport as a function of varying cell postseeding times. Cells were seeded on collagen treated transwell inserts in this case. The cell postseeding time started from 3 days, as the literature suggests (Siflinger-Birnboim et al., 1987; Taylor et al., 1981), and extended to 14 days. As revealed in Figure 3-4, in the absence of carrier, the permeability coefficients of hGH through CAPE cells that are postseeded for less than 9 days are not distinguishable from the one through the blank filter based on statistical analysis with a 95% confidential level. In

other words, CAPE cells do not present a barrier function to hGH transport when postseeded for 9 days or less. However, when the cell postseeding time is prolonged to 14 days, we observe a significant difference between hGH permeability on the blank filter and that through cell monolayers, with hGH permeability coefficient through the blank filter about 2 times higher than that through the cell monolayer. Longer time incubation of CAPE cells in this experimental condition (21 days), resulted in a peeling off of cells from the filter membranes.

Human growth hormone transport through Caco-2 cells was also examined. Table 3-3 lists hGH permeability coefficient through CAPE cells, Caco-2 cells, and different regions of excised rabbit intestinal tissues. It can be seen that the Caco-2 cell monolayer, with a hGH permeability coefficient of 8.63×10^{-7} cm^2/sec, represents the tightest barrier for hGH transport among the above three categories. The permeability coefficient of hGH in different regions of excised tissues, varied from 1.02×10^{-6} cm^2/sec for the lower duodenum to 4.59×10^{-6} cm^2/sec for the upper colon, is about 1.2 to 5 times higher than hGH permeability in Caco-2 cells, indicating that the excised tissues are more leaky than the well developed *in vitro* Caco-2 cell monolayer in terms of macromolecular transport. The permeability coefficient of hGH through CAPE cells is the highest among these three categories, suggesting that the endothelial monolayer is very permeable toward macromolecules such as hGH.

Siflinger-Birnboim (Siflinger-Birnboimet al., 1987) examined the selectivity of the CAPE cells to different molecular weight molecules ranging from 182 to 340,000 Dalton. It was found that the cultured endothelial monolayer is a selective barrier to molecules with different size. The permeability coefficient of cytochrome C (MW 12, 000 Dalton) is 4.4×10^{-5} cm^2/sec and α-thrombin (MW 36, 600 Dalton) is 1.73×10^{-5} cm^2/sec. With a MW of 21,500 Dalton, hGH permeability coefficient measured in our study, 1.76×10^{-5} cm^2/sec, fits well into the range of their measurement.

We further studied the enhancement effect of carriers towards hGH transport, using 14 days postseeded CAPE cells. As shown in Figure 3-5, a slight enhancement in hGH transport, in the presence of 37.5 mg/ml of carrier E414, was observed. However, this enhancement is very limited in that the permeability coefficient of hGH, in the presence of carriers, is only 1.2 times higher than the one without carrier, although statistical analysis shows that this difference is significant with $P < 0.05$.

The barrier function of CAPE cells towards hGH transport, as a function of cell postseeding times in the presence of carrier, was also examined (Figure 3-6). It is shown that in the presence of 37.5 mg/ml carrier, even when cells were postseeded for 14 days, we do not observe a distinct difference between hGH transport on the cell monolayer and the one on the filter alone. This indicates that because of the limited barrier function of CAPE cells to hGH, as well as the enhancement effect of carrier, hGH permeability on CAPE cells in the presence of carrier has achieved an upper limit that is equal to the one on the blank filter.

Using the same cell line, but postseeded for only 3-7 days, Siflinger-Birnboim (Siflinger-Birnboim et al., 1986) observed that the flux across gelatinized filters alone was significantly different (about 8 times higher) than the fluxes across the endothelium monolayer. Our observation was a two-fold difference in hGH transport on filter alone and on cell monolayers when cells were postseeded for longer periods of time, i. e., 14 days. This difference, between their results and ours, could be explained by the different experimental details. One possibility is that the substrates of these two studies are different. The permeant that they studied was albumin. It has about three times higher molecular weight than the hGH used in our study, therefore, it is more selective. The other possibility may lay in the different experimental conditions. In our case, cells were seeded on collagen coated transwell filters whereas in their experiment, the cells were grown on gelatinized filters.

It was also suggested that basement membrane components appear to play an important role in the permeability of macromolecules (Del Vecchio et al., 1987). To check if an improved cell adhesiveness can be achieved by changing to different bedding materials, CAPE cells that were seeded on gelatinized transwell inserts and hGH transport on CAPE monolayer under this experimental condition was measured (Figure 3-7, Table 3-2). It is revealed that the permeability of hGH shows no difference on cells that were seeded using two different matrix materials, both in the presence and absence of carrier, indicating that these two matrix materials have no effect on the development of CAPE cell barrier function.

Transepithelial electrical resistance, TEER, has been commonly used to evaluate epithelial tightness. Endothelial cells usually have less tight paracellular junction complexes compared with epithelial cells. Brain endothelial cell monolayer is commonly known to represent tight paracellular junctions among all other endothelial monolayers. It has reported TEER value ranges from 140 – 160 Ω cm^2 (Bowman et al., 1983). Endothelial cells derived from retinal capillaries have been used for blood-retinal barrier studies, and the average TEER in these cell monolayers has been reported to range from 129 to 140 Ω cm^2 (Gardner et al., 1997; Gillies et al., 1995a; Gillies et al., 1995b). The mean TEER of endothelial cells from bovine adrenal microvessels was 69 Ω cm^2 when the cells were cultured on human amniotic membrane for eight days (Furie et al., 1987). The TEER value for aortic endothelial cells from bovine and rabbit tissues range from 14-20 Ω cm^2 (Albelda et al., 1988). As demonstrated by Figure 3-8, our results showed that the TEER of CAPE cells was maintained low throughout the four weeks of the experimental period, i. e., 11Ω cm^2 at day seven and 24 Ω cm^2 at day twenty, which is in agreement with literature values. On the contrary, the tightness of Caco-2 cells developed rapidly so that its TEER value achieved 500 Ω cm^2 in the first week of incubation and increased to 800 Ω cm^2 at week three. This result confirms that the Caco-2 monolayer is a tighter barrier for macromolecular transport whereas a CAPE cell monolayer is much more "leaky".

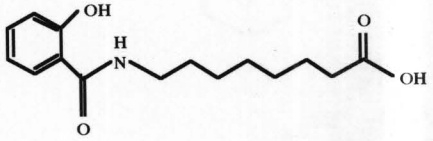
The reason why a CAPE cell is much more permeable than a Caco-2 cell in hGH transport could be accounted for by structural differences between endothelial and epithelial

cells. Endothelial cells are enriched by pinocytotic vesicles (also named caveolae, plasmalemmal vesicles or transport vesicles) (Figure 3-9). These vesicles have internal radii of ~ 250Å and are considered to be responsible for the uptake of macromolecules by pinocytosis. Experiments with macromolecular tracers with molecular diameters ranging from ~ 30nm to ~ 2nm demonstrate that molecules of this size pass through the continuous endothelium mainly via vesicles and channels (Simionescu et al., 1971;1972;1975;1978). A unique feature of an endothelial cell that makes it differ from an epithelial cell is that it possesses transendothelial channels (Renkin, 1977; 1985; Simionescu et al., 1984). Transendothelial channels are formed by one or more vesicles that open simultaneously on both sides of the endothelium (Figure 3-10). The maximum internal diameter of these transient channels approach that of vesicles, i.e., 500 Å, yet the channels have strictures at their necks and at the points of fusion between vesicles that reduce the internal diameter to 100-400 Å (Tayloret al., 1984). Macromolecules that transport through these channels bypass the lysosomal compartment. It is very likely that hGH, with a size of ~ 5nm in diameter, could be transported via the vesicles and transendothelial channels through CAPE cells.

Our preliminary studies using CAPE cell as an endothelial cell model have led us to the following conclusions: 1) The endothelial monolayer is more permeable than the epithelial monolayer; 2) The barrier function of the *in vitro* endothelial monolayer for macromolecule transport, like hGH, is limited; 3) The carrier can enhance hGH permeability, but this enhancement is restricted by an upper limit of the barrier function of CAPE

monolayer, i.e., in the presence of the carrier, the permeability coefficient of hGH on CAPE monolayer can be as high as the blank filter.

Since there is a upper limit for hGH transport in the presence of the carrier, and this limitation will have a restriction on further mechanistic studies of carrier enhanced protein absorption system, we later switched our focus to the uptake of hGH, rather than transport of hGH, for the further exploration of carrier enhanced protein absorption technology.



N-[8-(2-hydroxybenzoyl)amino]caprylic acid

Figure 3-1: Chemical structures of carrier E414.

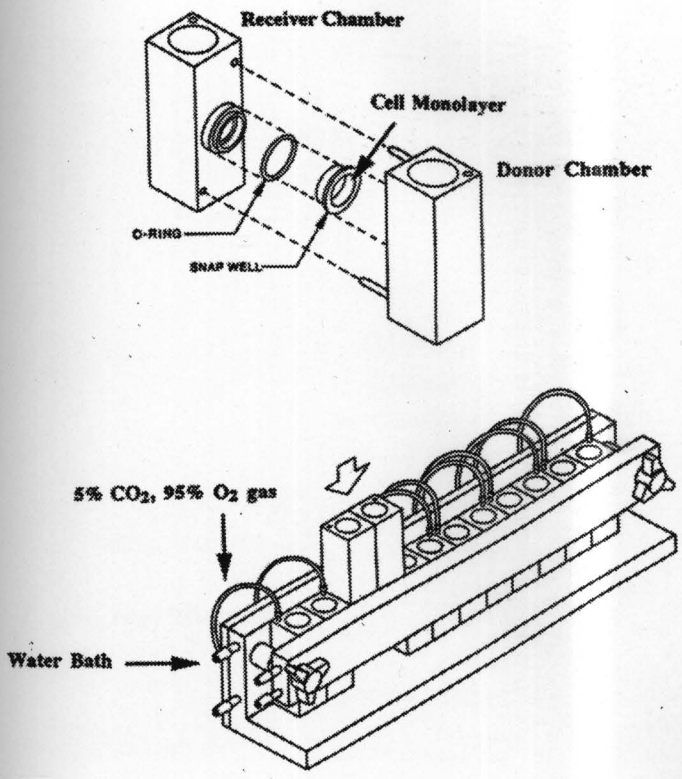


Figure 3-2: Diagram of acrylic diffusion chamber and insert used for *in vitro* permeability experiments. Reproduced from Costar Advertisement.

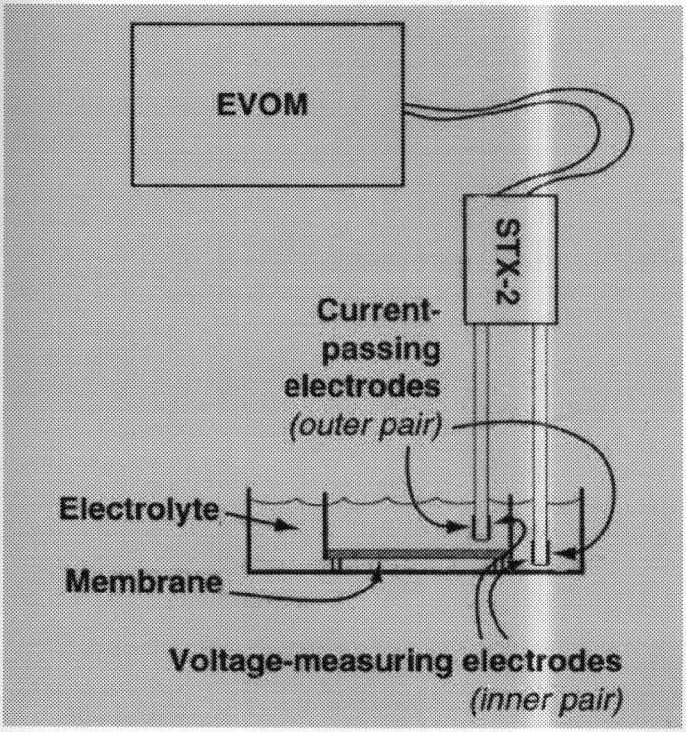


Figure 3-3: Cartoon depiction of EVOM epithelial voltohmmeter for transepithelial electrical resistance measurement. Copied from EVOM user's handbook.

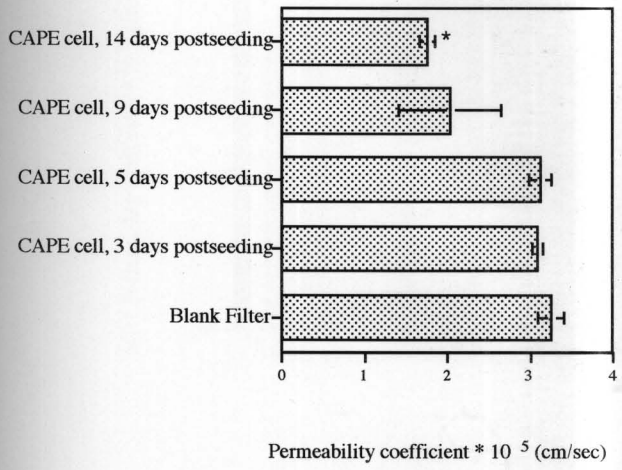


Figure 3-4: Human growth hormone permeability coefficient measured through blank filter and CAPE cells with different postseeding times. Experiments were conducted in the absence of carriers at 37°C (error bars indicate SEM, n=3-6, * denotes statistical significance of $p<0.05$ compared to blank filter).

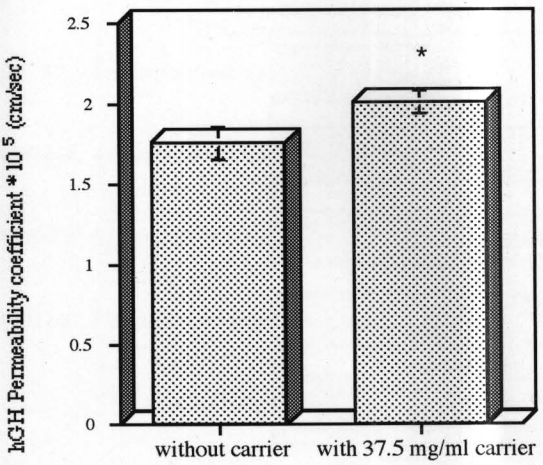


Figure 3-5: Human growth hormone permeability coefficient measured CAPE cells in the absence and presence of 37.5 mg/ml carrier. Cells were postseeded for 14 days. Experiments were conducted at 37°C (error bars indicate SEM, n=3-6, * denotes statistical significance of $p<0.05$).

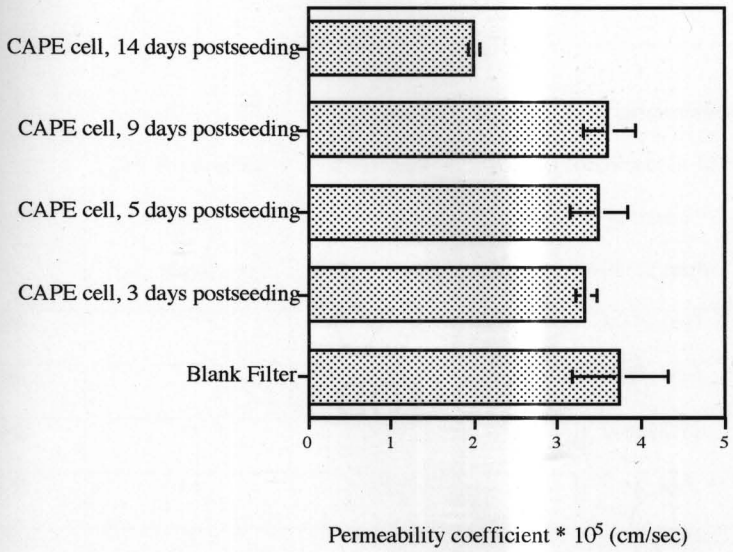


Figure 3-6: Human growth hormone permeability coefficient measured through blank filter and CAPE cells with different postseeding times. Experiments were conducted in the presence of 37.5 mg/ml carriers at 37°C (error bars indicate SEM, n=3-6, no statistical significance was seen).

	Cell Postseeding time (days)	hGH permeability coefficient ($\times 10^{-5}$) (cm/sec) With 37.5 mg/ml carrier	hGH permeability coefficient ($\times 10^{-5}$) (cm/sec) Without carrier
CAPE	3	3.34 ± 0.13	3.09 ± 0.07
CAPE	5	3.49 ± 0.35	3.13 ± 0.14
CAPE	9	3.62 ± 0.32	2.03 ± 0.63
CAPE	14	2.01 ± 0.08	1.76 ± 0.10 *, **
Blank Filter	0	3.74 ± 0.57	3.25 ± 0.15

* denotes statistical significance of $P< 0.05$ as compared to the result on blank filter;

** denotes statistical significance of $P< 0.05$ as compared to the result of the one with carrier treatment.

Table 3-1: Human growth hormone permeability coefficient measured under different experimental conditions. CAPE cells were seeded on collagen coated polycarbonate filter membranes, data show the permeability coefficient of hGH from luminal to abluminal direction.

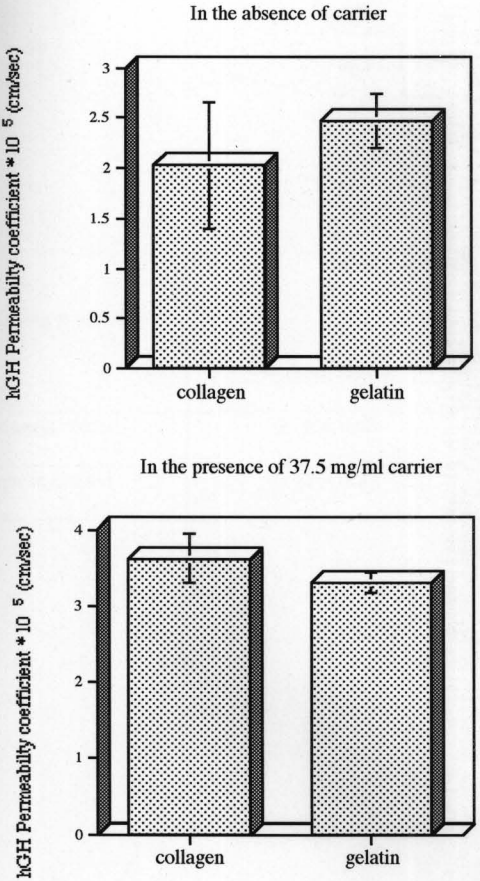


Figure 3-7: Human growth hormone permeability coefficient on CAPE cells grown on different supporting materials. Cells were postseeded for 9 days. Experiments were conducted at 37°C (error bars indicate SEM, n=3-6, no statistical significance was seen).

Supporting materials	hGH permeability coefficient ($\times 10^{-5}$) (cm/sec)	
	Without carrier	With 37.5 mg/ml of carrier
Collagen coated	2.03 ± 0.63	3.62 ± 0.32
Gelatin coated	2.47 ± 0.27	3.31 ± 0.13

Table 3-2: Comparison of human growth hormone permeability coefficient on CAPE cells grown on different supporting materials. Permeability coefficient was measured from luminal to abluminal direction in the presence and absence of carrier. Cells were postseeded for 9 days. Experiments were conducted at 37°C.

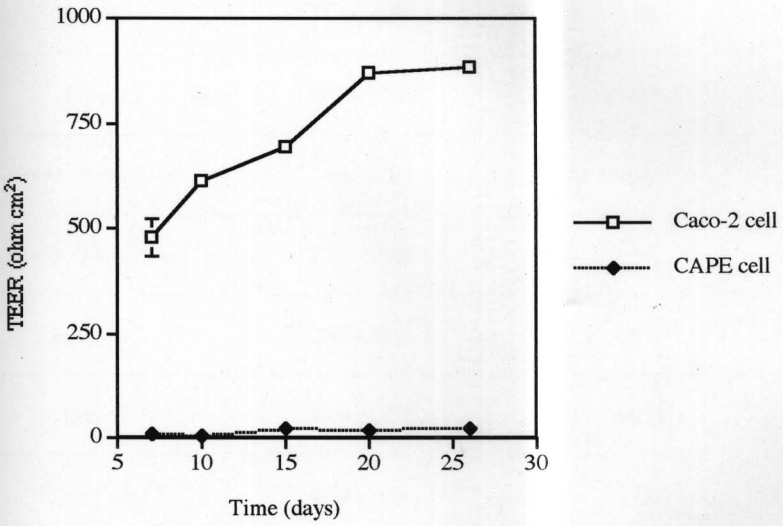


Figure 3-8: Transepithelial electrical resistance time profile of Caco-2 and CAPE cells (bars indicate SEM, n=3-6).

	hGH Permeability Coefficient at pH 7.4 ($\times 10^{-6}$, cm/sec)	Extraction Factor [a]
Blank filter	32.5 ± 1.53	----
CAPE	17.6 ± 3.33	1.85
Caco-2	0.863 ± 0.0263	37.66
Upper Duodenum [b]	1.64 ± 0.32	19.8
Lower Duodenum [b]	1.02 ± 0.18	31.9
Jejunum [b]	2.86 ± 0.28	11.36
Ileum [b]	3.06 ± 0.25	10.62
Upper Colon [b]	4.59 ± 0.84	7.08
Lower Colon [b]	4.13 ± 0.19	7.87

[a] Extraction factor is calculated by dividing the permeability coefficient on blank filter by the one on cell monolayer;

[b] Data obtained from reference (Mlynek, 1995).

Table 3-3: Comparison of human growth hormone permeability coefficient (± S. E. M.) across different biological barriers. Each value was an average of 3-6 repetitions.

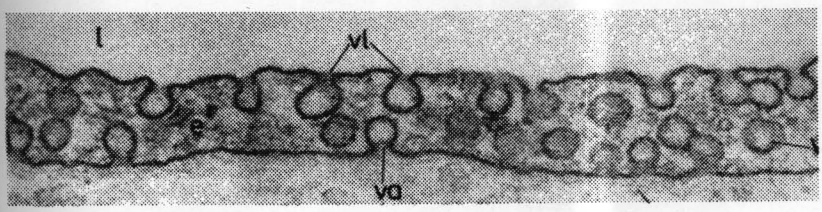


Figure 3-9: Capillary endothelium of continuous type (rat diaphragm). Flattened endothelial cell (e) contains numerous plasmalemmal vesicles open on cell fronts (vl, va) or apparently free in cytoplasm. l, lumen. × 95, 000. Modified from reference (Simionescuet al., 1984).

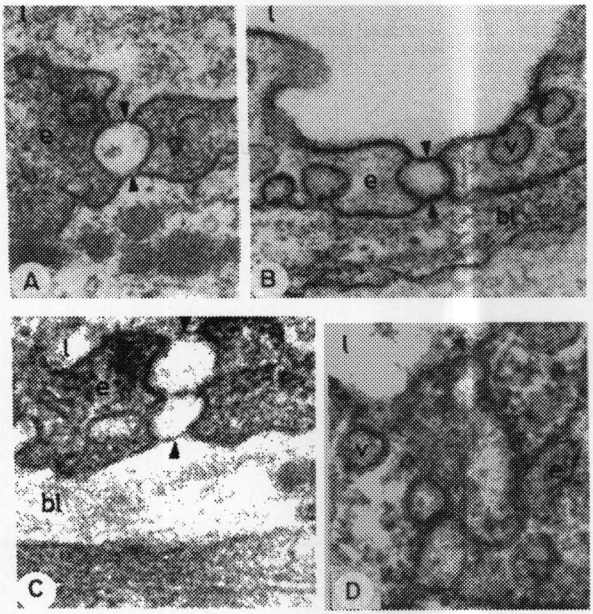


Figure 3-10: Microvascular endothelium. Gallery of transendothelial channels (arrowheads) made up of a single vesicle (A and B), 2 fused vesicles (C), or a chain of several vesicles (D). bl, Basal lamina; e, endothelium; l, lumen; v, vesicle. ×100, 000. Modified from reference (Simionescu et al., 1984).

CHAPTER 4: EVALUATION OF CARRIER CYTOTOXICITY USING VARIOUS TOXICITY INDICATORS

I. Experimental

A. Materials

In addition to the materials mentioned in the previous chapter, the following materials were used for this section: trypan blue solution, and neutral red were obtained from Gibco, Grand Island, NY. Propidium iodide and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical, St. Louis, MO. Human growth hormone was kindly supplied by Emisphere Technologies Inc., Tarrytown, NY.

B. Cell Culture

Caco-2 cells were cultured the same way as described in Chapter 3. Unless otherwise stated, cells were seeded on the collagen-treated transwell cell culture inserts (0.4 μm pore size) (Costar, Cambridge, MA.) at a density of 2×10^5 cells/ml, 0.5 ml each well, and allowed to grow for 3-4 weeks before experiments. The culture medium was changed every other day.

C. Trypan Blue Exclusion Test

Caco-2 cell monolayers grown on transwell cell culture inserts were washed with PBS. The apical chamber of the cell monolayers was treated with KRB, or KRB containing

carriers, or 0.1% (w/v) SDS. The basolateral chamber was filled with 2 ml of KRB. At different time intervals, the medium in the apical chamber was blotted, and 100 μL of trypan blue solution (0.4%, w/v) was added. Cell monolayers were incubated with trypan blue for 1-2 minutes and then gently washed three times with PBS. The monolayer with the filter was then carefully removed from the transwell insert. Trypan blue was extracted from the cell monolayer by adding 300 μL of a mixture of (1:1, v/v) acetone – Na_2SO_4 solution (0.5%, w/v), sonicated for 5 minutes and then left at room temperature overnight. Two hundred μL of supernatant was transferred to a 96 well plate and absorption measured at 610 nm with a microplate reader (Molecular Devices Corporation, Menlo Park, CA).

For recovery experiments, cells were treated with KRB, or with different concentrations of carrier for 60 minutes. The cell monolayers were washed with PBS three times. Cells were then incubated with a regular medium for 24 hours for recovery and membrane damage was tested according to the method described above.

D. Neutral Red Assay

After cell monolayers were treated with different testing solutions for different time intervals, the apical chamber and basolateral chamber were replaced by 0.5 ml neutral red (50 μg/ml) and 2 ml of KRB, respectively. Cells were further incubated overnight in an incubator. Cell monolayers were then fixed with 0.5 ml formaldehyde solution (1%, v/v) containing $CaCl_2$ (1%, w/v) on the apical side for 3 minutes. The cell monolayer with filter

was gently removed from the transwell insert and neutral red was extracted from the cell monolayer by adding 300 μL of a 1% (v/v) acetic acid solution containing 50% ethanol. Cells were left overnight at room temperature. Two hundred μL of supernatant was transferred to a 96 well plate and absorption measured at 490 nm. For recovery experiments, cells were treated the same way as in the trypan blue recovery experiments, and the neutral red assay was performed as described above.

E. DNA-Propidium Iodide Staining Test

A Biorad 1024 with Ar/Kr laser system (Bio-Rad Laboratories, Richmond, CA) linked to a Nikon Diaphot 200 inverted microscope (Fryer Company Inc. , Huntley, IL) was used. Images were taken with a Nikon Plan Apo *60 or *20 objective. The propidium iodide images were collected via the 605DF32 filter blocks (excitation at 568 nm). The images were means of nine scans. Autofluorescence of the Caco-2 monolayers was invisible at the settings used for imaging.

Cell growth medium was changed to a phenol free medium the day before the experiments. Medium on the apical side of the chamber was replaced by 0.5 ml of testing solution and cells were treated for different time intervals. Cells were washed with PBS three times, incubated with 0.5 ml of propidium iodide solution (50 μg/ml) on the apical side for 5 minutes, and then washed with cold PBS three times. Cell monolayers were removed, together with the filter membranes, carefully "sandwiched" between two No. 1 microscope

cover glasses (Fisher Scientific, Pittsburgh, PA), and mounted on the microscope stage. Images were taken within 20 minutes after positioning.

F. Transepithelial Electrical Resistance (TEER) Measurement

TEER values were measured by an EVOM epithelial voltohmmeter equipped with an adjustable tip spread "chopstick" electrode set (World Precision Instruments, Sarasota, FL) as shown in Figure 3-3. Caco-2 cell monolayers were grown on transwell inserts for 3-4 weeks. Immediately before the experiment started, the TEER of the cell monolayer, together with the filter, was measured. This reading was regarded as the initial value. Then cell monolayers were washed with PBS three times. Solution in the apical chamber was replaced with 0.5 ml testing solution whereas the solution on the basolateral side was replaced with 2 ml of KRB. TEERs were measured at different time intervals. To study recovery of the TEER, after treating cells with testing solutions for different time intervals, cell monolayers were washed with PBS three times. The apical and basolateral solutions were replaced with 0.5 ml and 2 ml of regular growth medium, respectively. The resistance of cells was then measured at different time intervals for up to 24 hours. The resistance of a blank filter, i.e., without cell monolayer, was measured the same way. The actual TEER of the cell monolayer was calculated by subtracting the value for the blank filter from that with cells in place at each time point. The resistance was expressed as the percentage of the initial value, i.e., the TEER value at time t divided by TEER at time zero.

II. RESULTS

A. Cell Membrane Damage Evaluated by the Trypan Blue Exclusion Test

Plasma membrane damage was assessed by the trypan blue exclusion test. Viable cells that have intact cell membranes exclude trypan blue whereas cells with damaged membranes permit trypan blue penetration. Two concentrations of carrier, 25 mg/ml and 37.5 mg/ml carrier, which have been shown to significantly enhance hGH uptake (Figure 5-11 and 5-12), were chosen for the study. KRB treated cells were used as the negative control and 0.1% (w/v) SDS treated cells were chosen as the positive control.

As shown in Figure 4-1, when cells were treated for 30 minutes, both the 25 and 37.5 mg/ml carrier treated cells show low trypan blue uptake, and statistical analysis indicates there are no significant differences between the results of treated cells and that of control cells. When treatment time is prolonged to 60 minutes, an increase in trypan blue uptake was observed for both concentration treated cells, and the results are significantly different as compared to that of the KRB control. In both cases, SDS treated cells result in very high trypan blue uptake, indicating severe damage to cell membranes.

The recovery of cell membrane damage is shown in Figure 4-2. Treating cells with 25 mg/ml carrier for 60 minutes, followed by incubating cells with cell growth medium for 24 hours shows a complete recovery from cell membrane damage. But when cells were

treated with 37.5 mg/ml carrier for 60 minutes, cell membrane damage could not be recovered completely.

Table 4-1 summarizes the results of the trypan blue exclusion test.

B. Cell Lysosomal Compartment Damage Evaluated By Neutral Red Assay

Neutral red is a weakly cationic supravital dye. It can bind to the lysosomal matrix of viable cells. Incubating cells with toxic agents will reduce this binding (Babich et al., 1990). Figure 4-3 shows cellular uptake of neutral red into the lysosomal compartment after treatment with testing solutions. After 30 minutes treatment, 0.1% (w/v) SDS caused a significant reduction in neutral red uptake, whereas both 25mg/ml and 37.5 mg/ml carrier treated cells did not show a statistically significant difference compared to KRB control cells. However, when the treatment time increased to 60 minutes, neutral red uptake for cells that were treated with both concentrations of carrier were reduced. The results are statistically different from the KRB control result.

The recovery of lysosomal compartment damage is shown in Figure 4-4. Again, a full recovery of lysosomal function was observed for cells that were treated with 25 mg/ml carrier for 60 minutes, followed by 24 hours incubation with cell growth medium. For 37.5 mg/ml carrier treated cells, the uptake of neutral red was increased about 2 times as

compared to cell uptake before recovery, but there was incomplete recovery as compared to the KRB control.

Table 4-2 summarizes the results of the neutral red assay.

C. Cell Nuclear Membrane Damage Tested by DNA-Propidium Iodide Staining Assay

The effect of the carrier on cell nuclear integrity was evaluated by the DNA-propidium iodide staining test and the results were observed by Confocal Laser Scanning Microscopy (CLSM). Propidium iodide is a nuclear acid probe that fluoresces after interacting with DNA. It has access to DNA only if the cell nuclear membrane is compromised. Figure 4-5 to 4-13 show the confocal images of Caco-2 cell nuclear staining when cells were treated with different testing solutions. As demonstrated by the images with both the horizontal and vertical views, the negative control, KRB treated cells, did not show any fluorescence signal, suggesting that the cell nuclear membrane is completely intact (Figure 4-5). On the contrary, the positive control, 10 minutes treatment with 0.1% (w/v) SDS, cells showed substantial staining of the cell nucleus by propidium iodide (Figure 4-6). Almost all cell nuclei were stained. Sectional images on top, middle, and bottom show that the apical membranes of some cells have been damaged (Figure 4-7). With 15 minutes treatment by SDS (0.1%, w/v), cell nuclei have been ruptured (Figure 4-8, vertical view). Ten minutes treatment with 37.5 mg/ml E414 caused very limited cell nuclear staining (Figure 4-9). Overall, nucleus staining is less than 1%. When treatment time increased to

40 minutes, nuclear stains were increased (Figure 4-10, 4-11). But for most stains, the fluorescence signals are weak, indicating slight damage to the cell nucleus. Only a small number (around 5-10%) of cell nuclei showed an intense fluorescence signal, indicating that these cell nuclei are severely damaged. Figure 4-12 summarizes the results of propidium iodide staining test on cell nuclear damage.

As compared to 37.5mg/ml E414, 25 mg/ml E414 causes less damage to the cell nuclei as observed under confocal microscopy. A representative nuclear staining image of cells treated with 25mg/ml E414 for 40 minutes is shown on Figure 4-13.

D. Paracellular Tight Junction Damage Measured By Transepithelial Electrical Resistance (TEER) Value

Transepithelial electrical resistance is a indicative of the integrity between cells, e.g., paracellular junctions. A lowering of TEER value indicates an opening of cell tight junctions. It is well known that calcium is an essential ion for maintaining the tight junctions of cells, therefore depleting Ca^{2+} from the cell medium causes an opening of the tight junction. EDTA is a chelating agent that chelates with Ca^{2+}, therefore also causing an opening of tight junctions. The control experiment (Figure 4-14) shows that the TEER of cells dropped 50% when cells were incubated with KRB without Ca^{2+}, or EDTA without Ca^{2+} solution. KRB itself with Ca^{2+} caused a small drop in TEER, but the effect was so slight that 60 minutes incubation only resulted in a 10% drop in TEER (Figure 4-15). Adding hGH (0.5

μM) to KRB caused a slight change in TEER and it dropped the TEER of cell monolayers maximally to 20% (Figure 4-15). The carrier had a significant effect on reducing the cell TEER value (Figure 4-16) in a concentration dependent manner. Five minutes incubation with 10 mg/ml E414 caused a 25% drop of TEER. With 37.5 mg/ml E414, 5 minutes incubation resulted in a 50% drop of cell TEER. By 60 minutes, cell TEER had dropped to its lowest level.

Recovery experiments showed that treating cells with E414 25 mg/ml and 37.5 mg/ml for a short time, e.g., 15 min, or 30 min, followed by incubating cells in a regular growth medium (Figure 4-17, 4-18), TEER demonstrated a quick recovery in the first few hours, then a gradual recovery. By the end of 24 hours, both concentration treated cells reached 75%-80% TEER of their original level. Cells incubated with EDTA without Ca^{2+} also showed a similar phenomena. However, if the cells were treated for 60 minutes (Figure 4-19) with both concentrations of carrier then allowed to recover, TEERs only showed a slight tendency to recover during the 24 hours recovery period. By 24 hours, for the 25mg/ml E414 treated cells, TEER has recovered to about 50%, but for 37.5 mg/ml treated cells, TEER only recovered to 20% of its original level.

III. Discussion

An overall assessment of carrier cytotoxicity, including damage to the cell plasma membrane, lysosomal compartment, cell nuclear membrane, and paracellular tight junction

was evaluated using Caco-2 cells as an *in vitro* model. In all cases considered, no substantial damage to the cells was detected when treating cells with effective concentration of carriers (results about carrier effective concentrations are discussed in Chapter 5) for 30 min or less, i.e., no statistically significant differences were observed for the carrier treated cells and the KRB treated cells. However, an increase in cytotoxicity caused by carriers is observed when treating cells for long times (e.g., 60 min). This toxicity to cells is not only time dependent but also carrier concentration dependent. Sixty minutes treatment with 25 mg/ml E414 result in little, but statistically significant toxicity as compared to the negative control. However, this toxicity is fully reversible. Both cell membrane and lysosomal perturbation are completely recovered after 24 hours. Meanwhile, damage caused by high concentration of carrier (37.5 mg/ml E414) seems unequal in its effect towards the cell membrane and lysosomal compartment. The cell plasma membrane, which has direct contact with the treating solution, is perturbed more severely so that for 60 minutes treatment followed by 24 hours incubation, no obvious recovery occurs. However, the function of the lysosomal compartment, a subcellular organelle, partially recovers as indicated by an increase in neutral red uptake.

The carrier effect to the cell nucleus was directly demonstrated by images obtained from CLSM. As shown by the images, 10 minutes treatment with 37.5 mg/ml E414 results in negligible damage to the cell nuclei. A small amount of damage to the cell nucleus, when treating cells for 40 minutes, was observed. The same study, using 25 mg/ml E414,

demonstrated similar results, but the fluorescence signal was much less in terms of intensity and density.

The carrier effect on the paracellular junction is marginal. A drop in TEER was observed for cells that were treated with low (10mg/ml), medium (25 mg/ml), and high (37.5mg/ml) concentrations of carrier E414. Recovery experiments showed that treating cells with carrier for 30 minutes or less, TEER of cells fully recovers, whereas when treating cells for 60 minutes, the recovery is much slower. Differing from the plasma membrane and lysosomal membrane recovery experiments, where 25 mg/ml carrier E414 treated cells demonstrated a full recovery, the paracellular integrity recovery results showed that TEER was only partially recovered when cells were treated with 25 mg/ml E414 for 60 minutes followed by 24 hours incubation with regular growth medium. This is possible because paracellular junctions, being directly exposed to the testing solution, can be damaged to a more severe extent. Meanwhile, TEER, as an indicator of the integrity of the paracellular junction, is a very sensitive parameter. This can be reflected by the drop of TEER when cells are incubated with KRB, a buffer that mimics normal physiological condition.

Opening of tight junction is one of the indicators of involvement of the paracellular route. In fact, the paracellular pathway involvement in carrier enhanced hGH absorption has been studied extensively by earlier investigators. Using Caco-2 as a model, it has been shown that the permeability of a group of paracellular markers with different molecular weight can be enhanced significantly in the presence of carrier E414, which strongly suggests

that the tight junctions were loosened by the carrier (Wu, 1999a). However, results obtained from rabbit intestinal tissues showed no difference in the transport of mannitol, a commonly used paracellular marker, in the presence of another carrier E198 (Mlynek, 1995). This could be because Caco-2 cells are much more sensitive in terms of changes in tight junctions as compared with rabbit intestinal tissue. Or it could be possible that different carriers possess different mechanisms when exerting transport enhancement functions.

Although data from the Caco-2 cell model illustrates evidence of the opening of tight junctions caused by the carrier, as indicated by the drop of TEER values, the opening of the tight junction is believed not enough to accommodate hGH permeation. This is supported by the experimental evidence obtained from the D-series carriers. D-series carriers are polyethylene glycol (PEG) derivatives of E-series compounds (structure shown in Figure 5-13). They caused a similar drop in the Caco-2 cell TEER value (Figure 5-14), but did not show the enhancement effect on hGH permeation in Caco-2 cell (Figure 5-15). This ensures that the involvement of cell paracellular tight junctions was not the main cause for enhanced hGH absorption. In addition, taking into account the limited surface area of the paracellular junction versus the transcellular membrane, the amount of hGH transport through the paracellular pathway should be much less than that using the transcellular pathway.

It should be noted that direct exposure of isolated cells to such high concentration of carrier for such an extended period of time is not likely to mimic what occurs *in vivo*. It has been shown by histological examination of rat ileal mucosa that after 5 hours exposure to

carrier, no evidence of significant intestinal toxicity occurred. However, transmission electron microscopy demonstrated noticeable damage to a Caco-2 cell monolayer (Brayden et al., 1997). Actually, intact intestinal membranes are often found to be more resistant to cytotoxic effects than are cell culture models. This difference could be because the intact tissue has mechanisms for recovery from trauma, which may not be present in cell cultures. Factors such as the protective effect of mucus, rapid cell turnover, short transit time and dilution of toxic agents in the intestine, may be important in preventing substrate-induced membrane damage *in vivo*. For example, dioctyl sodium sulfosuccinate (docusate) is used as a stool softener at suggested doses of up to 200 mg, which if dissolved in a volume of gastrointestinal fluids of 250 ml would give a concentration of 1.8 mM. Although this concentration was cytotoxic to Caco-2 cells (Anderberg et al., 1992), docusate has few side effects in clinical practice. It has been reported that some other compounds, such as sodium caprate and polysorbate, also display higher toxicity in Caco-2 cells than in whole animal tissue models (Anderberg et al., 1994).

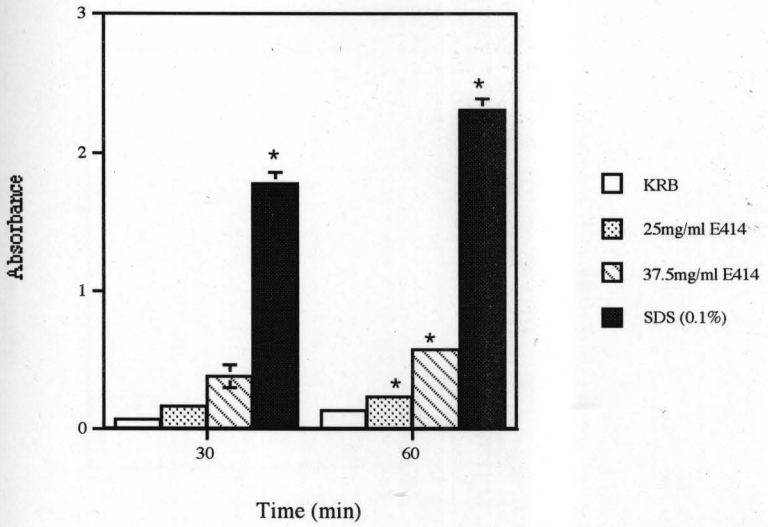


Figure 4-1: The effect of different reagents on integrity of the Caco-2 cell membrane evaluated by trypan blue exclusion (error bars indicate SEM, n=3-6, * denotes statistical significance of $p<0.05$ compared to control).

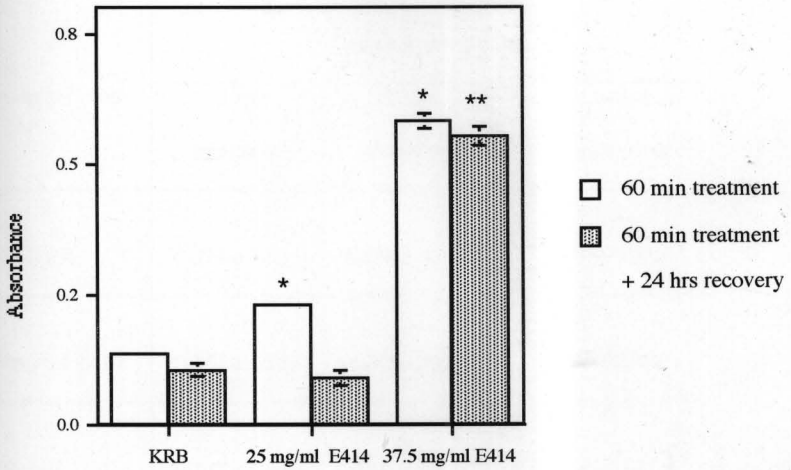


Figure 4-2: Comparison of the membrane damaging effect caused by 60 minutes carrier treatment and 60 minutes treatment followed by 24 hours recovery (error bars indicate SEM, n=3-6, * denotes statistic significance of $p<0.05$ compared to control before recovery, ** denotes statistical significance of $p<0.05$ compared to control after recovery).

Testing solutions	Absorbance at 610 nm		
	30 min treatment	60 min treatment	60 min treatment + 24 hrs recovery
KRB	0.074 ± 0.035	0.139 ± 0.005	0.108 ± 0.012
25 mg/ml E414	0.164 ± 0.033	0.231± 0.009 *	0.092± 0.014
37.5 mg/ml E414	0.387 ± 0.079	0.583 ± 0.014 *	0.555 ± 0.017 *
0.1% SDS (w/v)	1.778 ± 0.077 *	2.309 ± 0.082 *	NA

Table 4-1: Values of trypan blue absorbance in Caco-2 cell monolayers measured at 610 nm (values are average of 3-6 determinations. * denotes statistical significance of $p<0.05$ compared to KRB control).

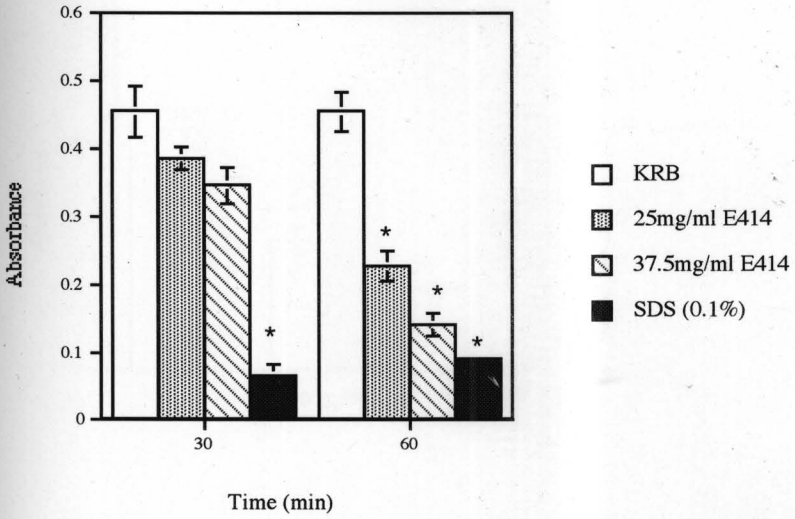


Figure 4-3: The effect of different reagents on integrity of the Caco-2 cell lysosomal compartment evaluated by neutral red assay (error bars indicate SEM, n=3-6, * denotes statistical significance of $p<0.05$ compared to KRB control).

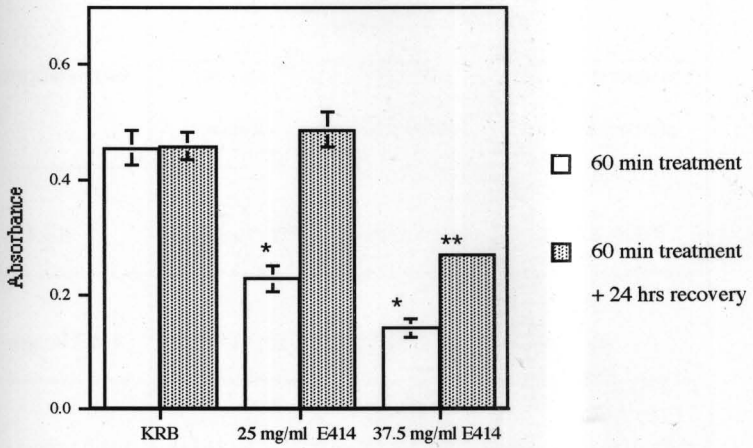


Figure 4-4: Comparison of the lysosomal compartment damaging effect caused by 60 minutes carrier treatment and 60 minutes treatment followed by 24 hours recovery (error bars indicate SEM, n=3-6, * denotes statistic significance of $p<0.05$ compared to control before recovery, ** denotes statistical significance of $p<0.05$ compared to control after recovery).

Testing solutions	Absorbance at 490 nm		
	30 min treatment	60 min treatment	60 min treatment + 24 hrs recovery
KRB	0.455 ± 0.038	0.455 ± 0.030	0.459 ± 0.025
25 mg/ml E414	0.387 ± 0.017	0.228± 0.022 *	0.487± 0.031
37.5 mg/ml E414	0.346 ± 0.026	0.141 ± 0.016 *	0.269 ± 0.002 *
0.1% SDS (w/v)	0.065 ± 0.016 *	0.090 ± 0.008 *	NA

Table 4-2: Values of neutral red absorbance in Caco-2 cell monolayers measured at 490 nm (values are average of 3-6 determinations. * denotes statistical significance of $p<0.05$ compared to KRB control).

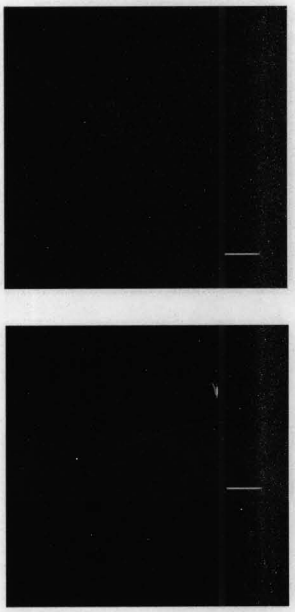


Figure 4-5: Confocal images of Caco-2 cell nuclear stain by propidium iodide assay. Cells were treated with KRB for 40 minutes. Upper: horizontal view (XY view) of cells; Lower: vertical view (XZ view) of cell. Bar length=20 μm. No propidium iodide stain was observed.

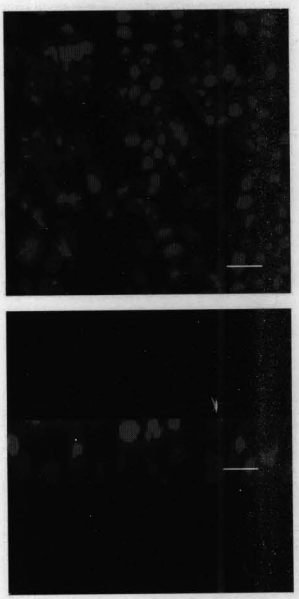


Figure 4-6: Confocal images of Caco-2 cell nuclear stain by propidium iodide assay. Cells were treated with SDS (0.1%, w/v) for 10 minutes, most cell nuclei are stained. Upper: horizontal view (XY view) of cells; Lower: vertical view (XZ view) of cell. Bar length=20 μm.

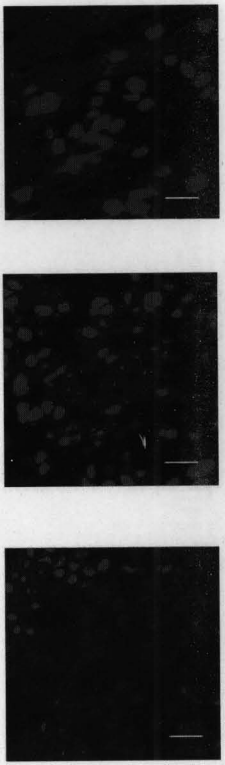


Figure 4-7: Sectional view of Confocal images of Caco-2 cell nuclear stain by propidium iodide assay. Cells were treated with SDS (0.1%, w/v) for 10 minutes, most cell nuclei are stained. Upper: top section of cells; Middle: middle section of cell; Lower: bottom section of cell. Bar length=20 μm.



Figure 4-8: Confocal images of Caco-2 cell nuclear stain by propidium iodide assay. Cells were treated with SDS (0.1%, w/v) for 15 minutes, most cell nuclei are stained. Upper: horizontal view (XY view) of cells; Lower: vertical view (XZ view) of cell. Bar length=20 µm.

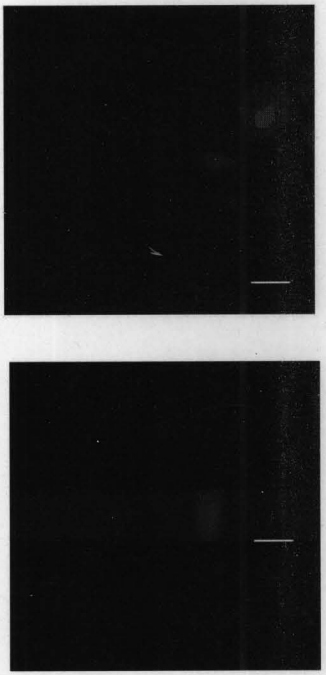


Figure 4-9: Confocal images of Caco-2 cell nuclear stain by propidium iodide assay. Cells were treated with 37.5 mg/ml E414 for 10 minutes, only few nuclei are stained. Upper: horizontal view (XY view) of cells; Lower: vertical view (XZ view) of cell. Bar length=20 µm.

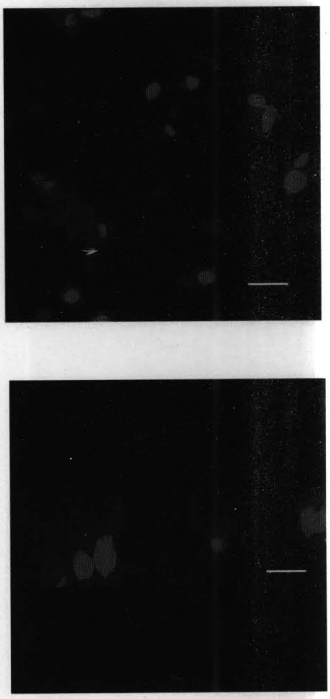


Figure 4-10: Confocal images of Caco-2 cell nuclear stain by propidium iodide assay. Cells were treated with 37.5 mg/ml E414 for 40 minutes, a few nuclei are stained. Upper: horizontal view (XY view) of cells; Lower: vertical view (XZ view) of cell. Bar length=20 μm.

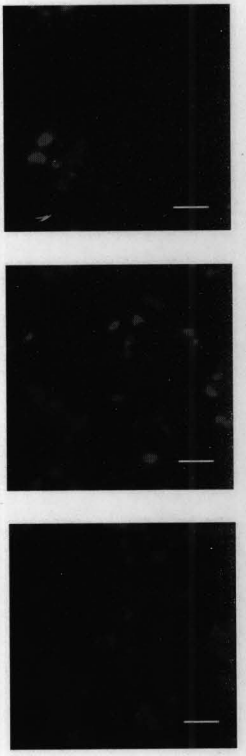


Figure 4-11: Sectional view of Confocal images of Caco-2 cell nuclear stain by propidium iodide assay. Cells were treated with 37.5 mg/ml E414 for 40 minutes, a few nuclei are stained. Upper: top section of cells; Middle: middle section of cell; Lower: bottom section of cell. Bar length=20 μm.

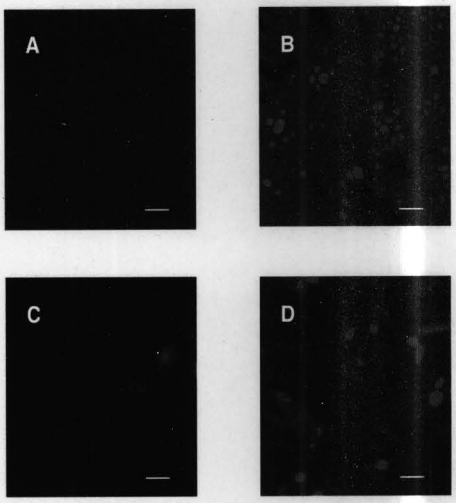


Figure 4-12: Comparison of the confocal images of Caco-2 cell nuclear stain by propidium iodide assay under different experimental conditions. A, cells were treated with KRB; B, cells were treated with SDS (0.1%, v/w) for 10 minutes; C, cells were treated with 37.5 mg/ml E414 for 10 minutes; D, cells were treated with 37.5 mg/ml E414 for 40 minutes. Bar length=20 μm.

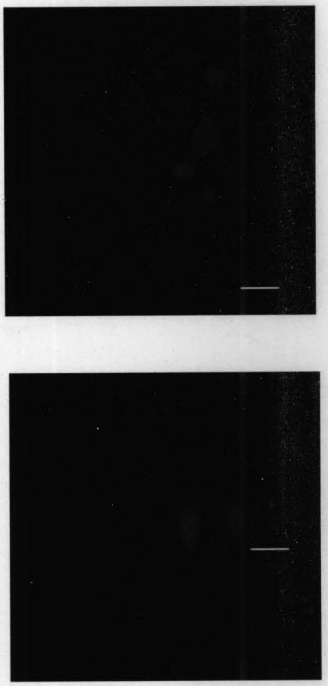


Figure 4-13: Confocal images of Caco-2 cell nuclear stain by propidium iodide assay. Cells were treated with 25 mg/ml E414 for 40 minutes, a few nuclei are stained. Upper: horizontal view (XY view) of cells; Lower: vertical view (XZ view) of cell. Bar length=20 µm.

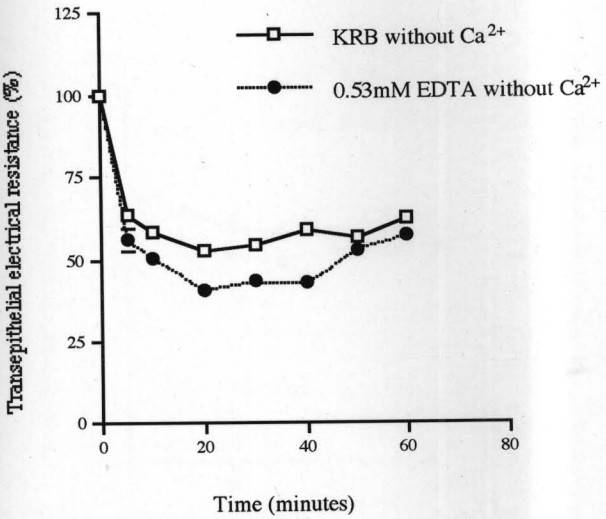


Figure 4-14: The effect of KRB and EDTA without calcium ion on the transepithelial electrical resistance time profile on Caco-2 cells (error bars indicate SEM, n=3-6).

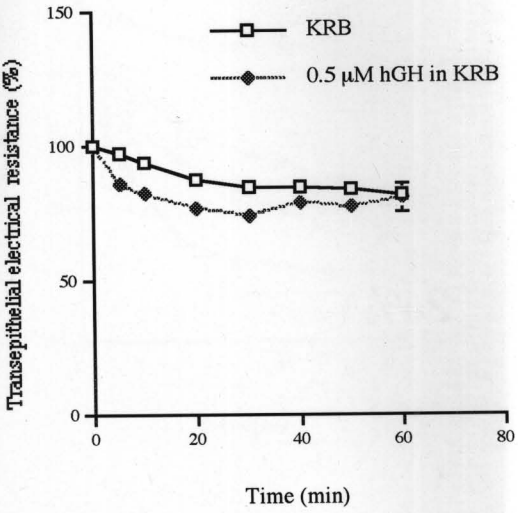


Figure 4-15: The effect of KRB and hGH on the transepithelial electrical resistance time profile on Caco-2 cells (error bars indicate SEM, n=3-6).

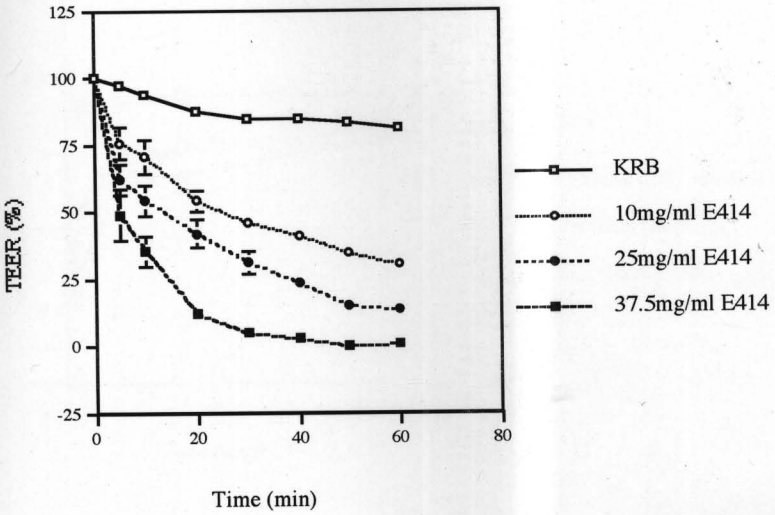


Figure 4-16: The effect of different concentrations of E414 on the transepithelial electrical resistance time profile on Caco-2 cells (error bars indicate SEM, n=3-6).

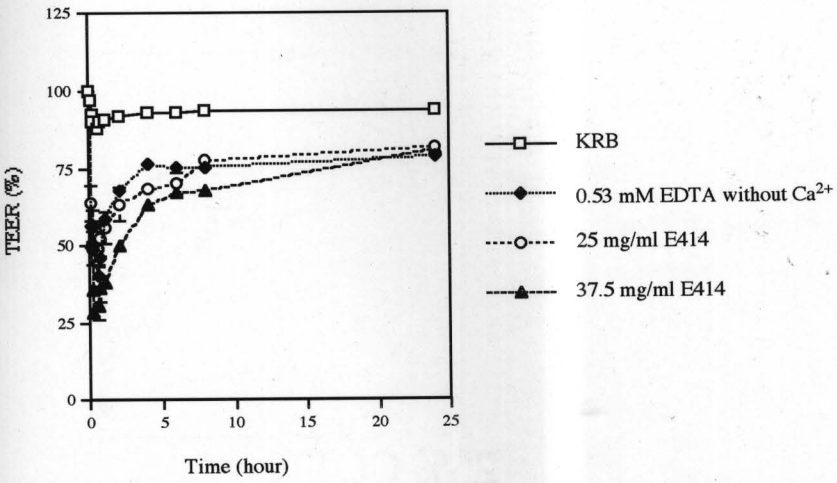


Figure 4-17: Recovery of transepithelial electrical resistance of Caco-2 cells after 15 minutes treatment with different reagents (error bars indicate SEM, n=3-6).

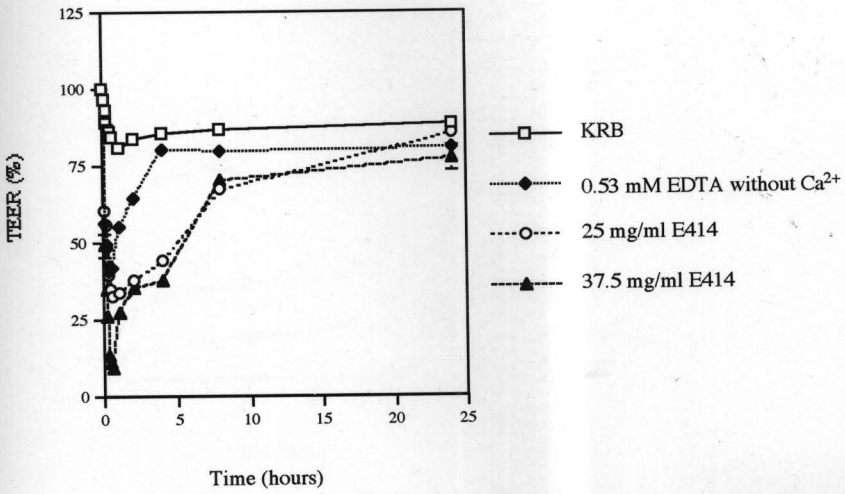


Figure 4-18: Recovery of transepithelial electrical resistance of Caco-2 cells after 30 minutes treatment with different reagents (error bars are not shown under selected axis scale).

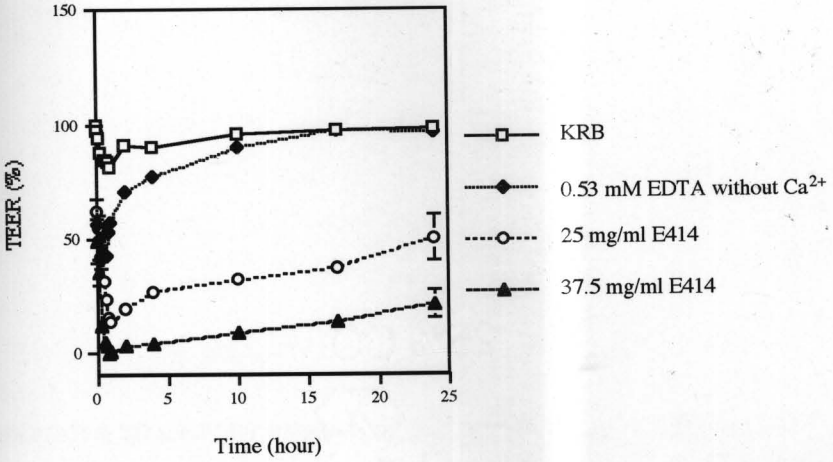


Figure 4-19: Recovery of transepithelial electrical resistance of Caco-2 cells after 60 minute treatment with different reagents (error bars indicate SEM, n=3-6).

CHAPTER 5: TRANSPORT PATHWAYS AND SUBCELLULAR LOCALIZATION OF THE CARRIER ENHANCED hGH ABSORPTION SYSTEM

I. Experimental

A. Materials

In addition to materials mentioned in previous chapters, the following materials were used for this section: Butanol and acetic acid were obtained from Aldrich, Milwaukee, WI. Lissamine rhodamine B sulfonyl chloride (lrho) was obtained from Molecular Probes, Eugene, OR. Fluorescein isothiocyanate labeled human growth hormone (FITC-hGH) was kindly supplied by Emisphere Technologies, Inc. Tarrytown, NY.

B. Labeling and Characterization of Lissamine rhodamine B Labeled Human Growth Hormone (lrho-hGH)

Human Growth Hormone was labeled with lissamine rhodamine B employing the method described by Vangala (Vangala, 1998). Fifty microliters of lissamine rhodamine sulfonyl chloride (lrho) (1 mg/940 μl DMF) was added to 470 μl of hGH (2 mg hGH/470 μl 0.24 M phosphate buffer, pH 8.0) at 20°C (water bath) for 20 minutes. Thirty microliter of 1 M Tris HCl (pH 8.0) was added immediately afterward and the mixture was kept on ice to quench the reaction. The protein was dialyzed twice against previously chilled 50 mM TRIS HCl (pH 8.5) and 300 mM TRIS HCl (pH 8.5) in a cold room, for at least 6 hours each time. Dialysis was carried out using Pierce Slide-A-Lyzer® 10K dialysis cassettes (Pierce Rockford, IL).

The labeling efficiency and purity was checked by thin layer chromatography (TLC) using solvent containing Butanol : Acetic acid : Water in the proportions of 4 : 2: 1 and UV-VIS spectrophotometer (Varian, Victoria, Australia).

The labeling ratio of lrho versus hGH was determined using the following equation:

$$\text{Labeling ratio} = (A_{574}/\varepsilon_{574lrho})/((A_{280}-0.17 \times A_{574})/\varepsilon_{280hGH}) \qquad \text{(Eq. 2)}$$

Where

$\varepsilon_{574lrho} = 93{,}000\ cm^{-1}M^{-1}$

$\varepsilon_{280hGH} = 17{,}000\ cm^{-1}M^{-1}$ per monomer

C. Confocal Laser Scanning Microscopy (CLSM)

The features of confocal laser scanning microscopy are the same as described in the experimental section in Chapter 4. The fluorescein isothiocyanate (FITC) fluorescence images were accumulated via a 522DF32 filter blocks (excitation at 488 nm) and the rhodamine images were collected via the 605DF32 filter blocks (excitation at 568 nm).

The inserts containing Caco-2 monolayers were placed in a side-by-side diffusion chamber. Five milliliters of KRB buffer (pH 7.4) containing labeled-hGH, with or without

carriers, was placed in the donor chamber whereas 5 ml KRB (pH 7.4) was placed in the receiver chamber. The solution was constantly bubbled with 95% O_2 plus 5% CO_2, and the temperature was maintained at 37°C with a water circulating system. At different time intervals, transport was stopped. Cell monolayers were washed with cold PBS three times, removed together with the filter membranes by carefully cutting the membrane edge with a surgical blade (Becton Dickinson, Lincoln Park, NJ). The cell monolayer together with membrane filter was then gently "sandwiched" between two No.1 microscope cover glasses (Fisher Scientific, Pittsburgh, PA), and mounted on the microscope stage. Images were taken within 20 minutes after positioning.

D. Uptake Measurement by Flow Cytometry

FACScan Instrument (Becton & Dickinson, San Jose, CA) equipped with a 15 mW argon ion laser tuned to 488 nm and a nozzle aperture of 200 microns was used for fluorescence uptake measurement. Caco-2 cells were plated in 24 well plates (Fisher Scientific, Pittsburgh, PA.), 1×10^5 cells per well and grown about 7-10 days until reaching 90% confluence. For high carrier concentration treated cells, since cells tend to peel off from the plate after treatment, cells were then seeded on the transwell inserts instead of plates. The regular cell culture medium was changed to KRB buffer (pH 7.4) containing either FITC-hGH alone or FITC-hGH plus carrier with a hGH concentration of 0.44 μM. Cells were incubated for various time intervals, gently washed three times with PBS, harvested by trypsinization, transferred into ice-cold PBS and immediately analyzed by flow cytometry.

Cells without any fluorescence treatment were used as reference cells. Forward and side scatter were used to exclude debris, dead cells and doublets. A 530/30 BP filter was used for fluorescence detection. The cell uptake of fluorescence was expressed as the mean fluorescence intensity in cells. Data were analyzed with LYSYS-II program.

E. Transport Studies

The transport experiments were conducted the same way as described in the experimental section in Chapter 3. Caco-2 cells were used for the transport study in this section and the membrane filter was coated with collagen only.

II. Results

A. Labeling and Characterization of lrho-hGH

To make hGH visible under microscopy, lissamine rhodamine B was conjugated to hGH and the labeled product was characterized by Thin Layer Chromatography (TLC) and UV spectroscopy.

Figure 5-1 is the TLC result. It is shown that hGH is successfully labeled with lissamine rhodamine B. No free rhodamine is found in hGH samples either before or after

the transport study. This indicates that rhodamine does not dissociate from hGH under the transport experiment condition, thus validating the confocal microscopy study.

The UV spectra of synthesized lissamine rhodamine B labeled hGH (Figure 5-2) shows two peaks at a wavelength of 280 nm and 574 nm, representing hGH and rhodamine, respectively. The molar ratio of lrho versus hGH calculated according to equation 2 is about 1:8.

B. Confocal Laser Scanning Microscopy (CLSM) Observation

Figure 5-3 through 5-6 are the images of lrho-hGH distribution on Caco-2 cells obtained from confocal microscopy observation. It can be seen from both the horizontal (Figure 5-3) and vertical view (Figure 5-4) that in the absence of carrier, hGH alone cannot be taken up by Caco-2 cells, at least cannot be taken up to a visible extent which can be detected by confocal laser scanning microscopy, whereas hGH uptake is greatly enhanced in the presence of carrier. The sectional view (Figure 5-5) demonstrated that hGH deposits throughout the entire cell monolayer, from cell top to cell bottom, indicating that in the presence of carrier, hGH is not only taken up into cells through the apical side of the cell, but also exits through the basolateral side of the cell. A large quantity of hGH is located in the cytosol indicating that the transport of hGH is mainly through the transcellular pathway.

The enlarged picture (Figure 5-6) of localization of lrho-hGH on Caco-2 demonstrate that the deposition of hGH is highly concentrated in the lipid bilayer of the cell membrane. This suggests that hGH preferentially locates in the hydrophobic cell membrane bilayer.

The above results were further confirmed with another labeled hGH, FITC-hGH. Confocal images of the time dependent appearance of FITC-hGH revealed that at 15 minutes treatment with 37.5 mg/ml E414, hGH has partitioned into the cellular membrane bilayer (Figure 5-7, panel A). As time increases, hGH gradually gets into the cell cytosol (Figure 5-7, panel B-D). It is important to note that the enhancement of hGH uptake happens as early as 15 minutes after treatment with the carrier. At this time point, detectable carrier cytotocixity has not occurred, i.e., toxicity of the carrier treated cells show no statistically significant difference compared to buffer treated cells.

Pretreating cells with E414 alone for 30 minutes followed by applying hGH alone, that is, treating cells with carrier and protein separately, there is no noticeable increase in hGH uptake (Figure 5-8, panel B). In comparison, if protein and carrier are applied together, the enhancement of hGH is obvious (Figure 5-8, panel C).

C. Uptake Measurement by Flow Cytometry

To confirm the above direct microscopic observation, quantitative measurements utilizing a flow cytometer were made to determine hGH uptake by individual cells. Figure 5-

9 demonstrates two examples of typical flow cytometry histograms in our studies. The peak that shifts to the right indicates a higher hGH fluorescence uptake measured in the presence of carrier. Figure 5-10 shows the kinetic uptake of hGH into Caco-2 cells in the presence and absence of 37.5 mg/ml carrier. It shows that without carrier, the fluorescence signal is low, indicating a low hGH uptake/transport. Whereas in the presence of carrier, hGH uptake is greatly enhanced. This is consistent with the direct confocal observation. It is also shown that at short time treatments, i. e., 15 minutes, where cytotoxicity caused by carrier has not yet emerged to a statistically different level compared to buffer treated cells, hGH uptake has been enhanced substantially.

Figure 5-11 and 5-12 show hGH uptake at different E414 concentrations in Caco-2 and CAPE cells. As revealed by the graph, the carrier concentration dependency on hGH uptake shows a unique pattern. For both cells, without the carrier, uptake for hGH alone is very limited. In the presence of low concentration of carrier (below 25 mg/ml), hGH uptake was only slightly enhanced. But when the E414 concentration reaches 25 mg/ml, hGH uptake is significantly enhanced. A further increase in carrier concentration does not result in an increase in hGH uptake. Statistical analysis shows no significant difference between 25, 30 and 37.5 mg/ml of carriers as to hGH uptake based on 95% confidential level.

D. Transport Study

Previous transport studies have shown that carrier E414 and E352 (Figure 5-13) increased hGH transport in Caco-2 cells at a concentration of 37.5 mg/ml and 25 mg/ml, respectively (Wu, 1999a). Based on this, three D-series compounds (polyethylene glycol (PEG) derivatives of E414 and E352), D51, D54, and D48 (Figure 5-13) were synthesized. The enhancement efficiency of the D-series carriers to hGH and their effect on the paracellular tight junctions were examined in Caco-2 cells. Figure 5-14 shows the effect of D-series carriers on the transepithelial electrical resistance time profile of Caco-2 cells. Table 5-1 and Figure 5-15 show the permeability coefficient of hGH on Caco-2 cells in the presence of these carriers. As can be seen, similar to E-series carriers, the D-series carriers also caused a drop on Caco-2 TEER. A 25% drop on TEER was observed when cells were treated with carriers for 5 minutes only. When cells were treated for 1 hour, TEER has dropped to 30% of its original level. However, although an opening of tight junction was detected, as reflected by a drop of TEER, the enhancement on hGH permeability in the presence of D-series carriers was not shown. It is revealed that with equal or higher concentrations, in which the parent compounds (E-series carriers) are effective, all three D-series compounds fail to enhance hGH permeability (Figure 5-15). However, they were tested to be able to deliver horse radish peroxidase (HRP) at carrier concentrations that are equal to or less than the ones required for the parent compounds (Smart, 1998).

III. Discussion

A. Labeling and Characterization of lrho-hGH

There are two major criteria to select a proper labeling procedure to label hGH in our study. First, the labeling should not affect the unfolding behavior of hGH. Secondly, the labeling compound should not interfere with the properties of the carrier. Based on these requirements, rhodamine was selected as a marker for hGH. Rhodamine has an absorbance maximum at 574 nm that has no interference with E414 absorbance (190-360nm). It has also been established to have minimal effect on hGH conformation, especially on the unfolding profile of hGH (Vangala, 1998). The additional advantage of using rhodamine is its high extinction coefficient (93,000 $cm^{-1}M^{-1}$) and high quantum yield. UV spectrophotometer and TLC results show that hGH was successfully labeled with rhodamine and the labeled product is stable under the transport experimental conditions. Therefore, we made clear that the confocal images represent hGH rather than free rhodamine signals.

B. Transport Pathways and Subcellular Localization of Enhanced hGH

We utilized confocal laser scanning microscopy to obtain direct observation on the cellular transport pathways and subcellular localization of enhanced hGH. Confocal microscopy has been widely used to study a variety of aspects in the area of cell biology. Thin optical sections parallel or perpendicular to the axis of the microscope allow new insight into a three dimensional view of the tissue or intracellular structures (Paddock, 1991). In confocal microscopy, light focuses only on one confocal spot inside the specimen,

therefore it excludes the out-of-focus light (Weinlich et al., 1994). It also permits the study of cells under growth conditions, i.e., cells can be maintained as viable while performing the confocal experiments (Bacallao et al., 1990).

In our study, two different labeled hGHs, rhodamine labeled hGH and fluorescein labeled hGH, were chosen to follow the transport pathways and subcellular localization of hGH. Taking advantage of confocal microscopy, hGH transport pathways and localization were visualized from different directions, e. g., vertical view, horizontal view, and a detailed sectional view. In addition, all the images were taken under the condition that the cells were viable.

The confocal microscopy observation in our study has led us to the following conclusions:

1. Confocal microscopy studies on two different labeled hGH molecules directly demonstrate that in the absence of carrier, hGH alone cannot be taken up into the cell to a visible extent as detected by confocal laser scanning microscopy. However, in the presence of carrier, hGH uptake/transport is significantly enhanced;
2. The fact that a large quantity of hGH locates intracellularly indicates that carrier enhanced hGH is mainly transported through the transcellular pathway;
3. The strong and intense signals observed in the cell membrane bilayers suggest that carrier enhanced hGH is preferentially concentrated in the lipophilic membrane bilayers;

4. Time-dependent appearance of fluorescence shows that significant enhancement of hGH can be observed before a detectable cytotoxicity occurs.

To further validate the conclusions obtained from confocal microscopy, quantitative measurement on the cellular uptake of hGH was conducted using flow cytometry. Flow cytometry is remarkable and unique among biomedical analysis instruments because it provides dynamic measurement of signal uptake by individual cells at a high rate. Having the advantage of high sensitivity and accuracy, flow cytometry has been applied to the pharmaceutical field including pharmacodynamics, pharmacokinetics and drug delivery (Ramanathan, 1997). In our study, we utilized this instrument as a main method to quantify the cellular uptake of fluoreseinated hGH. The peaks resulting from a plot of cell number versus FITC-hGH fluorescence were single, sharp and homogeneous (Figure 5-9). Thus, an increase in cell-associated FITC-hGH fluorescence reflected unimodal averages of FITC-hGH content in cell populations rather than averages arising from multiple cell populations exhibiting distinctly different FITC-hGH content. Kinetic uptake of hGH in Figure 5-10 also showed that in the absence of carrier, hGH uptake is limited. Whereas in the presence of carrier, even with short time treatment where no detectable cytotoxicity emerges, hGH uptake is significantly enhanced. This agrees well with direct confocal observation.

C. The Interaction between Carrier and Protein

It has been mentioned earlier that classical penetration enhancers exert their enhancement function by creating damage to tissues or cells, whereas the proposed mechanism of carrier enhanced protein delivery system is that the carrier can interact with the protein and make the protein more transportable. To distinguish these two mechanisms, we have conducted a series of cytotoxicity studies and examined carrier toxicity towards different cellular components as discussed in Chapter 4. Here, we discuss some of the biological evidence that supports the existence of an interaction between carrier and protein.

The unique pattern of carrier concentration dependency on hGH uptake has drawn our attention. Unlike most classic penetration enhancers where enhancement is directly proportional to concentration, there is a critical concentration (25 mg/ml E414) above which the carrier substantially enhances hGH absorption, but has little or no effect below that. This same phenomena has also been observed in a previous transport study (Wu, 1999a). However, in that study, the critical concentration was found to be 37.5 mg/ml rather than 25 mg/ml for carrier E414. There are several reasons that could account for the different critical concentrations observed in the transport and uptake studies. First, different labeled hGH was used in these two distinct studies. In the transport study, radio-iodinated hGH was used and the quantity of drug transported was detected by a scintillation counter. In the uptake study, fluoresceinated hGH was used and cellular uptake was measured by flow cytometry. It is possible that the iodinated hGH behaved differently than the fluoresceinated hGH. It has been found that iodination of growth hormone often results in protein damage, most commonly from reducing agents used in the process (Hunter et al., 1983; Kirkham et al.,

1971). Secondly, the concentration of hGH in the two studies are quite different. The final concentration of hGH in the transport study was around 3×10^{-8} M, whereas in the uptake study, it was 4.4×10^{-7} M. It has been shown that E414, being a weak amphiphilic compound, is surface active. Meanwhile, hGH itself is a surface active compound. When these two molecules are put together, the surface activities such as the critical micelle concentration (CMC) of each component could be changed. Therefore, different concentrations of hGH in the two studies may have a different impact on the surface activity of the carrier. Indeed, it has been shown in the literature that protein surfactant binding shifts the onset of micelle formation to a lower surfactant concentration (Jones et al., 1999). It is reasonable because in the presence of protein, less surfactant is required to form a micelle-like environment. Very likely, this change in the carrier surface activity may further influence its absorption enhancement activity. Thirdly, these two studies were conducted from different points of view. Uptake studies emphasize drug internalization. In this case, drug only penetrates through one side of the cell membrane (apical side in our study) and accumulates inside of the cell cytosol. On the other hand, transport studies focus drug permeation through cell monolayers. In this case, drug needs to penetrate both sides of the cell membranes (apical and basolateral). It is possible that, facilitated by the presence of 25 mg/ml E414, hGH can penetrate the apical membrane of the cell and accumulate in cells (uptake). However, once in the cells, due to environmental changes, e. g., dilution factor, the influence of subcellular organelles, etc., hGH cannot further penetrate through the other side of the cell. It is therefore trapped inside of cells. Only when E414 concentration increases to 37.5 mg/ml, hGH is able to be further delivered to the other side of the cell (transport).

If the enhancement of hGH caused by the carrier is simply due to carrier cytotoxicity, we should observe a higher hGH absorption due to the more severe damage caused by higher concentration of carrier. However, our results show that at or above 25 mg/ml E414, hGH uptake reaches a plateau, e. g., no statistical differences in hGH uptake between 25 mg/ml E414 and higher concentration treated cells. But the carrier cytotoxicity is concentration dependent once it emerges. E414 with concentration of 37.5 mg/ml caused more severe cytotoxicity than 25 mg/ml E414 in the cell membrane, cell lysosomal function, cell nuclei and paracellular junctions. More importantly, at 30 minutes or less, where the hGH uptake has been enhanced substantially, as demonstrated by direct observation obtained from CLSM images and quantitative kinetic measurement, both 25 mg/ml and 37.5mg/ml E414 show no statistically detectable cytotoxicity.

It is obvious that enhancement at this early stage is not mainly due to cytotoxicity of the carriers. But the reason for this enhancement is not yet entirely clear. DSC data has shown that the carrier interacts with the protein and induces a reversible denaturation of the protein molecule as reflected by a lowering of protein Tm. But further studies to prove the existence of this interaction are limited by the intrinsic physico-chemical properties of the carrier. The current evidence from biological studies have provided indirect, but valid, evidence that can establish the existence of this interaction. First, the confocal pictures clearly demonstrate that pretreating cells with carrier alone for 30 minutes, followed by applying hGH alone, do not result in enhanced hGH absorption. Using other carriers, earlier

studies in our research group on isolated rabbit tissues also showed that hGH transport cannot be enhanced when the carrier and hGH were applied separately (Mlynek, 1995). Normally, when using classical penetration enhancers, damage to the cells happens within 30 minutes of treatment. Therefore, even if it is washed away, the damage that has resulted to the cells should still lead to an enhancement of drug absorption. The evidence, that no obvious enhancement occurred when protein and carrier were applied separately, suggests that there is no significant damage to cells caused by carrier that allows hGH to be taken up, and furthermore, both carrier and protein have to be applied together to permit enhancement. Secondly, the carriers that enhance protein absorption function in a very specific way. Studies on D-series of carriers showed that while horseradish peroxidase (HRP) absorption was enhanced, with the same concentrations of carriers, absorption of hGH was not enhanced. Likewise, carrier E414 enhances hGH absorption significantly but fails to enhance lysozyme absorption with the same concentration (Wu, 1999a). While absorption of salmon calcitonin and α-interferon was increased by another specific carrier, heparin absorption, with the same carrier, was not enhanced (Brayden et al., 1997). These results, again, provide evidence to distinguish the mechanism of carrier enhanced protein absorption from the mechanism of classical penetration enhancers. Assuming the enhancement is due to damage to the cells, then it should non-selectively enhance many molecules and enhancement efficiency should be molecular weight dependent. That is, the tissue damage should allow low molecular weight compounds to be more permeable than high molecular weight compounds. However, our experimental results demonstrated that the same carrier enhances high molecular weight protein absorption, whereas it fails to enhance low

molecular weight protein absorption. All of the above evidence suggest that unlike classical penetration enhancers, which enhance drug absorption in a non-selective way, in the present case, there is a specific interaction between carrier and protein that causes enhancement.

The next step is to further characterize this interaction. We first examined whether this interaction had any relationship with the targeted cells. A pulmonary artery endothelial cell line, CAPE cell, that has different uptake/efflux mechanisms than Caco-2 cells, was purposely chosen and the carrier concentration dependency to enhance hGH absorption was compared with the results from Caco-2 cells. It is shown that although these two cell lines demonstrated different characteristics in terms of uptake/transport mechanisms, the enhancement occurred with the same carrier concentration. This suggests that the interaction simply occurred between carrier and carrier, or carrier and protein, most likely the latter, and it has no relationship with cell type. Detailed mechanistic studies further demonstrated that the uptake of hGH in the presence of carrier is a passive process that does not involve an active transport system and endocytosis (discussed in Chapter 6).

The next question is whether this interaction leads to a more hydrophobic form of the protein. This can be indirectly examined by the pathways the protein selects when it is transported through cellular barriers. There are generally two major pathways for a molecule to transport through a cell monolayer: paracellularly and transcellularly. The paracellular pathway is composed of aqueous pores that usually allows small and hydrophilic molecules to pass through. On the other hand, to employ the transcellular pathway by passive diffusion,

e.g., not through active transport or endocytosis, molecules have to partition into the lipophilic cell membrane bilayer, traverse through the cell cytosol and cross the cell membrane on the other side. This requires molecules to be in a hydrophobic form. Proteins, in their native state, usually have a hydrophobic core shielded by a hydrophilic outerlayer, thus they are quite hydrophilic. Theoretically, they prefer the paracellular pathway that is favored by hydrophilic compounds. It is difficult to envisage how a folded, water soluble protein could straddle two (or even one) lipid bilayer while retaining its native three dimensional conformation. However, proteins can become more hydrophobic when they are partially denatured, with their hydrophobic core exposed. This enhanced hydrophobicity makes passive transport through the transcellular pathway a possibility. Our confocal pictures clearly show that in the presence of carriers, hGH first partitions into the cell membrane bilayer, with increasing time, it enters the cell cytosol. The fact that protein transports through the transcellular pathway indicates that it maintains a hydrophobic form.

Other evidence that reveals that hGH becomes more hydrophobic in the presence of carriers, rely that it becomes a P-glycoprotein (P-gp) substrate (discussed in Chapter 6). P-gp is a "hydrophobic vacuum cleaner" that transports a variety of cationic and hydrophobic drugs out of cells. Proteins are usually hydrophilic and thus are not P-gp substrates. The only possibility for a protein to be recognized by a P-gp is that it is not in its native form, but in its intermediate state where the hydrophobic side chains or domains are exposed. It has been shown that hGH itself is not a P-gp substrate, but it becomes a P-gp substrate when

complexed with carrier, indicating formation of a more hydrophobic form of hGH (Wu, 1999b).

On the basis of results obtained, we believe that because protein absorption enhancement occurs before detectable carrier toxicity emerges, carrier enhanced protein absorption mainly takes place through a mechanism other than cell or tissue damage. In addition, in the presence of carrier, the protein maintains a hydrophobic form, and thus transport occurs primarily through the transcellular pathway.

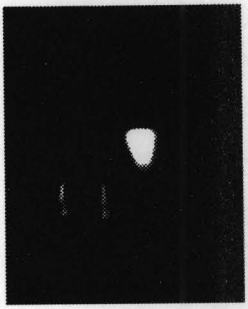


Figure 5-1: Thin Layer Chromatography (TLC) results of free rhodamine sample and lrho-hGH samples before and after transport study.

Lane 1: Lissamine rhodamine B labeled hGH before transport study;

Lane 2: Lissamine rhodamine B labeled hGH after transport study;

Lane 3: Free lissamine rhodamine B sulfonyl chloride.

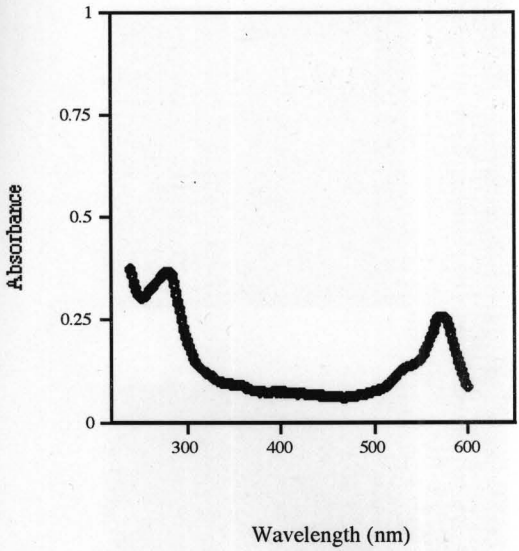


Figure 5-2: UV/Vis spectrum of rhodamine labeled human growth hormone.

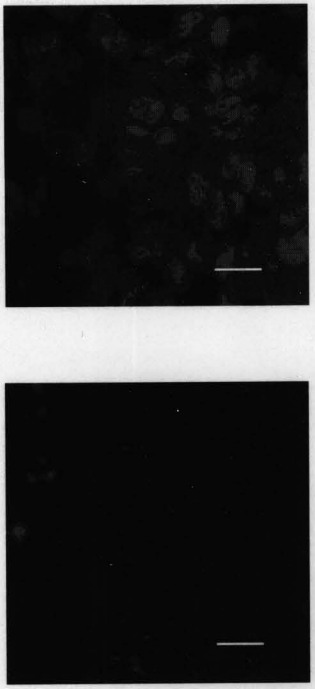


Figure 5-3: Confocal images of lrho-hGH distribution on Caco-2 cells. Upper, an XY section view, showing the distribution of lrho-hGH on Caco-2 cells treated with 37.5 mg/ml E414. Lower, control experiment, showing some fluorescence particles floating around in the absence of carrier, but no cellular uptake. Bar length=20 μM.

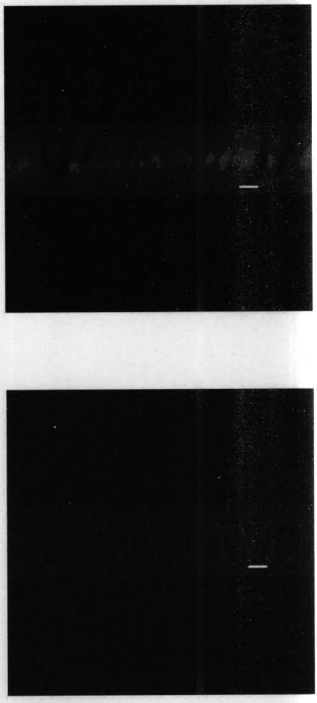


Figure 5-4: Confocal images of lrho-hGH distribution on Caco-2 cells. Upper, an XZ section view, showing the distribution of lrho-hGH on Caco-2 cells treated with 37.5 mg/ml E414. Lower, control experiment, no carrier treatment. Bar length=20 μM.

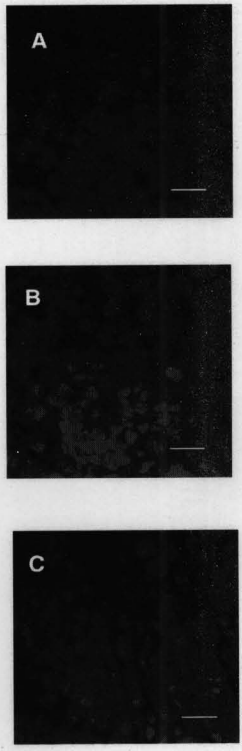


Figure 5-5: Confocal image of sectional XY view on different sections of Caco-2 cells, showing the distribution of lrho-hGH throughout the entire cell monolayer. Cells were treated with 37.5 mg/ml E414. A, cell top; B, cell middle; C, cell bottom. Bar length=20 μm.

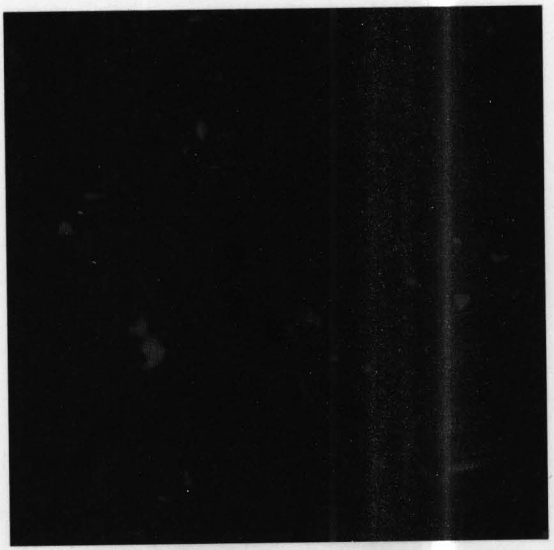


Figure 5-6: An enlarged picture of a confocal image of Caco-2 cells, showing that lrho-hGH is preferentially accumulating in the cell lipophilic membrane bilayers. Cells were treated with 37.5 mg/ml E414 for 30 minutes.

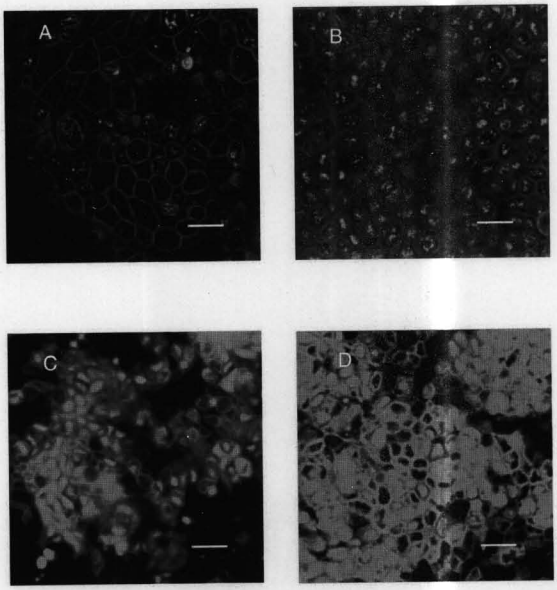


Figure 5-7: Confocal images of time dependent appearance of fluorescence within Caco-2 cells. Cells were treated with FITC-hGH in the presence of 37.5 mg/ml E414 for different length of time, showing hGH first partitions into the cell lipid bilayers, and enters to cell cytosols as time increases. A, 15 minute; B, 30 minutes; C, 60 minutes; D, 120 minutes. Bar length=20 μm.

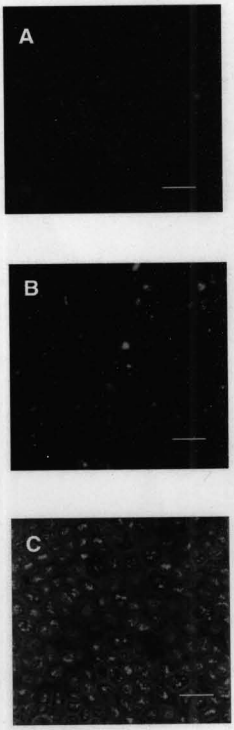


Figure 5-8: Confocal images of cellular uptake of FITC-hGH on Caco-2 cells under different experimental conditions. A, control experiment, cells were pretreated with KRB for 30 minutes, followed by FITC-hGH for another 30 minutes; B, cells were pretreated with 37.5 mg/ml E414 for 30 minutes, followed by FITC-hGH for another 30 minutes; C, cells were treated with 37.5 mg/ml E414 together with FITC-hGH for 30 minutes. Bar length=20 μm.

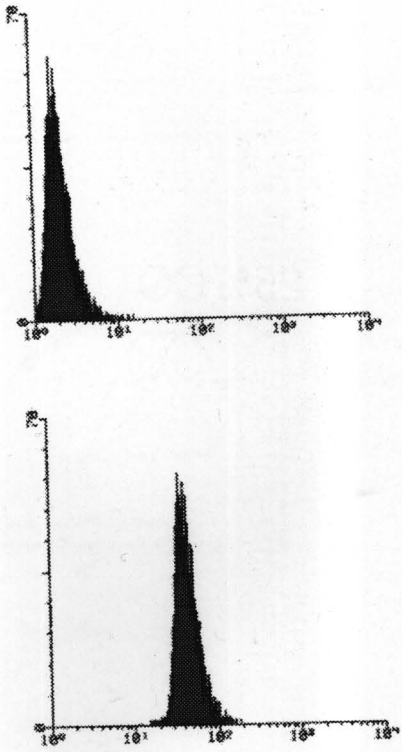


Figure 5-9: Examples of typical flow cytometry histogram. Peak shifted to the right means higher fluorescence uptake.

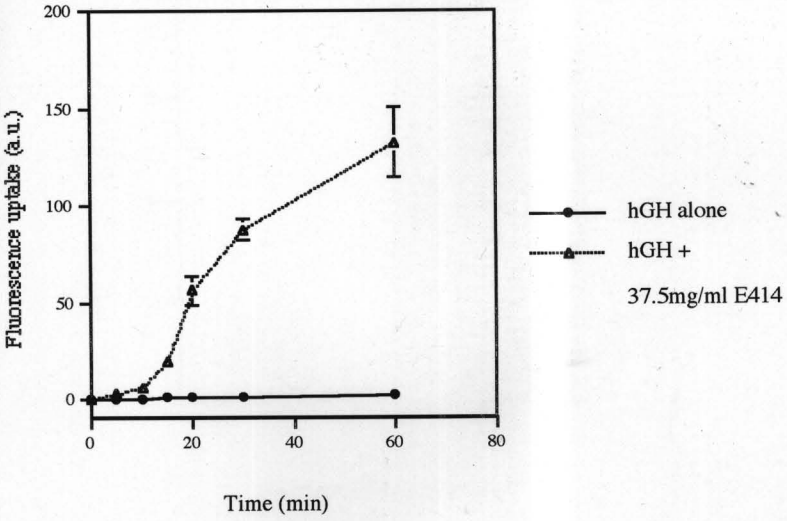


Figure 5-10: Kinetic uptake of hGH in Caco-2 cells in the presence and absence of 37.5 mg/ml E414.

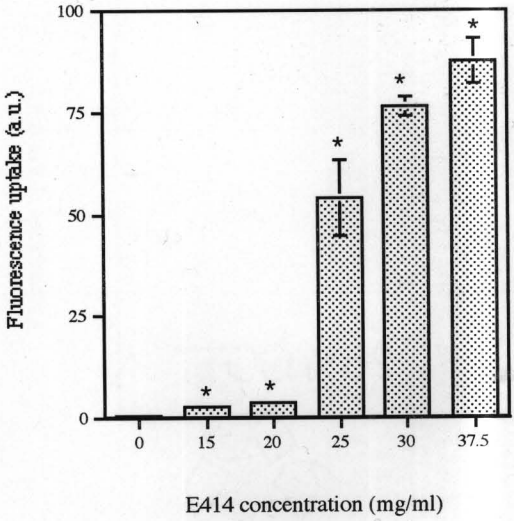


Figure 5-11: Concentration dependency of carrier enhanced human growth hormone uptake in Caco-2 cells. Cells were treated with different concentrations of E414 for 30 minutes. Experiments were done at 37°C (error bars indicate SEM, n=3-9, * denotes statistical significance of $p<0.05$ compared to cells without carrier treatment. No statistic difference was found for 25 mg/ml, 30 mg/ml, and 37.5 mg/ml carrier treated cells).

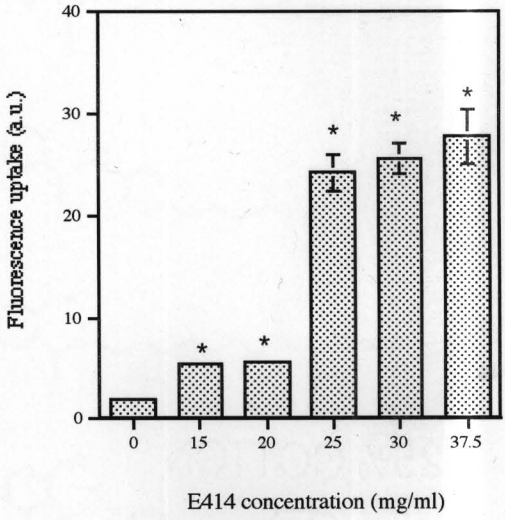
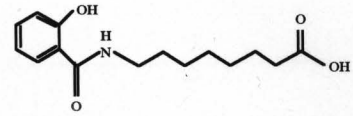
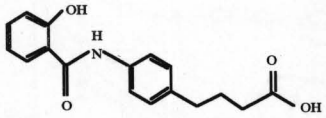


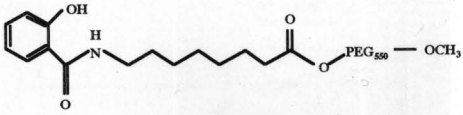
Figure 5-12: Concentration dependency of carrier enhanced human growth hormone uptake in CAPE cells. Cells were treated with different concentrations of E414 for 30 minutes. Experiments were done at 37°C (error bars indicate SEM, n=3-9, * denotes statistical significance of $p<0.05$ compared to cells without carrier treatment. No statistic difference was found for 25 mg/ml, 30 mg/ml, and 37.5 mg/ml carrier treated cells).



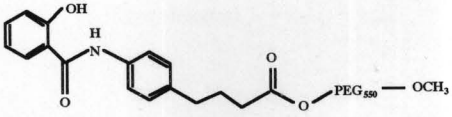
E414



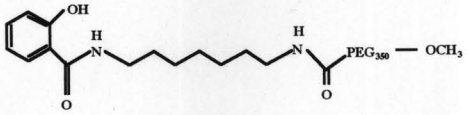
E352



D51-01



D48-01



D54

Figure 5-13: Chemical structures of carriers.

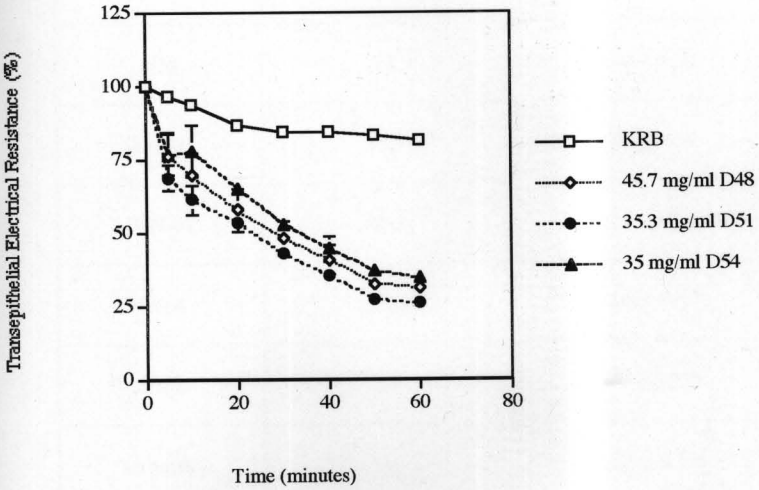


Figure 5-14: The effect of D-series carriers on the transepithelial electrical resistance time profile of Caco-2 cells (error bars indicate SEM, n=3-6).

Carrier	Carrier Concentration (mg/ml)	hGH Permeability Coefficient at pH 7.8 ($\times 10^{-6}$ cm/sec)
D54	35	1.54 ± 0.50
D51-01	35.3	1.87 ± 0.24
D48-01	45.7	1.02 ± 0.13
E414	37.5	16.3 ± 0.99*
E352	25	12.6 ± 2.68*
No carrier	---	1.65 ± 0.16

* Data obtained from reference (Wu, 1999a).

Table 5-1: Human growth hormone permeability coefficient (± S.E M) in Caco-2 cells in the absence and presence of D-series and E-series carriers.

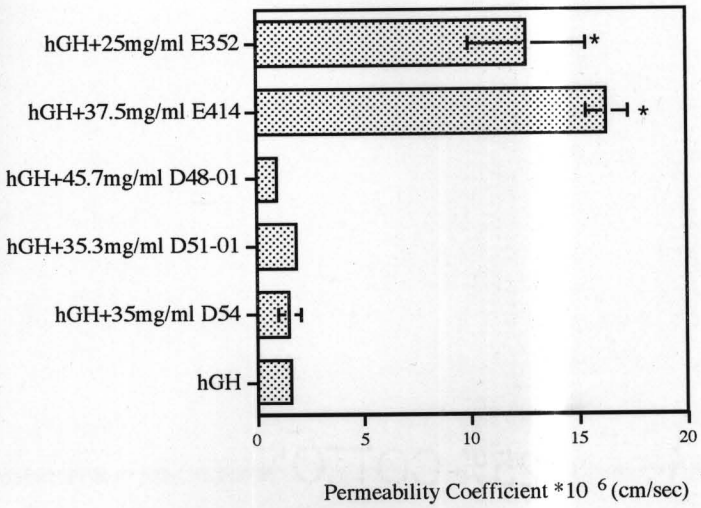


Figure 5-15: Human growth hormone permeability coefficient in Caco-2 cells in the absence and presence of D-series and E-series carriers. Experiments were done at 37°C (error bars indicate SEM, n=3-9, * denotes statistical significance of $p<0.05$ compared to cells without carrier treatment).

CHAPTER 6: MECHANISTIC STUDIES ON THE CELLULAR INFLUX AND EFFLUX OF CARRIER ENHANCED hGH ABSORPTION

I. Experimental

A. Materials

In addition to the materials mentioned in previous chapters, the following materials were used for this section: monensin, amiloride, ammonium chloride (NH_4 Cl), chloroquine, nocodazole, colchicine, cytochalasin D, verapamil, cyclosporin A were obtained from Sigma Chemical, St. Louis, MO. Ouabain and formaldehyde were obtained from Aldrich, Milwaukee, WI. Bovine serum albumin and goat serum were obtained from Gibco, Grand Island, NY. Signet 4E3 monoclonal antibody was obtained from Signet Pathology Systems, Dedham, MA. Fluorescein-5-isothiocyanate (FITC "isomer I") – conjugated rabbit IgG fraction to mouse IgG was obtained from ICN Pharmaceuticals, Inc, Aurora, Ohio.

B. Uptake Measurement by Flow Cytometry

Except for the P-gp expression and P-gp inhibitor experiments which will be discussed separately below, all the other uptake studies conducted in this section were carried out the same way as described in Chapter 5 with minor modifications. Inhibitors for each particular mechanism were added to the solution and kept in the solution until cells were harvested. All experiments were conducted in the presence of 37.5 mg/ml E414 for Caco-2 cells and 25 mg/ml for CAPE cells. For control experiments, only FITC-hGH and E414, but

no inhibitors, were added. FITC-hGH concentration was maintained as 0.44 μM of hGH unless otherwise specified.

C. Human Growth Hormone Uptake Measurement in the Presence and Absence of P-gp Inhibitors

Human growth hormone uptake in the presence and absence of P-gp inhibitors, e.g., cyclosporin A, verapamil, was conducted in two different flow cytometry instruments owning the un-availability the first instrument, and data were analyzed by different programs. For CAPE cells and Caco-2 cells postseeded for short periods of time, e. g., 7-10 days, P-gp inhibitor experiments were conducted the same way as for all other inhibitor experiments. The experimental procedure is described in experimental section B of this Chapter. The only exception is that cells were pretreated with P-gp inhibitors for 30 minutes prior to experiments.

For Caco-2 cells that were postseeded for longer periods of time, e.g., 21-28 days, the procedures of P-gp inhibitor experiments were conducted as follows: Caco-2 cells were seeded on transwell inserts with a density of 2×10^5 cells/ml, 0.5 ml each well, and allowed to grow for 21-28 days. Cells were washed with PBS three times and the apical side of the cells was pretreated with P-gp inhibitors for 30 minutes at 37°C. The apical solution was then replaced with KRB containing FITC-hGH, 37.5 mg/ml E414 plus P-gp inhibitors and further incubated for different time intervals. For control experiments, only FITC-hGH, 37.5

mg/ml E414 but no inhibitors were added. Cells were washed with PBS three times, trypsinized, and suspended in 1ml cold PBS. Cells without any fluorescence treatment were used as reference cells. Data were acquired and analyzed on a FACSCalibur flow cytometer (Becton Dickson, San Jose, CA) using CellQuest acquisition and analysis software. For the purpose of comparison, data for P-gp inhibitor experiments were presented in percentage and the control was set as 100%.

D. Immunohistochmical Detection of P-gp Expression

Two batches of Caco-2 cells, 7-10 days postseeding cells and 21-28 days postseeding cells, were used. Cells were seeded on the transwell inserts for different periods of time. The apical side of the cells was fixed with 1% formaldehyde plus $CaCl_2$ (1%, w/v) for 20 minutes at room temperature. Cells were incubated with Bovine serum albumin (1% in PBS) for 1 hour at room temperature. This is to block non-specific binding sites. Cells were then treated with 5 μg/ml 4E3 monoclonal antibody for 1 hour followed by FITC-conjugated rabbit anti-mouse secondary antibody (0.12 mg/ml) for another hour. For control experiments, a mouse IgG2a isotype-matched control antibody was used to replace 4E3 antibody. Cell monolayers were washed with cold PBS three times, removed together with the filter membranes by carefully cutting the membrane edge with a surgical blade. The cell monolayer together with membrane filter was then gently "sandwiched" between two No.1 microscope cover glasses, and mounted on the microscope stage. Immunofluorescence was

observed using confocal microscopy as described in Chapter 5, at an excitation wavelength of 488nm.

E. Quantitative Measurement of P-gp Expression by Flow Cytometry

Caco-2 cells were seeded on the transwell inserts with densities of 2×10^5 cells/ml. Cells were allowed to grow for different periods of time: 7-10 days period and 21-28 days period. Cells on transwell inserts were washed twice with cold PBS and incubated with 1% bovine serum albumin at 4°C for 30 minutes. After washing twice with cold PBS, 0.5 ml of PBS solution containing 5 μg/ml 4E3 mAb plus 2% goat serum were added to the apical side of cells. The basolateral side of cells was maintained with 2ml of PBS solution. For control experiments, 10 μg/ml IgG2a isotype-matched control Ab was used to replace 4E3 antibody. Cells were incubated with the above solutions for 30 minutes at 4°C, followed by incubating with a solution containing 2% goat serum plus FITC-rabbit anti-mouse Ab (1:30) for 30 minutes at 4°C in the dark. Cells were then washed with PBS, trypsinized, and resuspended in 1 ml cold 0.36% formaldehyde solution prior to analysis. Data was acquired and analyzed on a FACSCalibur flow cytometer (Becton Dickson, San Jose, CA) using CellQuest acquisition and analysis software.

II. Results

Two time points, 15 minutes and 30 minutes, were chosen to study the mechanism(s) of hGH uptake/efflux in the presence of carriers. According to previous cytotoxicity studies (discussed in Chapter 4), there is no toxicity at these time points. For this Chapter which emphasizes mechanistic studies, we chose 25 mg/ml E414, which is the lowest effective carrier concentration, for CAPE cell studies. This tends to minimize any possible toxicity effect. For Caco-2 cells, we chose 37.5 mg/ml E414 as the carrier concentration. This concentration has been used for earlier transport studies on Caco-2 cells. We therefore chose it for the purpose of comparing the uptake/efflux mechanism(s) with the transport mechanism(s).

A. Human Growth Hormone Influx in the Presence of Carriers

a. Transporter-Mediated Influx

The involvement of two well-known transporter-mediated uptake systems, sodium gradient dependent and proton gradient dependent transporter systems, on enhanced hGH absorption, was investigated. As shown in Figure 6-1 and 6-2, a Na^+/K^+-ATPase inhibitor, ouabain (5 mM), has no effect on enhanced hGH uptake on both Caco-2 and CAPE cells at the time points studied, suggesting that a sodium gradient dependent transporter system is not involved in hGH uptake in the presence of carriers. Another inhibitor, amiloride (0.5 mM), which can inhibit Na^+/H^+-ATPase, also showed no effect on hGH uptake, indicating that the enhanced hGH uptake is also not through a proton gradient dependent transporter system.

Besides the above two transporter-mediated uptake systems, there exists other transporter-mediated uptake systems. To investigate the involvement of other possible transporter-mediated uptake systems in enhanced hGH influx, 100 fold excess unlabeled hGH was added to the system to block potential transporters. Figure 6-3 and 6-4 show the effect of excess unlabeled hGH on labeled hGH uptake. For 7-10 days postseeded Caco-2 cells and CAPE cells, we observed no changes in labeled hGH uptake when adding excess unlabeled hGH. This indicates that there is no saturability involved in hGH uptake, which further supports the result that transporter-mediated uptake is not involved in enhanced hGH uptake on cells postseeded for short time periods.

b. Endocytosis

To check whether enhanced hGH uptake is through an endocytosis process, three different categories of endocytosis inhibitors were applied to the system and their effect on enhanced hGH uptake was examined. Monensin, ammonium chloride and chloroquine are weakly basic compounds, and block the process of endocytosis by increasing the pH and inhibiting acidification of endosomal vesicles. Colchicine and nocodazole are microtubule disrupting agents, and prevent endocytosis by disrupting the fusion of early endosomes to late endosomes. Cytochalasin D belongs to the third category of endocytosis inhibitor, and prevents endocytosis by depolymerizing actin filaments. Figure 6-5 through 6-10 show the effects of these endocytosis inhibitors on enhanced hGH uptake for Caco-2 and CAPE cells.

As can be seen from these figures, all three categories of endocytosis inhibitors cause no change in hGH uptake for both cells, indicating that endocytosis is not involved in carrier enhanced hGH uptake.

B. Human Growth Hormone Efflux in the Presence of Carriers

a. P-glycoprotein

Two P-gp inhibitors, cyclosporin A and verapamil, were applied to the system and their effects on hGH efflux in the presence of carriers were examined. Figure 6-11 and 6-12 show the P-gp inhibitor effects on enhanced hGH uptake in CAPE cells. As can be seen from the figures, pretreating cells with inhibitors for 30 minutes followed by 15 and 30 minutes treatment together with hGH and E414, both cyclosporin A (10 μM) and verapamil (300 μM) do not cause an increase in hGH uptake. This indicates that for CAPE cells, P-gp is not involved in hGH efflux in the presence of carriers.

For Caco-2 cells, P-gp involvement in enhanced hGH uptake was tested on two groups of cells. For cells that were postseeded 7-10 days, verapamil (300 μM) caused a slight increase in hGH uptake (Figure 6-13), and this increase barely falls at the borderline of statistical difference as compared to the control, i.e., $p=0.03$ for 15 minutes treatment and $p=0.045$ for 30 minutes treatment. Ten μM cyclosporin A did not cause a change in hGH uptake that is statistically different from control (Figure 6-14). However, for cells that are

postseeded for longer time, i. e., 21-28 days, both cyclosporin A and verapamil treatment resulted in significant increases in hGH uptake (Figure 6-15).

We further measured P-gp expression level in Caco-2 cells with different postseeding times. As can be seen in Figure 6-16, there is a distinct difference in the P-gp expression level between 10 days postseeded Caco-2 cells and 23 days postseeded cells. The late stage cells give a much higher P-gp expression level than the early stage cells. To further verify this result, confocal laser scanning microscopy was employed and the P-gp expression level in different staged cells were directly demonstrated. Figure 6-17 shows confocal images of P-gp expression in Caco-2 cells with different postseeding times. As shown by the images, for cells that were postseeded for short periods of time (7 days), only a small number of cells exhibits fluorescence staining and the fluorescence signals are weak and diffusive, indicating a low P-gp expression (Figure 6-17, panel B). On the other hand, the majority of the long time postseeded cells (28 days) exhibits an intense and bright fluorescence signal, suggesting a high P-gp expression level. For control experiments (Figure 6-17, panel A), we used an antibody that does not recognize P-gp, so no fluorescence signal was detected.

b. Multidrug Resistant Associated Protein

Indomethacin is a MRP inhibitor. It can block the MRP efflux pump, thus increasing the uptake of compounds that are subject to an MRP efflux system. As can be seen in Figure 6-18 and 6-19, indomethacin (10μM) has no effect on enhanced hGH uptake for both Caco-2

and CAPE cells, suggesting that MRP is not involved in hGH efflux in the presence of carriers.

III. Discussion

Earlier studies have shown that enhanced hGH absorption is mainly through the transcellular pathway. To further investigate the detailed mechanism(s) through which enhanced hGH absorption occurs, a series of mechanistic studies on hGH uptake/efflux in the presence of carrier were conducted and the possible pathways of hGH/carrier uptake/efflux explored.

To investigate mechanisms, drug uptake studies offer advantages over traditional drug transport studies. Uptake studies focus on drug permeation through individual membranes, e.g., either apical or basolateral membranes for polarized cells, whereas transport studies measure the overall permeation of drug through both membranes simultaneously. Indeed, many cells have different absorption mechanisms through their different membranes. In epithelial cells, such as those involved in absorbing nutrients from the gut, transporter proteins are distributed asymmetrically in the plasma membrane. For example, Na^+ linked symporters located in the apical (absorptive) domain of the plasma membrane actively transport nutrients into the cell, building up substantial concentration gradients, while Na^+-independent transport proteins in the basolateral domain allow nutrients to leave the cell passively down these concentration gradients (Alberts et al., 1994). The

transferrin receptor (TfR) is present predominantly on the basolateral surface of the cell (Banerjee et al., 1986; Podbilewicz et al., 1990) and the majority of internalized TfR is recycled back to the basolateral membrane (Fuller et al., 1986). In Madin-Darby canine kidney (MDCK) cells, the number of TfR's on the basolateral membrane is about 800-fold higher than that on the apical surface (Fuller et al., 1986). Transport studies cannot differentiate these two membranes since it evaluates the overall results of a molecule penetrating through both cell membranes. On the other hand, uptake and efflux studies allow focus on one side of the cell membrane and as a consequence, provide a more detailed and accurate explanation of the phenomena. For example, using uptake studies, Kawazu and coworkers (Kawazu et al., 1999) showed the existence of a transporter-mediated system for levofloxacin absorption in cultured rabbit corneal epithelium, which is difficult to characterize by transepithelial transport studies. In our particular case, measurement of uptake rather than transport is especially necessary because there may be a quantity of hGH that transports through the paracellular pathway. This amount of hGH, although small, might be included in the total amount in the transport study, and may cause interference in clarifying the different transport mechanisms. Therefore, we chose uptake measurements and focused on mechanisms on the apical side of the plasma membrane.

In general, there are three major mechanisms for drug influx into cells: transporter-mediated uptake, endocytosis and passive diffusion. Once a drug enters cells, there are three possible mechanisms that can efflux it out the apical membrane from the cell cytosol: P-

glycoprotein efflux system (P-gp), multidrug resistance associated protein efflux system (MRP) and passive diffusion (Figure 6-20).

Two *in vitro* models, an endothelial cell model, Caco-2 cell, and an epithelial cell model, CAPE cells, were chosen for this part of the study. In general, endothelia and epithelia possess certain similarities and differences. Structurally, the endothelium is derived from the mesoderm and lacks the typical epithelial cytokeratins. Vimentin is the predominant intermediate filament (Bowman et al., 1991). Desmosomes, which are prominent in all epithelia, are absent in endothelial cells. Endothelial cells do exhibit zonula adherens type junctions, as do epithelial cells. Morphologically, the cytoplasm of the endothelial cell, in contrast to most epithelial cells, is remarkably attenuated, generally less than 0.3 um. The endothelium is subject to substantial hydrostatic as well as osmotic forces and is relatively more permeable to large, lipid-insoluble molecules than the epithelium. Endothelium and epithelium may be distinct in terms of uptake/efflux mechanisms. For the two *in vitro* models chosen in our study, Caco-2 cells have been shown to have a number of transporter-mediated transport systems, for example, sodium gradient dependent and independent transporters, proton gradient dependent transporter, etc., and have proved to have P-gp overexpression on its apical side of the cell membrane (Peters et al., 1992; Anderle et al., 1998; Hunter et al., 1993). Recent studies have identified that the MRP efflux system is also present on the apical side of Caco-2 cell membranes (Walgren et al., 2000; Hirohashi et al., 2000; Gutmann et al., 1999; Makhey et al., 1998). However, being an epithelial cell line, the Caco-2 cell has very limited endocytotic activity. On the other hand, the endothelial

cell line, CAPE cell, has an active endocytotic process, but there is no reported evidence showing the existence of a P-gp and an MRP efflux system in the CAPE cell line. Table 6-1 compared the different mechanisms associated with these two *in vitro* models. We purposely chose these two cell models because their distinction in uptake/efflux systems allows for good comparison.

A. Transporter-Mediated Uptake

Drugs, depending on their nature, can be absorbed through certain specialized transporters into cells. Some di/tripeptides, such as glycylsarcosine, cephradien (Inui et al., 1992) or peptide mimics are absorbed through an oligopeptide transporter system. Amino acid transport systems are responsible for transporting a number of amino acids such as ß-alanine, L-alanine, and proline (Thwaites et al., 1995). Certain short chain fatty acids, e. g., lactic acid, have been shown to be absorbed through an acid transport system. Most transporter systems require an energy supply either from a chemical reaction, such as ATP hydrolysis, or the energy stored in electrochemical gradients, e.g., sodium gradient, or proton gradient. One method to block the transporter-mediated transport system is to deplete their energy source. This can be achieved by either disrupting the chemical reaction or breaking the electrochemical gradient.

It is known that many transporter-mediated uptake systems rely on a sodium gradient as their energy source, e. g., some amino acid transporters and glucose transporters.

Ouabain, a Na^+/K^+-ATPase inhibitor, can compete for K^+-binding sites and therefore prevent formation of Na^+ and K^+ electrochemical gradients. If the uptake of a compound is involved in sodium dependent transporters, the addition of ouabain should result in a decrease in its uptake. However, for Caco-2 cells which have been identified as having a number of Na^+-dependent transport systems, and CAPE cells which have no reported sodium dependent transporter systems, 5 mM ouabain showed no effect on carrier-enhanced hGH uptake (Figure 6-1 and 6-2). It is, therefore, suggested that a sodium dependent transporter system is not involved in hGH uptake in the presence of carrier.

There are also other transporters, such as a di/tripeptide transporter, which functions by utilizing the energy stored in a proton gradient. Amiloride is a HCO_3^- ionophore that permits HCO_3^- to flow through and therefore interfere with the proton gradient. The addition of amiloride, as a consequence, results in a decrease in compound uptake if this compound is utilizing a proton dependent transporter as its uptake route. In our study, we observed no change in hGH uptake in both cells in the presence of 0.5 mM amiloride. This indicates that carrier enhanced hGH uptake is not through a proton dependent transporter system.

One of the general features of transporter-mediated uptake is its saturability. As stated before, the process by which a transporter protein transfers a solute molecule across a lipid bilayer resembles an enzyme-substrate reaction, and the transporter involved behaves like a specialized membrane bound enzyme. Each type of transporter protein has one or more specific binding sites for its solute (substrate), and the total number of binding sites are

limited. Therefore, these sites can be saturated when adding an excess amount of solute. Based on this, we added a100 times higher concentration of unlabeled hGH to the system and checked its effect on labeled hGH uptake. If hGH is taken up through any transporter-mediated system, the excess unlabeled hGH should competitively occupy the binding sites of labeled hGH, causing a reduction of labeled hGH uptake. However, in our study, we observed no change in the labeled hGH uptake upon adding an excess amount of unlabeled hGH to both cells (Figure 6-3). This further strengthens our finding that carrier enhanced hGH uptake is not through a transporter-mediated transport for cells that are postseeded for short time period, which is in agreement with the previous observation on the excised tissue (Mlynek, 1995) and the results obtained from the transport studies on Caco-2 cells (Wu, 1999).

B. Endocytosis Involvement

Endocytosis is considered a major pathway for uptake of macromolecules. Generally speaking, endothelial cells such as CAPE cells have a large number of vesicles that transport macromolecules. Most macromolecules such as proteins appear to use this endocytotic pathway. It is possible that hGH, as a macromolecular, could be endocytosed into CAPE cells. On the other hand, Caco-2 cells, as is an epithelial cell line, have limited endocytosis activity. Previous transport studied on Caco-2 cells have suggested that no endocytotic activity is involved in the carrier enhanced hGH transport (Wu, 1999).

One common experimental approach to test involvement of the endocytotic pathway is the use of inhibitors that block endocytosis. There are several compounds that can inhibit the endocytotic process. One group of compounds includes weakly basic compounds, such as ammonium chloride, chloroquine, acridine orange, and neutral red. Due to their basic nature, they increase the pH of the endosomal vesicles and thus prevent endocytosis (Jessup et al., 1983; Ohkuma et al., 1981; Hwang et al., 1986; Burkhardt et al., 1993; Hastings et al., 1994). Many lysosomotropic amines have been shown to be effective in increasing the accumulation of transferrin inside of cells (Glass et al., 1986). It has been shown that 20mM ammonium chloride can completely abolish the acidification of endosomes and thus result in a 4- fold increase in transferrin intracellular accumulation and a 7-fold increase in transferrin transcytosis in the basal-to apical direction in MDCK cells (Shen et al., 1995). The other compound that belongs to this category is monensin. Monensin is an Na^+- ionophore which elicits a variety of effects on mammalian cells (Mollenhauer et al., 1990). Besides causing similar effects as lysosomotropic amines in elevating lysosomal and endosomal pH by interfering with vesicular Na^+/H^+ exchange across organelle membranes, the most important effect of monensin is the inhibition of normal functions of the trans-Golgi network (TGN) (Tartakoff, 1983). As a result, it disrupts the sorting pathways involving acidic compartments in the endocytotic process (Deffebach et al., 1996). Treating MDCK cells with monensin can result in a 24-fold increase in transferrin transcytosis and a 4-fold increase in cellular accumulation of protein (Shen et al., 1995).

Another experimental approach to verify the involvement of endocytosis is the use of microtubule disrupting agents. Microtubules seem to be involved in the transport of early endosomes from the cell periphery to a region close to the cell nucleus where fusion of early endosomes and late endosomes occurs. Studies have shown that treatment of the intestinal epithelium and Caco-2 cells with colchicine and nocodazole, two typical microtubule disrupting agents, results in the appearance of mis-sorting, indicating an inhibition of transcytosis (Eilers et al., 1989; Matter et al., 1990).

There are also studies suggesting that actin filaments of the cytoskeleton can effect the endocytotic process. It has been shown that cytochalasin D can depolymerize actin filaments of the cytoskeleton and block endocytosis of some polarized epithelial cells, such as MDCK cells (Gottlieb et al., 1993).

The involvement of endocytosis in carrier enhanced hGH uptake was examined by applying the above three categories of endocytotic inhibitors. If hGH is taken up by an endocytotic process, the addition of these inhibitors should cause an increase in hGH uptake as a result of accumulation of hGH entrapped inside of cells. In our study, for both cells, CAPE cells that possess active endocytosis activity and Caco-2 cells whose endocytosis is less active, all the inhibitors mentioned above caused no change in hGH uptake. Therefore, it is concluded that endocytosis is not the pathway through which carrier enhanced hGH uptake occurs.

C. P-gp Involvement

It has been suggested that enhanced hGH absorption is subject to an efflux system on Caco-2 cells. This efflux system, because functions similar to P-gp, was termed P-gp-like efflux system (Wu, 1999b). But there lacks of evidence to verify that it is indeed a P-gp. Since both P-gp and MRP function similar and locate on the apical side of the Caco-2 cells, so we chose uptake study, and focused on the apical side of the cells, to further investigate the efflux systems that may involved in the enhanced hGH absorption.

As stated earlier, P-gp is a plasma membrane protein which functions as an energy dependent drug efflux pump. It decreases drug influx and increases drug efflux. P-gp substrates are usually functionally unrelated. But in general, they possess an amphiphilic structure, and seem to favor lipid over aqueous solubility with a logarithm of partition coefficient greater than 2 in an octanol/water system (Germann et al., 1993). Figure 6-21 depicts the possible mechanism of P-gp action as a "hydrophobic vacuum cleaner" (Gottesman, 1993). There might be two ways through which a P-gp pump functions: drugs can enter cells by passive diffusion through the lipid bilayer and bind to P-gp on the cytoplasmic side of the membrane, or alternatively, bind to P-gp binding sites within the lipid bilayer of the cell membrane. In the later case, drug will be pumped out before it gets a chance to access the cell cytosol. The exact site of which location drug binding occurs is not clear yet, but in both cases, drug needs to partition into at least one layer of the plasma membrane to access to P-gp.

The action of the P-gp efflux pump can be inhibited by specific agents, referred to as membrane transport modulation agents, e. g., cyclosporin A and verapamil. These compounds interact specifically with high affinity with the drug binding sites of P-gp and competitively inhibit drug binding to P-gp. Consequently, they inhibit the active efflux of drug and increase drug accumulation inside of cells.

Figure 6-11 and 6-12 show the effect of verapamil and cyclosporin A on carrier enhanced hGH uptake in CAPE cells. As shown in these figures, both verapamil and cyclosporin A caused no change in hGH uptake when pretreating cells for 30 minutes with inhibitors followed by15 and 30 minutes treatment together with carrier and protein. This result may be due to two possible reasons. One is that since CAPE cells do not have P-gp overexpressed therefore we don't observe the effect of P-gp inhibitors. Another possible reason is that the hGH/carrier complex is not a P-gp substrate, therefore, its uptake bypasses the P-gp efflux pump.

We then used Caco-2 cells, which have been reported to have P-gp overexpression, to further test the involvement of P-gp in hGH efflux in the presence of carrier. The results obtained from Caco-2 cells are very interesting. Initially, we chose Caco-2 cells that were postseeded for 7-10 days to conduct the inhibitor experiments since all other uptake experiments were conducted using cells at this stage. As demonstrated in Figure 6-13 and 6-14, the effect of two P-gp inhibitors on hGH uptake gives confusing results: cyclosporin A

seemed to cause no change in hGH uptake, whereas verapamil caused a slight increase in hGH uptake. Repeating the same experiment with an enlarged sample size gave the same results.

The fact that Caco-2 cells have P-gp overexpressed has been established and studied extensively (Augustijnset al., 1993; Hunteret al., 1993; Peterset al., 1992; Anderleet al., 1998). Using Caco-2 cell postseeded for 21-28 days, earlier studies in our laboratory had shown that vinblastine, a P-gp substrate, demonstrated a lower permeation rate from the mucosal to serosal side compared with that from the serosal to mucosal side. Furthermore, when competitive inhibitors were applied to the system, the unequal fluxes of vinblastine disappeared (Wu, 1999a). This indirectly suggests that 21-28 days postseeded Caco-2 cells have overexpression of a P-gp or a P-gp-like system on the apical side of the plasma membrane. It was called P-gp-like efflux pump because it has many features that are in common with P-gp, yet we lack direct evidence to prove it is indeed a P-gp. In the transport studies, Caco-2 cells that were postseeded for longer time (21-28 days) were used, we then tested the P-gp inhibitor effect on enhanced hGH uptake using the same stage of cells, i. e., Caco-2 cells that are postseeded for 21-28 days. The results, shown in Figure 6-15, demonstrated that both cyclosporin A and verapamil caused significant increases in hGH uptake in Caco-2 cells that were postseeded for longer periods of time.

To further investigate the reason for the above observation, P-gp expression on two different stages of Caco-2 cells was detected using a immunohistochemical technique.

Signet 4E3, a mouse monoclonal antibody, is an IgG2a antibody and designed for detection of P-gp external epitope. It can be used to determine the level of P-gp expression in tissue specimens and on intact, viable cells. It specifically recognizes human MDR1 P-gp, but not the MDR3 gene product. It does not affect intracellular drug accumulation or cytotoxicity in cells and does not inhibit cell growth (Arceci, et al. 1993). We chose it for the detection of P-gp in our study. A mouse IgG2a isotype-matched antibody was used as control antibody. Flow cytometry was employed to quantitatively measure P-gp expression and confocal laser scanning microscopy was utilized to provide visual observation. As shown in Figure 6-16 and 6-17, both quantitative measurement and direct observation showed that there is a distinct difference in P-gp expression level in Caco-2 cells with different postseeding times. That is, cells that were postseeded for longer periods of time (21-28 days) have much higher P-gp expression than cells with shorter postseeding times (7-10 days). This finding clearly explains our previous observation on P-gp inhibitor experiments. Indeed, in the presence of carriers, hGH is subject to a P-gp efflux pump. But when Caco-2 cells are postseeded for short periods of time, P-gp expression level on the cell apical surface is low, so we don't observe an obvious effect of P-gp inhibitors on hGH uptake. However, P-gp becomes highly expressed once Caco-2 cells are developed for longer period of time, thus the effect of P-gp inhibitors becomes significant.

The variability of P-gp expression level on different staged Caco-2 cells was also observed by other investigators. Using western blot analysis, Hosoya (Hosoya et al., 1996) found that Caco-2 cells appear to express P-gp early on in culture, but do not become fully

functional until about day 17. Liang and coworkers (Liang et al., 2000) examined P-gp expression in a traditional Caco-2 cell model (21-25 days postseeding) and an accelerated 3-7 day cell model and observed that P-gp in the accelerated model was significantly less than that in the traditional model. These conclusions are in agreement with our findings. Contrary to these findings, Wil (Wil et al., 1994a; 1994b) reported that the expression level of P-gp in Caco-2 cells keeps constant from day 7 through 22 of culturing time. In addition, the authors observed that the capacity to express P-gp may be lost during long term cultivation. Furthermore, the influence of cell culture conditions and previous drug exposure on P-gp expression levels in Caco-2 cells were also investigated by Anderle and coworkers (Anderle et al., 1998). They showed that experimental conditions such as the cell passage number and timing for trypsinization have contributed to different P-gp expression levels.

The observed variability of P-gp expression level as a function of cell culture conditions, e. g., cell postseeding time, the way of cultivating cells, etc., should be considered by researchers when studying P-gp related issue. Our experience is that one may need to develop a system to standardize P-gp expression, or carefully monitor P-gp expression level for each specific study when coupling P-gp expression with related results.

D. MRP Involvement

It is known that MRP functions as a drug efflux pump, similar to P-gp, to exclude drugs that binds to it. Distinct from P-gp, MRP is a primary active transporter of many

organic anions. Typical substrates for MRP1 that have been studied include cysteinyl leukotriene LTC(4), (Loe et al., 2000), [(3)H]17-beta-estradiol 17-beta-D-glucuronide, and [(3)2,4-Dinitrophenyl-S-glutathion (Hirohashi et al., 2000). MRP is also responsible for transporting a wide range of xenobiotics, including anti-cancer drugs and chemical carcinogens (Payen et al., 1999). Some compounds are found to be MRP but non-P-gp substrates, e. g., carboxy-2',7'-dichlorofluorescein (CF) and calcein, yet some are substrates for both MRP and P-gp. It has been shown that the MRP-overexpressing SW-1573 cells were resistant to doxorubicin, daunorubicin, vincristine, VP-16, colchicine, and rhodamine 123. Moreover, agents such as verapamil that reverse P-gp-mediated resistance are often poorly, or not at all, effective in MRP1-overexpressing cells. Walgren have reported that efflux of quercetin 4'-beta-glucoside was not altered by verapamil, the P-gp inhibitor, but was competitively inhibited by MK-571, an MRP inhibitor (Walgren et al., 2000).

The function of MRP efflux pump can be inhibited by a number of inhibitors. Using primary cultures of rat astrocytes, Decleves reported that indomethacin, probenecid, and sulfinpyrazone increased the uptake of fluorescein, a substrate of MRP but not of P-gp (Decleves et al., 2000). Rifampicins such as rifamycin B and rifamycin SV were found to be able to increase intracellular accumulation of calcein in GLC4/ADR cells by inhibiting its MRP-mediated efflux system (Courtois et al., 1999a). The efflux pump was further found to be sodium and proton independent and ATP dependent, and inhibited by anionic drugs such as indomethacin, indoprofen and rifamycin B (Courtois et al., 1999b; Makhey et al., 1998).

Recent study also suggested that Pluronic P85 block copolymer inhibit MRP (Batrakova et al., 1999; Miller et al., 1999).

The results of the involvement of MRP in carrier enhanced hGH absorption are shown in Figure 6-18 and 6-19. It can been seen the MRP inhibitor indomethacin demonstrated no effect on hGH uptake for both MRP associated cells (Caco-2 cells) and non-MRP associated cells (CAPE cells). So it is concluded that MRP is not involved in the efflux of hGH in the presence of carriers.

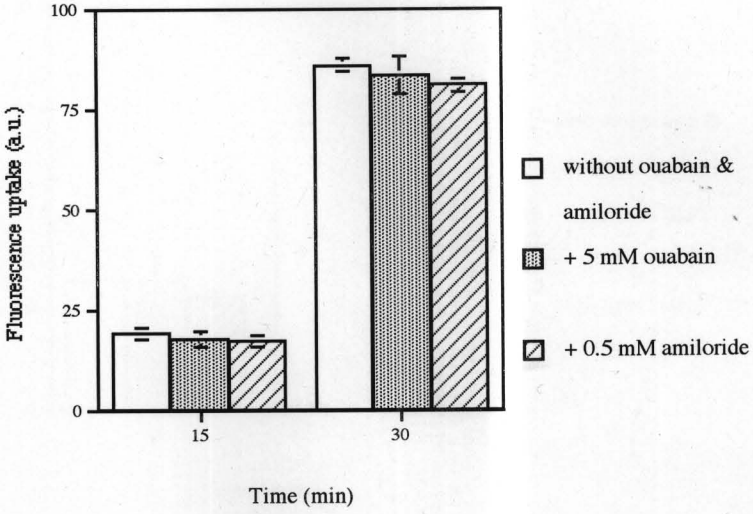


Figure 6-1: Effect of 5 mM ouabain and 0.5 mM amiloride on hGH uptake on Caco-2 cells in the presence of 37.5 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

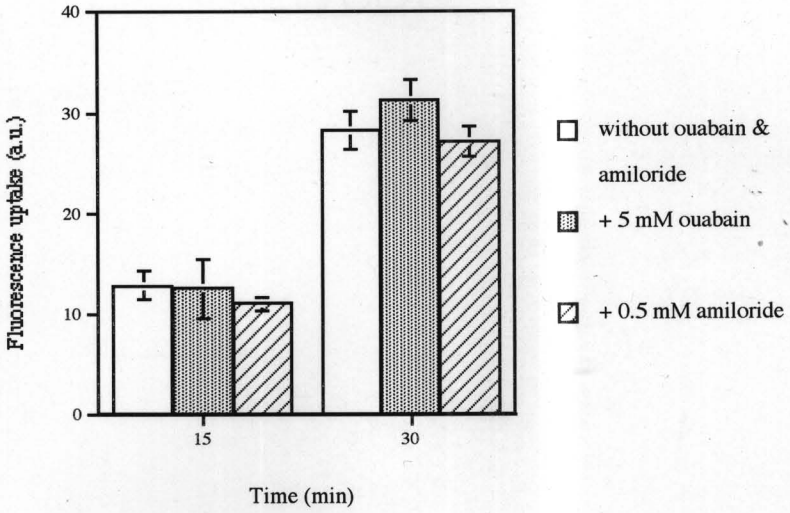


Figure 6-2: Effect of 5 mM ouabain and 0.5 mM amiloride on hGH uptake on CAPE cells in the presence of 25 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

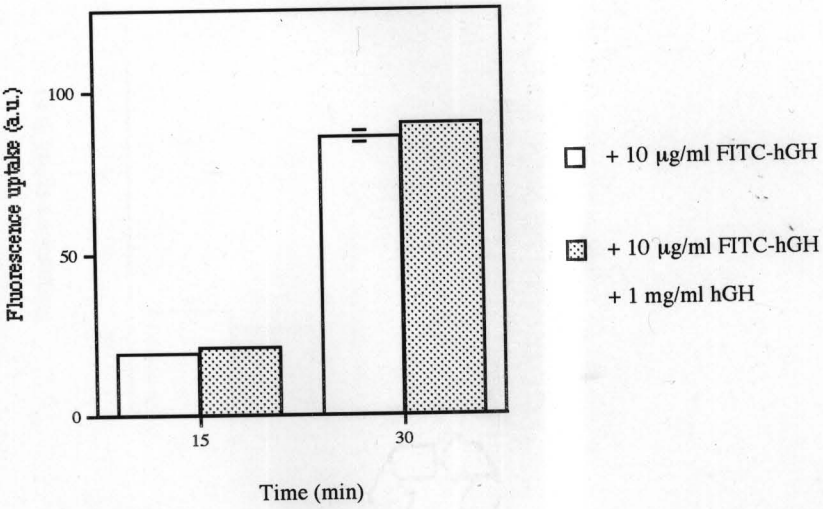


Figure 6-3: Effect of 100 fold excess unlabeled hGH on FITC-hGH uptake on Caco-2 cells in the presence of 37.5 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

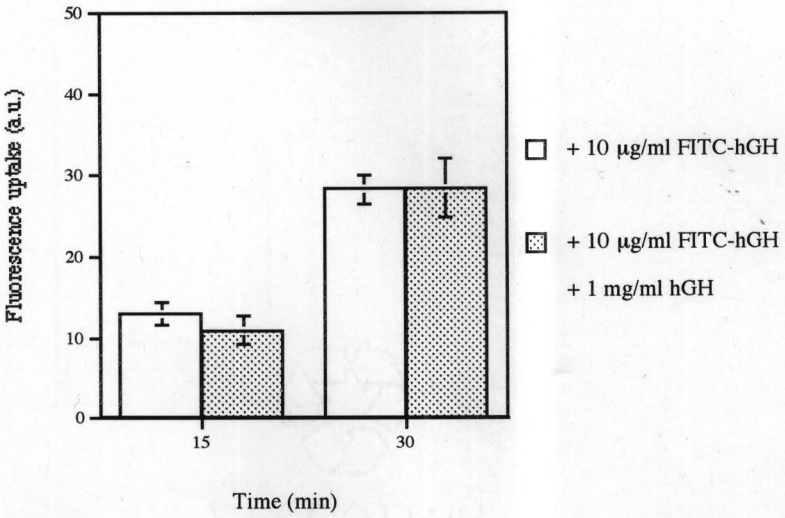


Figure 6-4: Effect of 100 fold excess unlabeled hGH on FITC-hGH uptake on CAPE cells in the presence of 25 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

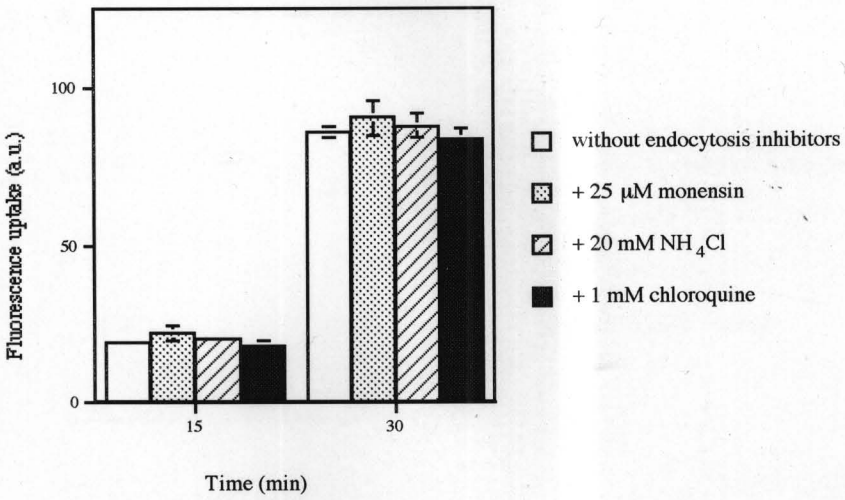


Figure 6-5: Effect of endocytosis inhibitors on hGH uptake on Caco-2 cells in the presence of 37.5 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

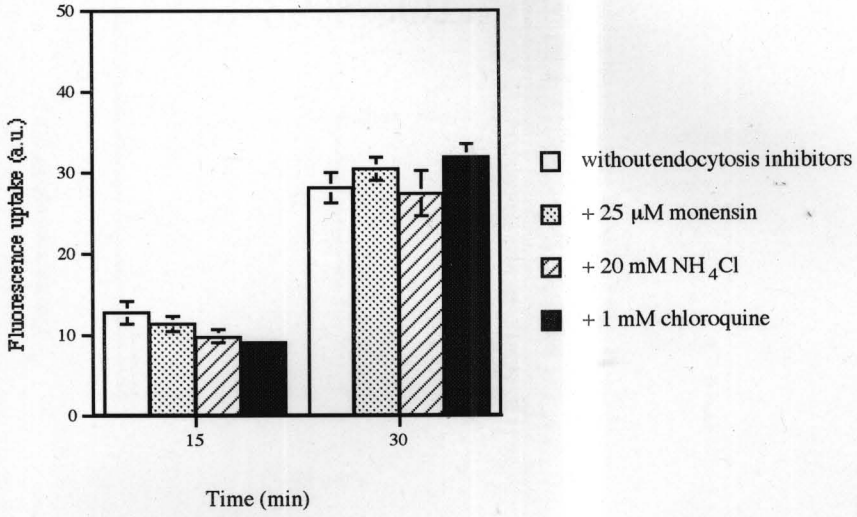


Figure 6-6: Effect of endocytosis inhibitors on hGH uptake on CAPE cells in the presence of 25 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

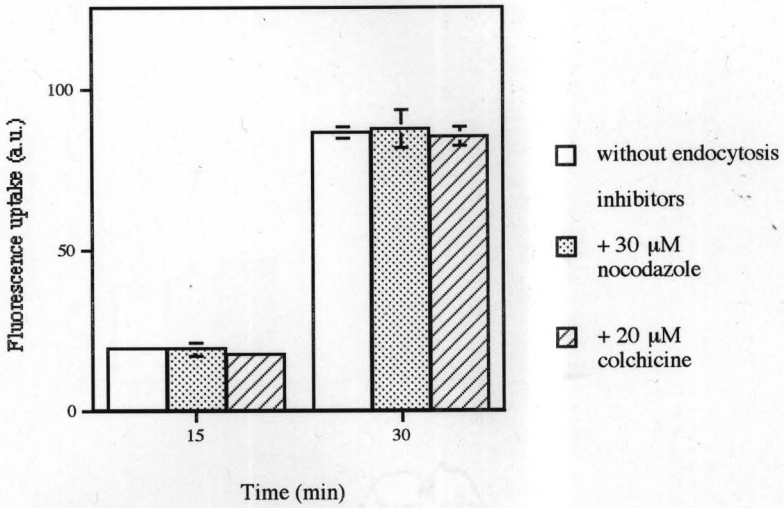


Figure 6-7: Effect of endocytosis inhibitors on hGH uptake on Caco-2 cells in the presence of 37.5 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

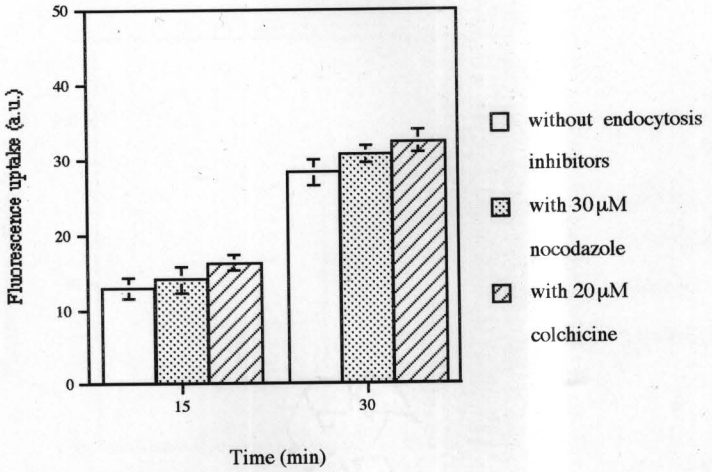


Figure 6-8: Effect of endocytosis inhibitors on hGH uptake on CAPE cells in the presence of 25 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

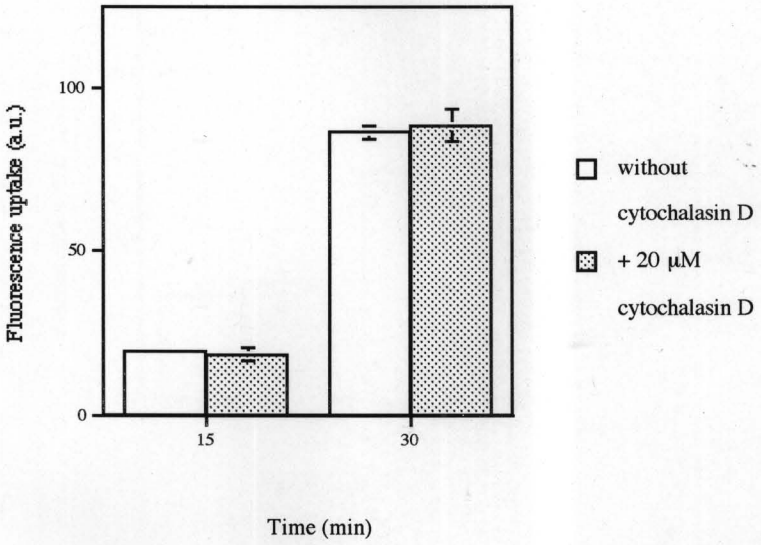


Figure 6-9: Effect of endocytosis inhibitors on hGH uptake on Caco-2 cells in the presence of 37.5 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

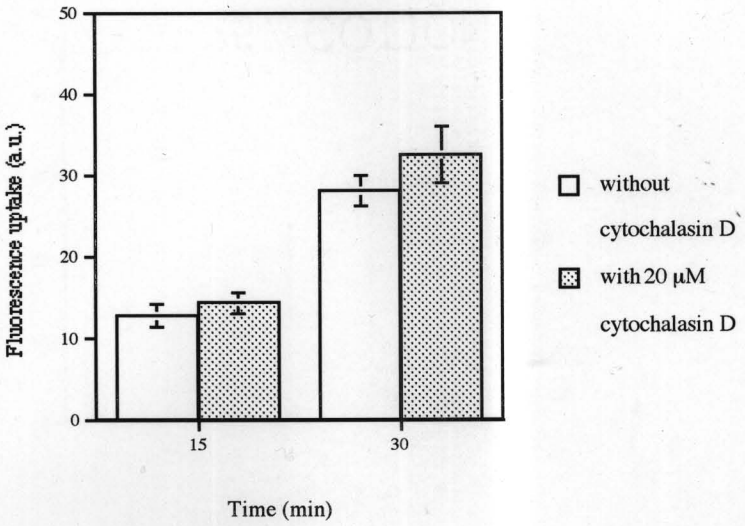


Figure 6-10: Effect of endocytosis inhibitors on hGH uptake on CAPE cells in the presence of 25 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

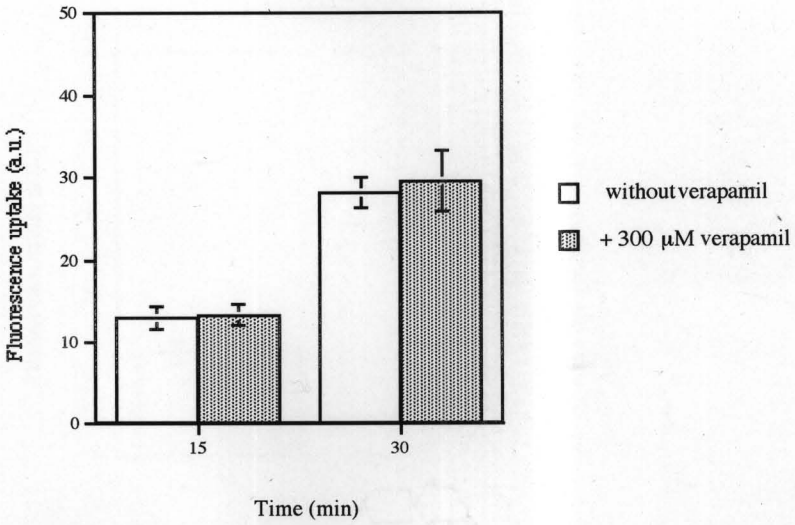


Figure 6-11: Effect of 300 μM verapamil on hGH uptake through apical side of CAPE cells in the presence of 25 mg/ml of E414. Cells were pretreated with inhibitors for 30 minutes followed by 15 and 30 minutes treatment together with hGH and E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

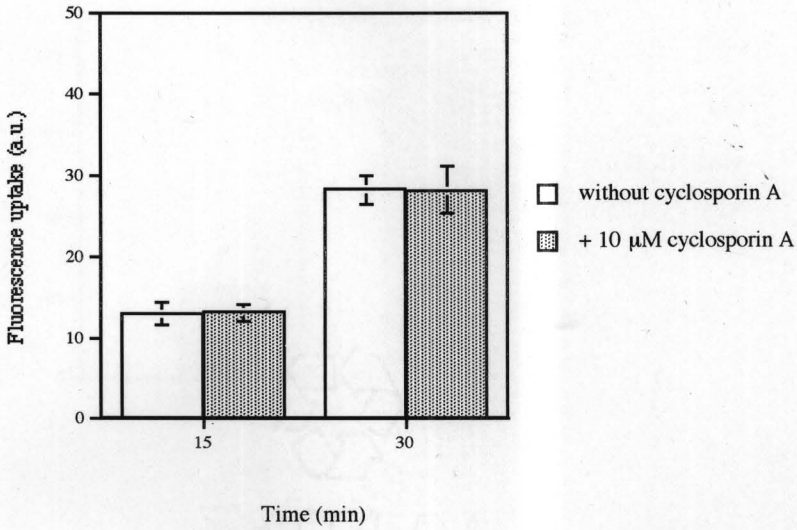


Figure 6-12: Effect of 10 μM cyclosporin A on hGH uptake through apical side of CAPE cells in the presence of 25 mg/ml of E414. Cells were pretreated with inhibitors for 30 minutes followed by 15 and 30 minutes treatment together with hGH and E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

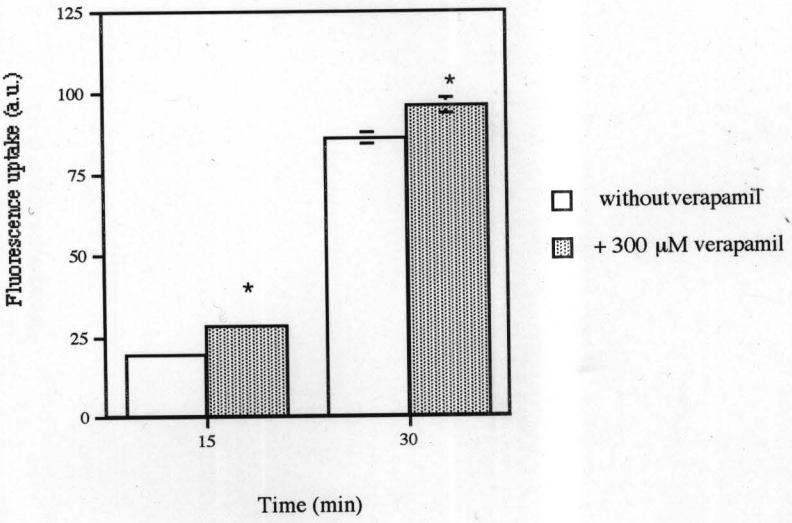


Figure 6-13: Effect of 300 μM verapamil on hGH uptake through apical side of 7-10 days postseeded Caco-2 cells in the presence of 37.5 mg/ml of E414. Cells were pretreated with inhibitors for 30 minutes followed by 15 and 30 minutes treatment together with hGH and E414 (error bars indicate SEM, n=3-6, * denotes statistical significance of $p<0.05$).

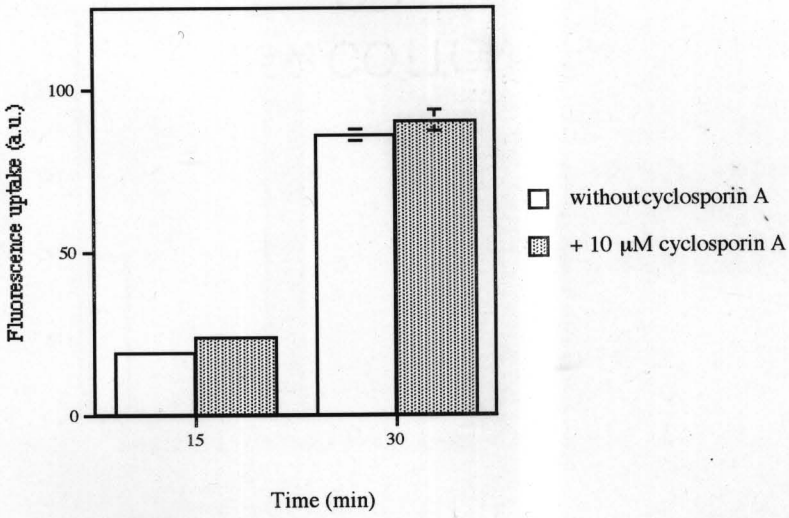


Figure 6-14: Effect of 10 μM cyclosporin A on hGH uptake through apical side of 7-10 days postseeded Caco-2 cells in the presence of 37.5 mg/ml of E414. Cells were pretreated with inhibitors for 30 minutes followed by 15 and 30 minutes treatment together with hGH and E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

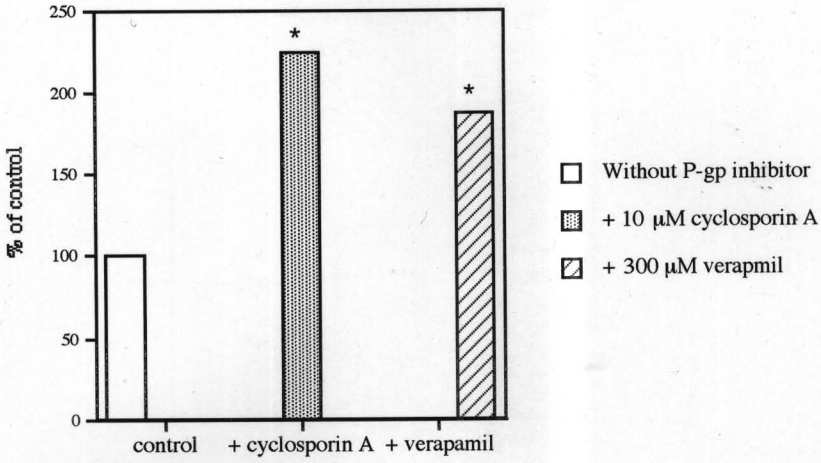


Figure 6-15: Effect of P-gp inhibitors on hGH uptake through apical side of 28 days postseeded Caco-2 cells in the presence of 37.5 mg/ml of E414. Cells were pretreated with inhibitors for 30 minutes followed by 15 and 30 minutes treatment together with hGH and E414 (error bars indicate SEM, n=3-6, * denotes statistical significance of $p<0.05$).

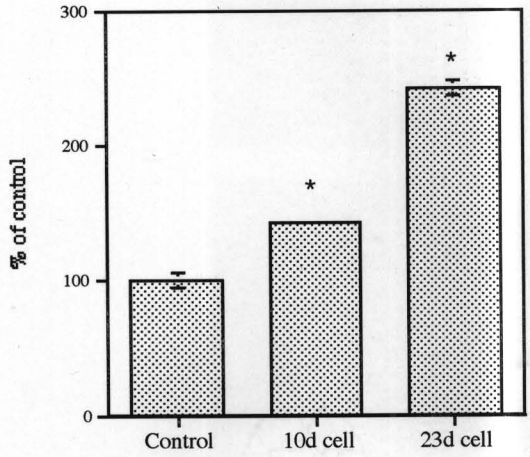


Figure 6-16: P-gp expression on different stages of Caco-2 cells. Cells were postseeded on polycarbonate filters for 10 days and 23 days. P-gp expression was detected by flow cytometry (error bars indicate SEM, n=3, * denotes statistical difference of $p<0.05$).

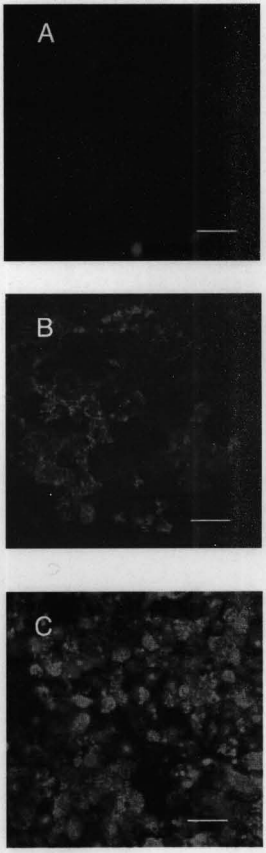


Figure 6-17: Immuohistochemical detection of P-gp expression level on different stages of Caco-2 cells shown by confocal laser scanning microscopy images. A, control experiment; B, 7 days postseeded Caco-2 cells; C, 28 days postseeded Caco-2 cells. Bar length = 20 μm.

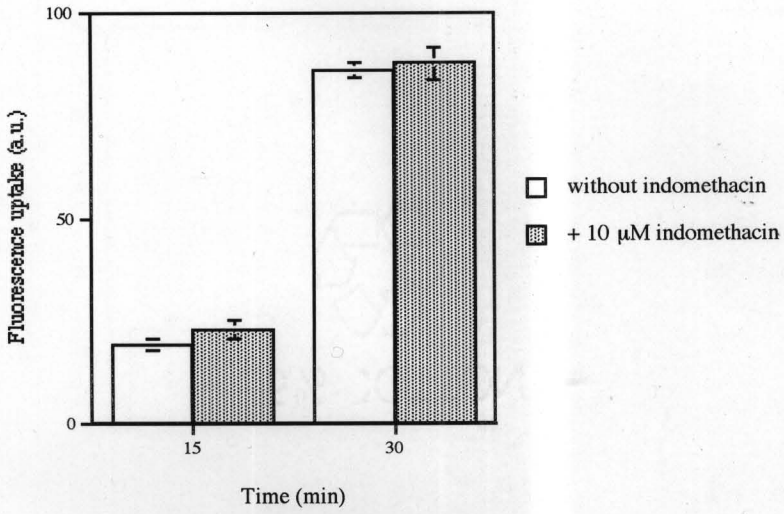


Figure 6-18: Effect of 10 μM indomethacin on hGH uptake through apical side of Caco-2 cells in the presence of 37.5 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

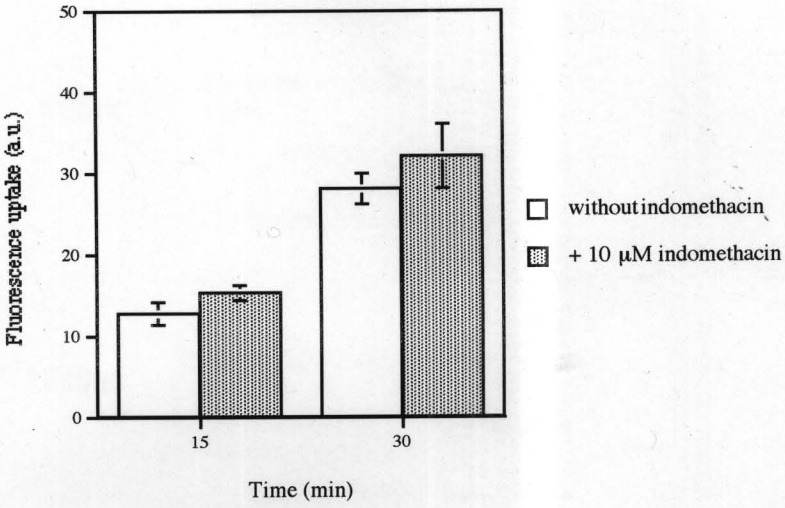


Figure 6-19: Effect of 10 μM indomethacin on hGH uptake through apical side of CAPE cells in the presence of 25 mg/ml of E414 (error bars indicate SEM, n=3-6, no statistical significance was seen between the inhibitor treated cells and control cells).

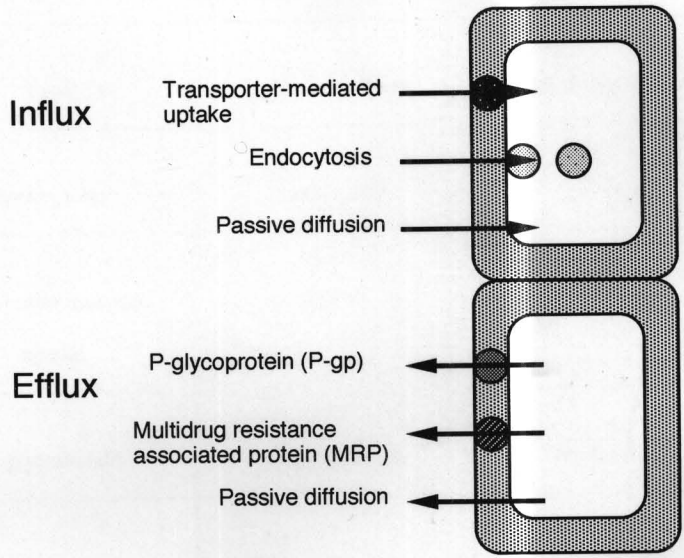


Figure 6-20: Schematic diagram of cellular influx and efflux mechanisms.

	Caco-2 cell	CAPE cell
Type	Epithelial cell line	Endothelial cell line
Endocytosis	Less active	Active
Transporter-mediated uptake	Active	Less active
P-glycoprotein	Overexpressed	No evidence
Multidrug resistance-associated protein	Overexpressed	No evidence

Table 6-1: Comparison of uptake/efflux mechanisms associated with two different *in vitro* models.

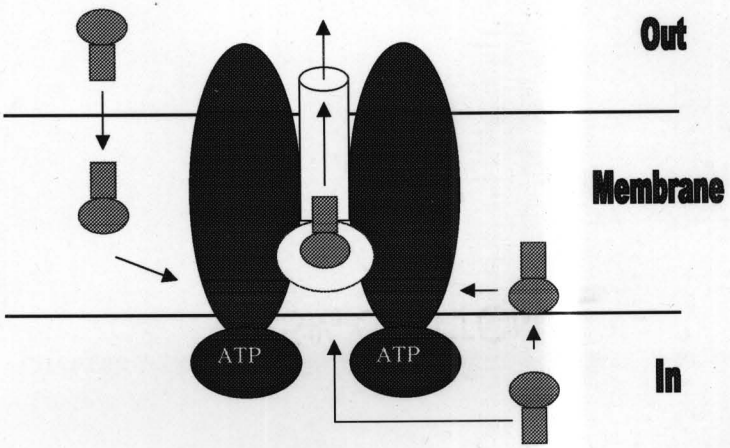


Figure 6-21: Possible mechanism of action of P-glycoprotein as a "hydrophobic vacuum cleaner." The two features of the model, the ability of the multidrug transporter to remove drugs directly from the plasma membrane and the association of both halves of the transporter to form a single transport channel, are illustrated. Modified from reference (Gottesman, 1993).

CHAPTER 7: CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

I. Conclusions

A comprehensive study that investigated the mechanism(s) of a carrier enhanced protein absorption technology has been carried out. Utilizing two *in vitro* cell models, a series of studies including carrier toxicity towards major subcellular components, protein transport pathways and subcellular localization, and the mechanism(s) of enhanced protein uptake and efflux, were conducted. The quantitative measurement results, coupled with direct microscopy observations, have provided insight as to the mechanism(s) through which carrier enhanced protein absorption occurs. The major conclusions that can be derived from this work are the following:

1. Carrier N-[8-(2-hydroxybenzoyl)amino]caprylic acid, the main model carrier chosen in this study, functions effectively in enhancing human growth hormone absorption at a concentration above 25 mg/ml. It demonstrates no substantial damage to major subcellular components such as the cell membrane, cell lysosome compartment and cell nucleus when treating cells for a short period of time. However, extended period of cell treatment with this carrier results in some perturbation to the subcellular components. This cytotoxicity is both time dependent and carrier concentration dependent. The damage to cells is completely reversible for cells that were treated with low, but effective, concentrations of carrier. Yet a high concentration of carrier treatment results in apparently irreversible damage to cells. Meanwhile, the carrier effect on cells is also pertinent to the relative location of these subcellular components to carrier. Components

that are directly exposed to carrier, such as paracellular tight junctions and cell membranes are damaged to a greater extent. On the other hand, subcellular organelles such as the cell nucleus and lysosome compartment, were affected less.

2. Following two labeled hGH molecules, confocal microscopy studies directly demonstrate that hGH alone cannot be taken up to a visible extent as detected by confocal microscopy whereas in the presence of carrier, hGH absorption is significantly enhanced. Quantitative measurements of hGH uptake in cells using flow cytometry show the same phenomena.

3. Differs from classical penetration enhancers, enhanced hGH uptake by carrier is not predominately through compromising tissue or cell membranes. Both visualized evidence and quantitative measurements demonstrate a significant enhancement of hGH uptake occurring before cytotoxicity emerges to a detectable level.

4. There are interactions between carrier and protein in order for the carrier to exert its enhancement function. This is illustrated by two experiments. First, no obvious hGH enhancement happens when cells were treated with carrier and protein separately whereas the enhancement is significant when both protein and carrier were applied together. Secondly, carrier enhanced protein absorption occurs in a selective and specific manner.

5. The interaction between carrier and protein has no relationship with cell type. The same carrier concentration, which causes an abrupt enhancement in hGH uptake, was demonstrated in two different cell lines that possess different uptake/efflux mechanisms.

6. Facilitated by carrier, proteins maintain a hydrophobic form while transporting. This is demonstrated by three experiments. First, enhanced hGH preferentially uses the transcellular pathway that is favored by hydrophobic molecules. Secondly, an intensive signal observed in the periphery of cell membrane delineation indicates that a quantity of hGH, being hydrophobic, was entrapped in the lipophilic cell membrane bilayer. Thirdly, in the presence of carrier, hGH can be effluxed by P-gp, a drug efflux pump, which recognizes hydrophobic compounds.

7. The paracellular pathway is also affected when carrier enhanced protein absorption occurs. This is reflected by the drop of TEER values caused by carrier. However, this pathway should not be considered as a major pathway for enhanced hGH absorption. This is because that, while opening of the tight junctions was caused by D-series of carriers, they fail to enhance hGH absorption. Enlarged confocal microscopy image has shown only a limited amount of hGH in the space between cells.

8. Uptake of enhanced hGH is neither through transporter-mediated uptake, nor through endocytosis. It is likely a passive process. The efflux of hGH in the present of carrier is not through an MRP efflux pump, but through the P-gp efflux system.

In conclusion, hGH absorption can be significantly enhanced in the presence of carrier. The carrier presents little or no toxicity to cells while being effective. However, this cytotoxicity is not the major mechanism for carrier enhanced protein absorption. Human growth hormone maintains a hydrophobic state in the presence of carrier, therefore it transports mainly through the transcellular pathway. In the presence of carrier, hGH diffuses passively into cells, and can be effluxed out by a P-gp efflux pump.

Based on the above, we reaffirm that this carrier enhanced protein absorption can be pictured as the carrier interacting with certain regions of the protein. The initial recognition depends on whole protein conformation, or the local conformation of certain regions of protein, and thus is protein specific. This interaction then induces and further stabilizes a change of protein conformation to a form with more hydrophobic regions being exposed. As a consequence, this more hydrophobic protein is able to use the transcellular pathway and thus become more transportable.

II. Future Work Suggested

The invention of the carrier enhanced protein absorption technology started with the finding that certain synthesized amino acid derivatives enable an increase in oral bioavailability of certain proteins while presenting no obvious damage to the tissue. Two major approaches have been followed to understand the system. One focused on the

biological relevance of this system, i. e., investigation of the mechanisms of this technology and its application in a biological system. This part of the study has been carried out from animal level, to a tissue level, then to a cellular level, and finally to a subcellular level. The findings obtained from this series of studies have provided mechanistic insight to explain biological related phenomena of carrier enhanced protein absorption. On the other hand, the other part of this study, which focuses on the solution properties of the carrier and protein mixture, has been hindered by the physico-chemical properties of the carriers as well as the complicated structures of high molecular weight protein. Most methods commonly used to investigate the interaction between macromolecules and small compounds, such as circular dichroism and far-UV analysis, cannot be utilized in this system mainly because of carrier interference. So far, little information is available on the physical aspect of the proposed enhancement mechanism. Therefore, more work needs to be done to test the hypothesis and verify the proposed mechanism. Direct evidence must be obtained from physico-chemical characterization of protein and carrier interaction. To achieve this goal, one possible way is to select a model protein that has a relatively simple structure. In this case, certain methods for protein analysis, such as NMR, could be utilized. The other way is to develop new carriers that have no interference with protein analysis, i.e., carriers that are optical clear. In this way, most traditional analytical methods could be used in the study.

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