CHAPTER I

INTRODUCTION

1. Rationale for development of ester of NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) are generally used as anti-inflammatory, analgesic, and antipyretic agents. The indications of NSAIDs range from osteo-rheumatoid arthritis, various pain states such as biliary and colonic pain, to possibly colon cancer prevention (Day et al., 2000). In spite of its broad pharmacological activities, clinical use of NSAIDs is often limited. Adverse reactions associated with NSAIDs are always reported and shown to affect several biological systems including kidney, gastrointestinal (GI) tract, liver, and blood vessels (Day et al., 2000). Frequent adverse effects include renal dysfunction and nephrotoxicity (Day et al., 2000), liver damage (Day et al., 2000; Purcell et al., 1991; Wood et al., 1985; Zimmerman 1981), and neurological disorders (Day et al., 2000). Pharmacological activities of the NSAIDs exert through inhibition of cyclooxygenase (COX), an important enzyme involving in prostaglandin and thromboxane biosynthesis (Figure 1). As illustrated in Figure 1, phospholipase is activated upon receiving cellular stimulus and cleaves arachidonic acid from the membrane phospholipids. Free arachidonic acid is used as a substrate for the production of either leukotrienes or prostanoids. The leukotriene biosynthetic pathway is initiated by the activity of the lipoxygenase family of enzymes. Prostanoid biosynthesis is initiated by the action of prostaglandin synthase, also know as cyclooxygenase (COX). The COX enzyme carries out a two-step reaction; arachidonic acid is first converted, by a *bis* oxygenation, to prostaglandin G_2 (PGG_2). The short-lived PGG_2 product is subsequently converted, by a hydroperoxidase reaction that occurs at a distinct site on the enzyme, to PGH_2. PGH_2 serves as a substrate for the production, by various prostanoid synthases, to the alternative prostaglandins, thromboxanes, and prostacyclins. Cyclooxygenase activity is present in nearly all tissues, with only a few exceptions. The determination of which prostanoid is produced in various cell types is the result of the alternative expression of tissue and cell-type restricted prostanoid

synthases (e.g., PGD_2 synthase, PGE_2 synthase, etc.). Prostaglandins have been implicated, as regulators, in wound healing, platelet aggregation, leuteinization, ovulation, parturition, water balance, kidney filtration, and hemostasis (Herschman et al., 2003).

NSAIDs inhibit both isoforms of COX, i.e. COX-1 and COX-2, though with variable ability (Warner et al., 1999). Suppression of COX-1 activity can disrupt the protective effects of the prostanoids, i.e. PGI_2 and PGE_2, on gastric epithelium. Gastrointestinal reactions were thought to be related to inhibition of prostaglandin biosynthesis at intestinal mucosa by NSAIDs (Weber 1984), and partly due to intestinal mucosa irritation from acidic properties of NSAIDs (Warner 1999).

Several approaches have been developed to diminish adverse effects encountered with NSAIDs. Two most striking strategies include the development of selective COX-2 inhibitors and prodrugs of NSAIDs whose acidic functional group temporarily masked. Although selective COX-2 inhibitors (Coxibs) have demonstrated an improved GI tolerability profile compared with non-selective NSAIDs (Bombardier et al., 2000; Langman et al., 1999; Silverstein et al., 2000), cardiovascular side effects associated with Coxibs were well characterized. Rofecoxib, a first generation Coxibs approved by USFDA in 1999, was withdrawn from the market on September 2004 because of an excess risk of myocardial infarction and strokes (Topol, 2004). Moreover, the costly sulfur-containing Coxibs were contraindicated in situations where hypersensitivity to sulfur-containing products is concerned. Adverse manifestations of the Coxibs as well as its cost may prevent the clinical implications of these drugs.

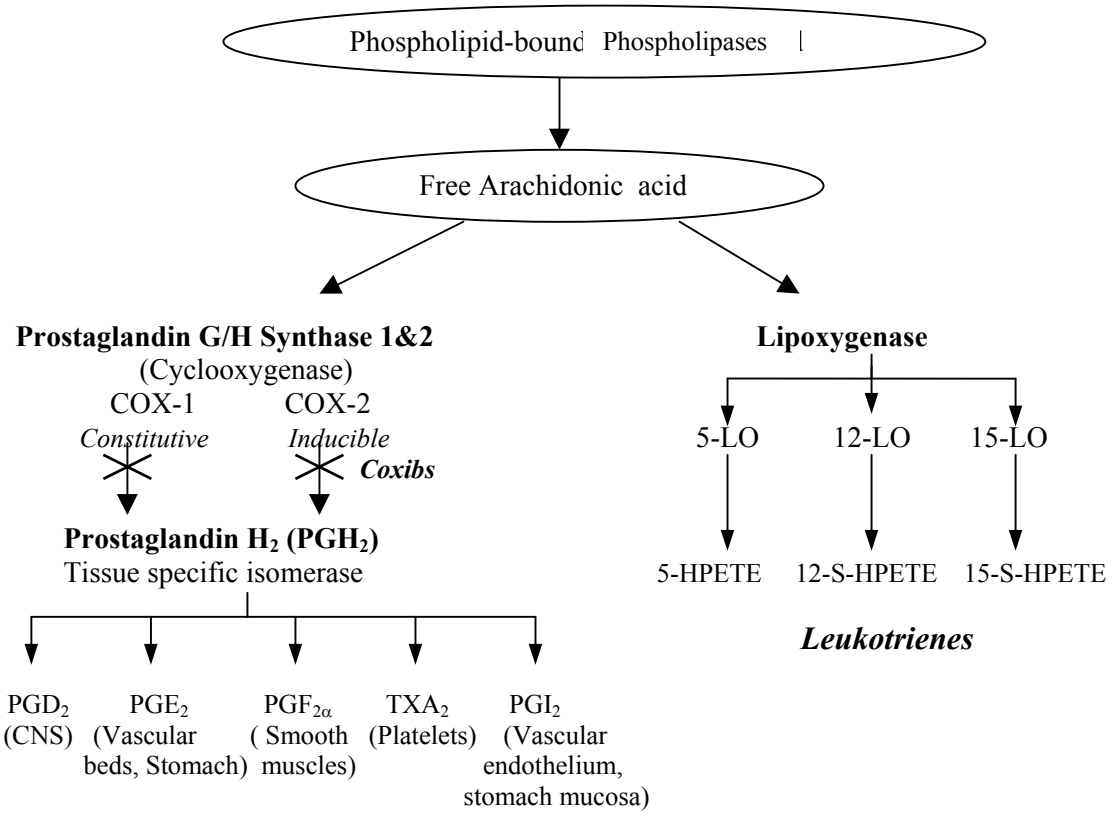


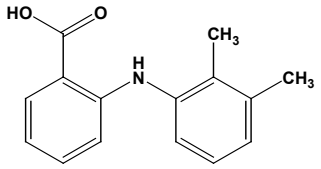
Figure 1 Arachidonic acid metabolism. The major metabolites of arachidonic acid produced by the cyclooxygenase (COX) and lipooxygenase (LO) pathways are indicated. Examples of tissues in which each prostanoids exert prominent effects are indicated in parentheses. (Thun et al., 2002)

Abbreviation: PGD_2: Prostaglandin D_2; PGE_2: Prostaglandin E_2; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; TXA_2: Thromboxane A_2; PGI_2: Prostaglandin I_2; HPETE: Hydroxyperoxyeicosatetraenoic acid; HETE: Hydroxyeicosatetraenoic acid.

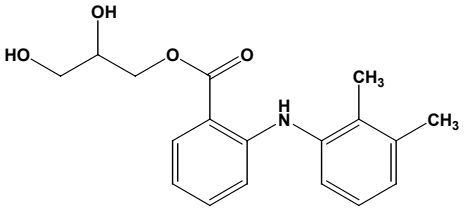
Prodrugs of NSAIDs have long been gaining considerable attentions in terms of reducing gastric irritation and bleeding caused by NSAIDs. Such prodrugs, common esters of carboxylic group, regenerated the parent compound either non-enzymatically or enzymatically and exhibited reduced GI toxicity and antiinflammatory properties. Due to ubiquitous distribution of the esterases in several organs and tissues, bioconversion of the ester pro-moieties could be easily obtained. For instances, amide and ester prodrugs of ibuprofen and naproxen were reported for their reducing GI irritation of these drugs (Shanbhag et al., 1992; Tozkoparan et al., 2000). Various

esters of indomethacin, made from conjugation of the parent acid to oligoethylene glycols, exhibited equivalent anti-inflammatory activity to that of parent indomethacin but with much less ulcerogenicity (De Caprariis et al., 1994). Some octyl ester of indomethacin were highly accumulated in the liver and slowly released parent drug (Ogiso et al., 1994). Another widely used NSAIDs, diclofenac, was derivatized to morpholinoalkyl esters (Tammara et al., 1994) and shown to be rapidly degraded in rat plasma and *in vivo*. Among alkyl esters of diclofenac studied, the ethyl ester was shown to possess 30% increase in oral bioavailability but reduced GI irritation (Tammara et al., 1994).

Mefenamic acid is a potent non-prescribed NSAIDs widely used for relief of mild to moderate pain including primary dysmenorhea. Similar to other NSAIDs, the most common adverse effects observed with mefenamic acid are nausea (1-10%), abdominal cramp (1-10%), diarrhea (1-10%), duodenal ulcer with bleeding or perforation, and gastritis (1-10%) (Lacy, 2004). Thus far, very few reports have been addressed for developing mefenamic acid as prodrugs, but a study by Ghariabeh and co-workers which only hydrolytic profiles were shown (Jiliani et al., 1997). In this study, various types of mefenamic acid esters were synthesized with masking the acidic functional group of mefenamic acid temporarily as the primary goal. These prodrugs, illustrated in Figure 2, include 2,3-dihydroxypropyl 2-(2,3-dimethy lphenylamino)benzoate (**1**), 2-(2-oxopyrroldin-1-yl)ethyl 2-(2,3-dimethylphenyl amino)benzoate (**2**), 2-morpholinoethyl 2-(2,3-dimethylphenylamino)benzoate (**3**), 2-(pyrrolidin-1-yl)ethyl 2-(2,3-dimethyl phenylamino)benzoate (**4**), 2-(piperidin-1-yl) ethyl 2-(2,3-dimethylphenylamino)benzoate (**5**), 4-acetamidophenyl 2-(2,3 dimethyl phenylamino) benzoate (**6**), and 5-quanidino-2-(2-(2,3-dimethylphenyl amino) phenyl amido)pentanoic acid (**7**).



Mefenamic acid



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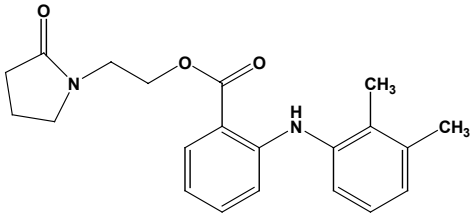

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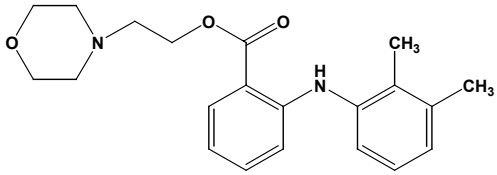

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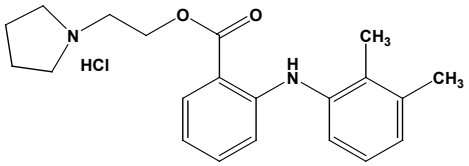

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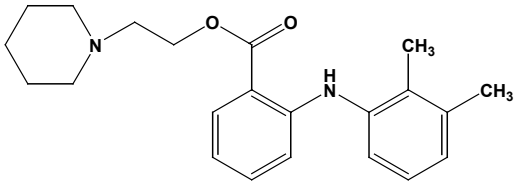

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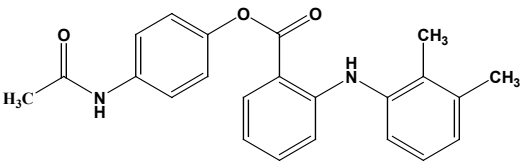

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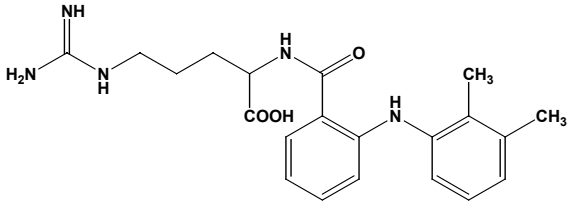

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Figure 2 Chemical structures of mefenamic acid and its esters used in this study.

Since oral administration is the most acceptable and convenient route of drug administration. Oral therapy of NSAIDs was found to be very effective. Therefore current study was initiated to select prodrugs of mefenamic acid possessing favorable physicochemical properties, high enzymatic bioconversion, and satisfactory transport characteristics for oral delivery. With this regards, esters **1**-**7** were examined for their solubility, lipophilicity, and stability toward chemical hydrolysis. Susceptibility of the compounds to esterase-mediated hydrolysis was also investigated. Permeability across intestinal epithelial were also evaluated using Caco-2 monolayer as the model.

2. Absorption across intestinal membrane

2.1 Mechanisms of intestinal membrane permeation

A drug molecule is subjected to a variety of physical and biological barriers during traverse process from the site of administration to the site of action. During the passage from the intestinal lumen into blood, a solute encounters sequential barriers, such as the mucus layer, the unstirred water layer, the single layer of epithelial cells, and the underlying tissues with blood and lymph vessels. The intestinal epithelium is comprised of two parallel barriers: the cell membrane and the tight junction. In addition, there is a metabolic barrier due to many metabolizing enzymes presented in the cell (Artursson, 1991). It is generally assumed that the permeation of the epithelial lining of the intestine is the rate-limiting step (Artursson and Karlsson, 1991; Fagerholm and Lennernas, 1995).

Transport of drugs across the intestinal epithelium may occur by one or more different routes, as illustrated in Figure 3. Two major mechanisms responsible in crossing the epithelial membrane are transcellular and paracellular routes. Since several active mechanisms for drug transport across intestinal epithelium have been discovered, it has been accepted that multiple transport system rather a single transcellular transport could be accounted for intestinal absorption. Transcellular process largely account for the transport, probably due to the enormous surface area available for the permeation to occur (Artursson et al., 2001), while paracellular route is a common transport pathway for small hydrophilic molecules. Passive diffusion is

generally accounted for both transcellular and paracellular, however, active processes may contribute to the permeability of drugs transported by transcellular pathway. Carrier-mediated active transport is evidenced for β-lactams (Dantzig and Bergin, 1990; Inui et al., 1992), ACE-inhibitors (Friedman and Amidon, 1989) and di/tri peptides (Friedman and Amidon, 1989; Inui et al., 1992). Moreover, intrinsic factor-cobalamin complexes could be transported across cellular barrier via vesicle by receptor-mediated endocytosis (Dix et al., 1990; Ramanujam et al., 1991).

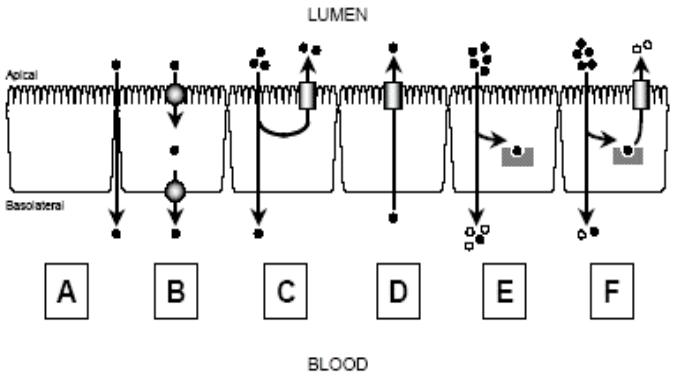


Figure 3 Transport pathways across intestinal epithelium : paracellular (A); carrier-mediated mechanisms at the apical and/or basolateral membranes (B); active secretion via efflux transporters at the apical membrane (C); apical efflux transport of compounds presenting in blood (D); metabolizing by intracellular enzymes prior to entry the blood (E); apical efflux transporters and intracellular metabolizing enzymes co-ordinate metabolizing and secreting compounds. (Chan et al., 2004)

2.1.1 Passive Transcellular Transport

In small intestine, the surface area of the brush border membrane is >1,000 fold larger than paracellular surface area (Pappenheimer and Reiss, 1987). It is likely that this route significantly contributes to the overall transport of most drugs *in vivo* (Artursson et al., 2001). The characteristics of passive diffusion process is energy independent, flux is proportional to concentration and rate is independent of direction (Burton et al., 1997). The first step of passive transcellular transport is the penetration through the apical membrane, diffusion through the cytoplasm and finally exits through the basolateral membrane. Diffusion of small molecule in the cytoplasm is

normally a rapid process, thus the rate of passive transcellular permeability is mainly determined by rate of transport across the apical membrane (Muranishi 1990). Factor to be considered in passive transcellular transport is that epithelial cells are polarized. There are appreciably different membrane properties between the apical and basolateral sided, such as protein and lipid compositions, and thus different permeability properties. While contributions of active transporters and efflux mechanisms to *in vivo* absorption are still to be determined, numerous studies have indicated that most of rapidly and completely absorbed drug are transported by passive transcellular route (Artursson et al., 2001; Stenberg et al., 2000).

2.1.2 Passive Paracellular Transport

Paracellular transport, taken up by hydrophilic molecules occurring via water-filled pores between the cells, is generally considered to be a passive process that follows Fick's Law (Burton et al., 1997), although a saturable and substrate specific mechanism were reported by some investigators (Gan et al.,1998; Lee and Thakker, 1999). The surface area of the water filled pore constitute only a small fraction (0.01-0.1%) of the total intestinal membrane surface area (Artursson et al., 2001; Nellans 1991), tight junctions further restrict the solute passage from a seal between adjacent epithelial cell (Madara 1988). The limitation of solute transport by this route which mainly exists at the distal part of the intestine (Artursson et al., 1993; Ungell et al., 1998), made the paracellular an inefficient pathway of epithelial transport.

2.1.3 Carrier mediated transport and efflux

Active and facilitated transport. Characteristics of carrier mediated transport are energy dependent, substrate specificity, competitive, and saturability (Burton et al., 1997). Intestinal absorptive cells express a number of carrier systems which are responsible for the absorption of vitamins, bile acids, amino acids, nucleosides, and di/tripeptides (Hidalgo et al., 1996). These transport proteins embedded in the cell membrane therefore helps in extracting nutrients and other essential compounds from the luminal side. This transport route is available to a limited numbers of drugs, such as β-lactam antibiotics, ACE-inhibitors and phosphate analogs, all of which are

structurally similar to the endogenous substrates of transport proteins (Hidalgo et al., 1996; Lee 2000). Substrate specificity is not absolute and saturation occurred at high drug or nutrient concentrations will result in a decreased absorbed fraction, especially when the drug has low passive permeability (Artursson et al., 2001; Chong et al; 1996; Lee et al., 1997). Due to variation in degree of expression of the transporters in different regions of the intestinal tracts (Ungell et al., 1998), the absorption of substrate should be better in the area with high expression of the carrier proteins. Apparently, carrier mediated mechanisms enhance the transcellular permeability to a limited number of drugs. The enhancement depends not only on the structural similarity between the drug molecule and endogenous substrate of the transporter, but also on concentration and physiological factors (Stenberg, 2001).

Receptor-mediated Transcytosis. This route can occur when solute binds to a receptor on cell membrane surface and is then internalized by endocytosis. The endocytotic vesicle formed is then proceeds to the opposite membrane surfaces. This pathway is mainly considered as a route for highly potent drugs (such as peptides) which are excluded from the other transport pathway due to their size (de Aizpurua and Russell-Jones, 1988). It is considered as a low capacity process and less contribution to the overall transport of the drugs. For example, transport of vitamin B12 which requires specific binding to an intrinsic factor followed by endocytosis/transcytosis across enterocytes from mucosal to serosal direction (Dix, 1990). Binding of vitamin B12-intrinsic factor complex to a specific receptor was very efficient, however, only 1-2% of the oral dose of vitamin B-12 is absorbed. The fact that only 1,000 receptors found per enterocyte may explain the low extent of absorption of vitamin B12 (Donaldson, 1987). Thus, the potential utility of this pathway to achieve carrier-mediated enhancement of intestinal drug absorption appears to be very low.

Efflux mechanisms. In contrast to the carrier mediated mechanisms that promote drug permeability, efflux proteins have the potential to limit absorption by extruding drugs from serosal to mucosal direction. Drug efflux in intestinal tract is often attributed to ATP-biding cassettes (ABC) transport protein, P-glycoprotein (P-gp) and multidrug resistance protein (MRP), located at the apical membrane of epithelial tissues. These proteins are termed multidrug resistance (MDR) proteins. The outward

orientation of these ABC transporters suggest the importances of these transporters in protecting oral absorption of xenobiotics, helping removal of xenobiotics from the body, and limiting the penetration of compounds through the blood brain barrier (Ayrton and Morgan, 2001). These proteins are highly expressed in pharmacological barriers or tissues involving drug disposition, such as the brush border membrane of intestinal epithelial cell, the biliary cannalicular membrane of hepatocytes, and the luminal membrane of proximal tubule of the kidney (Bodo et al., 1993). The significance of these drug efflux transporters on in vivo disposition has been receiving attention and addressed for some clinically important drugs. For example, P-gp limits the intestinal absorption of digoxin, talinolol, and cyclosporin after oral dosing (Lown et al, 1997; Schwarz. et al, 2000; Sparrheboom et. Al., 1997; Verschraagen, 1999), excrete paclitaxel into the intestine, and limits penetration into central nervous system of some HIV protease inhibitors (Kim et al., 1998; Polli et al., 1999). Since MDR transporters play key roles in transport processes, the interaction between pharmaceuticals and MDR is essential information for new drug development.

2.2 Factors affecting gastrointestinal absorption

Low and variable bioavailability is often the main reason for terminating further development of oral pharmaceutical products. Fraction of the drug reaching the systemic circulation or site of action in intact molecule or unchanged form after oral administration is a result of a rather complex series of events (Figure 4). Factors affecting these procedures can be divided into 2 categories: physicochemical and physiological factors. Physicochemical parameters include solubility, molecular size, partition coefficient, and chemical and enzymatic stability (Grass, 1997). These parameters depend largely upon the physicochemical properties of the compound. Aqueous solubility or dissolution in GI fluid dictates the sufficiency of the drug molecule being solubilized in certain time for absorption. Molecular size and partition coefficient governs the interaction of a compound with intestinal membrane. The stability of a compound to chemical degradation will determine if the molecule is intact long enough for absorption to occur.

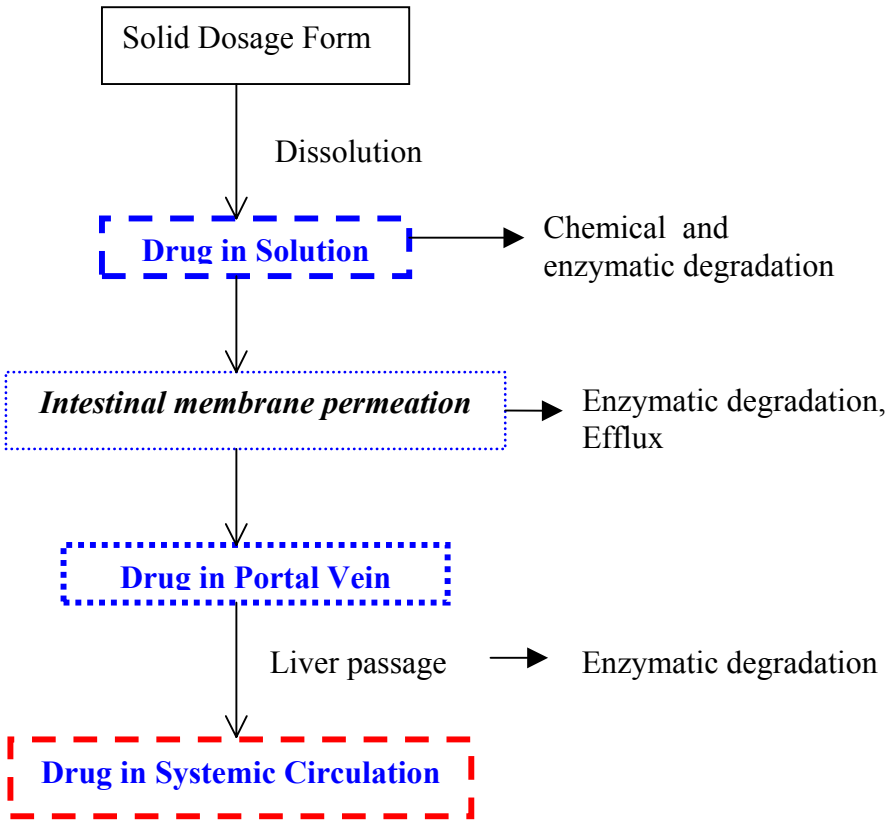


Figure 4 Schematic representation of drug transport from a solid dosage form into systemic circulation.

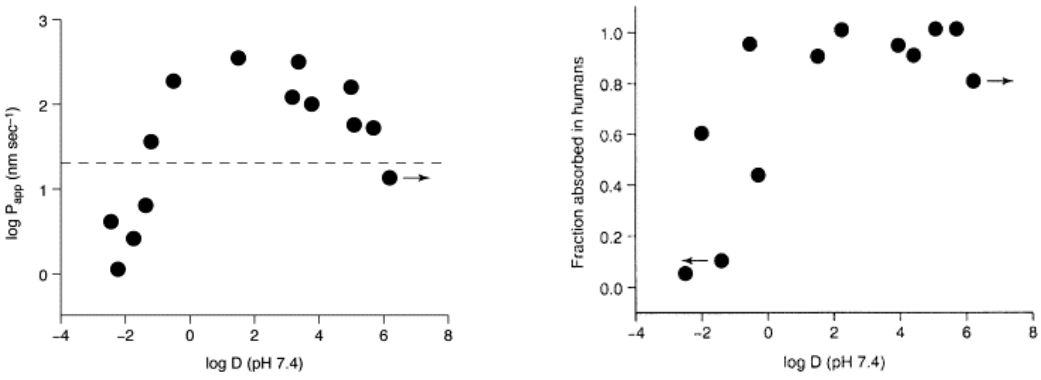
2.2.1 Physicochemical parameters: pKa and Lipophilicity

According to pH-partition theory, only the uncharged species of protolytic compounds will be partitioned into the membrane (Palm et al., 1999). Membrane permeability of lipophilic molecules with ionizable groups is normally dependent upon pKa of the ionizable groups (Muranishi, 1990). Thus, it has been proposed that molecules with pKa close to pH of the GI lumen will gain enhanced absorption compared to the ionized species. Transport of an ionized molecule across cell membrane though is insufficient, the process is not totally ceased (Burton et al., 1997). Passive transport across the cell membrane were governed by not only charge of the solute, but size, lipophilicity, and hydrogen boding capacity of the molecule all interplay to passive membrane permeability (Burton et al., 1997).

Lipophilicity is one of the most important physicochemical parameters that measure drug membrane interaction and describe the ability of a drug molecule to partition into the lipid phase. This property can be obtained experimentally using octanol/water partition system, as partition coefficient or logP, or computational method. In octanol/water system, hydrated octanol molecules are arranged in spherical aggregates, with polar center and apolar outward. The arrangement provides a range of localized environment similar to the lipid bilayer (Franks et al., 1993). In the case of ionizable drug, apparent distribution coefficient (logD) is often used instead of logP. Computational method to estimate lipophicity can be conducted using clogP (Pomona College Medicinal Chemistry Program), or Mlog P (Morigichi calculated logP) (Artursson et al., 2001). Several reports suggested that log P values provide information of the extent of passive transcellular transport. Reasonably good correlation between logP and permeability coefficients in cell monolayers (Artursson et al., 2001) was observed and thus logP has been used to predict rate of membrane transport (Artursson, 1990; Burr et al., 1996; Martin 1996; Palm et al., 1996; Rubas and Cromwell, 1997; Yazdanian et al., 1998). For compounds which transport is largely mediated by a passive mechanism, the relationship between permeability and lipophilicity is generally bell-shaped, as shown in Figure 5A (Kramer, 1999). Generally, permeability in intestinal epithelium increase roughly with the lipophilicity of the molecule, until reaching the plateau at logP ~ 2 (Martin,1981). Drug displaying logP closed to 2 are predicted to be completely absorbed in humans. As logP increase to a value greater than 4, the permeability starts to decrease (Wils et al.,1994) since very hydrophobic drug generally have poor water solubility and partition at a slower rate from cell membrane to extracellular fluid (Raub et al., 1993).

Correlation between the fraction absorbed in humans and logD (Figure 5B) was weaker than the correlation between the apparent permeability coefficient (P_{app}) (Caco-2 values) and log D (Kramer, 1999). Some investigations observed no correlation between fraction absorbed and logP (Chong et al., 1996; Lee et al., 1997). Lack of this correlation may partly due to the presence of the multiple pathways for intestinal absorption which parallel passive diffusion, such active carrier mediated pathways for influx and efflux (oligopeptide transporter and Pgp) across intestinal membrane (Lee et al., 1997). In addition, nutritional status, age, and actual pH at the

absorption site can affect overall extent of absorption. Permeability coefficients were found to correlate with fraction absorbed in human in sigmoidal relationship (Figure 6) (Artursson and Karlsson, 1991; Camenisch et al., 1998; Kramer, 1999).



(A) **(B)**

Figure 5 Relationship between logD at pH 7.4 (*n*-octanol–0.15 M KCl) and the logarithm of apparent permeability across Caco-2 cell monolayers,(A) and fraction absorbed in human, (B). (Kramer, 1999).

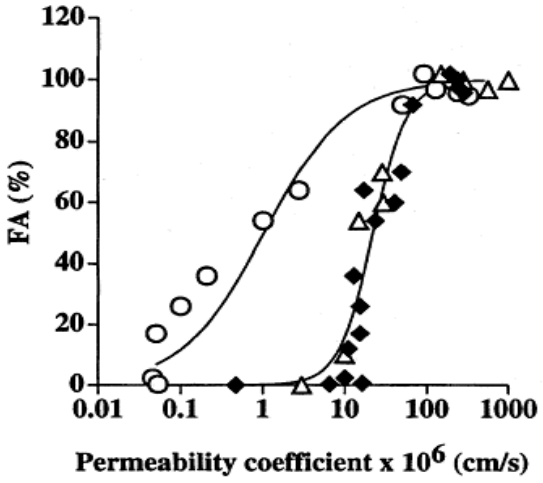


Figure 6 Relationship between the absorbed fraction of structurally diverse sets of orally administered drugs and permeability coefficients obtained in cell monolayers. ♦ 2/4/A1, ○ Caco-2, △ in vivo perfused human jejunum. (Kramer, 1999)

A potential limitation of logP as a predictor of membrane transport is that it does not describe hydrogen bond properties (Young et al.,1988). Since solute must pass through the outer hydrated polar part of the lipid bilayer and the more hydrophobic membrane interior. Octanol, a hydrogen bonding solvent, mainly models the affinity of the molecule for the polar membrane interface rather than the transport through the entire lipid bilayer. In contrast, cyclohexane shows no hydrogen bonding property, it is therefore more similar to the more hydrophobic membrane interior. Differences between $logP_{oct}$ and log cyclohexane-water partition coefficient, Δlog P, should model the membrane interfaces to membrane interior transfer of the molecule, i.e. the desolvation energy required for transcellular transport. Therefore, Burton and co-workers suggested that ΔlogP may be a better predictor of drug permeability in certain circumstances (Burton et al.,1992).

2.2.2 Physiological parameters

These parameters include binding or complexation, regional pH, intestinal permeability, metabolism, gastric and intestinal transit. Drug molecules may be bound by intestinal contents thus reducing the amount available for absorption. Different in pH along the intestine can affect solubilization and degradation of the molecule. Intestinal permeability, which is the ability of the molecule to pass the primary barrier, may be varied within the location within the GI tract. Gastric emptying and intestinal transit represent the time interval that compounds reside at the absorption site. First pass metabolism affect the amount of unchanged molecule available for reaching systemic circulation. The small intestine represents the first line of defense against ingested substances. The intestinal mucosa has a high capacity for metabolism of certain xenobiotics mediated by enzymes residing in the mucosal epithelium. Those enzymes include either phase I or phase II metabolizing enzymes, such as cytochrome P450, alcohol dehydrogenase, epoxide hydrolase, esterases, glucuronosyl transferases, sulfotransferases (De Wazier et al., 1990). The expression and activity of these intestinal enzymes may lead to degradation, activation, or toxification of drugs or xenobiotics.

Among those physicochemical and physiological parameters mentioned, solubility and permeability of a drug molecule are considered to be two of the most important properties determining absorption, and their influences on bioavailability has then been received considerable attentions for drug candidate investigation (Grass 1997).

2.3 Cell culture model used in assessing intestinal absorption

Numerous *in vitro* techniques have been used for assessing intestinal absorption potential of a drug candidate. *In vitro* methods for assessment of permeability are less labor and cost-intensive compared to *in vivo* studies. However, with the *in vitro* systems, the effect of physiological factors such as gastric emptying rate, GI transit rate and pH, cannot be incorporated in the data interpretation.

Varieties of cell monolayer models that mimic human intestinal epithelium have been developed and widely accepted. The advantages offered by cell culture model are (1) rapid assessment of the potential permeability and metabolism of a drug; (2) elucidation of the molecular mechanisms of drug transport under controlled conditions; (3) evaluation strategies to improve absorption, e.g. by use of prodrugs, absorption enhancers, or other pharmaceutical additives; and (4) minimizing the expensive, time consuming, and controversial animal studies (Audus et al., 1990; Artursson, 1991). Unlike enterocytes, human immortalized (tumor) cells grow rapidly into confluent monolayers followed by a spontaneous differentiation providing an ideal system for transport studies (Balimane et al., 2000). A few of these cell line models are described in Table 1.

Table 1 Most commonly used cell culture models for estimating intestinal transcellular flux (Balimane et al., 2000)

Cells	Species/tissue origin	Cell type
Caco-2	Human/colon	epithelial
HT-29	Human/colon	epithelial
T-84	Human/colon	epithelial
MDCK	Canine/kidney	epithelial
LLC-PK1	Porcine/kidney	epithelial

Caco-2 cells are the most well-characterized and have been extensively employed in the field of drug permeability. As of human colon adenocarcinoma originated, Caco-2 cells undergo spontaneous enterocytic differentiation in culture. Differentiated Caco-2 cells have a well-defined brush border on the apical surface facing the culture medium. Tight junction and cell polarity are well developed when reaching confluency on a semipermeable porous filter. Electrical measurement of cells grown on microporous filter have confirmed the formation of tight monolayer with transepithelial electrical resistance of 150-300 Ω-cm^2. Not only morphologically resemble small intestinal absorptive cells, they also express brush border enzymes, i.e. alkaline phosphatase, sucrose isomaltase, amonopeptidase (Quaroni, A. and Hochman, J. 1996), and drug metabolizing enzyme, i.e. phenolsulfotransferase, glutathione-S-transferases, UDP-glucuronosyltransferases, cytochrome P450 1A1, 1A2 and 3A4 (Schmiedlin-Ren et al., 1997). Numerous intestinal transporters, for example transporters for peptide, amino acid, bile acid, vitamin, and organic cationic or anion (OCT and OAT), were expressed in this cell line. Three glucose carriers have been detected in confluent cells: GLUT1 (localized primarily in basolateral membrane), GLUT3 (in the apical membrane), and GLUT5 (in both domains). Energy-dependent drug efflux pumps (Pgp and MRPs) were also reported for their expression in Caco-2 cells (Makhey et al., 1998), and will be addressed in details in section 2.4.

In the last decade, there has been a tremendous growth in the use of Caco-2 cells for mechanistic studies and as a rapid screening tool for drug discovery. The variation between laboratory in compound permeability can be attributed to variable expression of transporters (i.e. Pgp and MRPs) (Gutmann et al., 1999 and Hirohashi et al., 2000), depending on culturing condition, sources of Caco-2 cell, and composition of cell subpopulation (Anderle et al., 1998). Nevertheless, within each individual laboratory, completely absorbed drugs can be easily separated from poorly absorbed drug by this model (Balimane et al., 2000).

2.4 Drug Efflux Transporters as an absorption barrier

The intestinal enterocyte, while presenting an optimal surface for nutrient absorption, provides a selective barrier to drugs and xenobiotics. Besides limiting the access of toxins and other xenobiotics including drug intake, intestinal epithelium is also an important site for excretion of specific classes of xenobiotics and endogenous toxins (Hunter and Hirst, 1997; Chan et al., 2004). In addition to intracellular metabolizing enzymes, the barrier function depends largely upon two principal drug efflux proteins, i.e. the multidrug resistance (MDR) and multidrug resistance-associated protein (MRP)-type transporters. Although these transporters tend to be overexpressed in tumors, they are widely distributed in many normal tissues, most notably in excretory sites such as liver, kidney, intestine, where they provide a barrier for drug permeation and thus a mechanism for drug elimination (Chan et.al., 2004).

P-glycoprotein (Pgp), a 170 kDa membrane glycoprotein encoded by multidrug resistance (Pgp, ABCB1) gene, is an ATP-dependent efflux transporter, firstly found in tumor cells (Juliano and Ling, 1976) and conferred resistance to a wide range of amphiphilic drugs. It is highly expressed in solid tumors of epithelial origins, including colon (Cordon-Cardo et al., 1990), kidney (Fojo et al., 1987a), and breast (Merkel et al., 1989), indicating the pivotal role of Pgp in resistance to cancer chemotherapy. Pgp is also constitutively expressed at high level in normal tissue such as kidney and adrenal gland; at intermediate levels in liver, small intestine, colon, and lung; and at low levels in prostate, skin, spleen, heart, skeletal muscle, stomach and ovary (Fojo et al., 1987b; Gatmaitan and Arias, 1993). Pgp is also found in brain

(Begley et al., 1996; Cordon-Cardo et al.,1989; Lee et al., 2000; Tsuji; 1992), choroids plexus (Rao et al., 1999) and placenta (Ushigome et al., 2000). Pgp is located at the apical membrane, for example intestinal Pgp is present at the villus tip (Hunter et al., 1993 a,b; Terao et al., 1996), thereby limiting access of xenobiotics into cells or across the cells by transporting the compound from the cytoplasm to extracellular space. It is currently known that Pgp extrudes many structurally and functionally unrelated molecules in addition to cytotoxic agents, such as vinblastine and daunomycin. Showing extremely broad substrate specificity, with a tendency toward lipophilic, cationic compounds, substrates/inhibitors of Pgp includes anticancers, antibiotics, antivirals, calcium channel blockers, and immunosuppressive agents (Table 3). With Pgp diverse substrate specificity, and its localization at the important sites of drug disposition, it is not surprising that Pgp has a major influence on drug absorption, distribution and elimination.

Role of other non-Pgp transporters such as MRP was also proved significant in determining pharmacokinetics of various clinically important drugs. Belonging to the same ATP-binding cassette transporter, MRP was discovered later than Pgp. MRPs consist of at least seven members, several of which can confer resistance to drugs (Larsen et. al., 2000). The MRP family has been shown to transport predominantly anionic substances, but nonionic organic drugs was also transported possibly either cotransport or conjugation with glutathione (Chan et al., 2004; Evers et al., 2000; Gerk and Vore, 2002). This contrasts with Pgp, which favor lipophilic compound of a cationic nature. MRP1, 4, and 5 mRNA showed relatively ubiquitous expression, for example MRP1 is expressed in lung and small intestine (Barrand et al., 1997), and choroids plexus removing xenobiotics via cerebrospinal fluid (Rao et al., 1999). MRP 2, 3 and 6 are more restricted and mostly found in renal, intestinal, and hepatic epithelia (Chan et al., 2004). MRP2 (cMOAT), is mainly expressed in liver, kidney, and gastrointestinal tract (Borst et al.,1999; Peng et al., 1999). While other MRP isoforms were expressed at the basolateral membrane, MRP2 or canalicular multispecific organic anion transporter (cMOAT) is mainly expressed at the apical membrane of the polarized cell (Borst et al.,1999,2000), where it was postulated to provide apical drug efflux, analogous to Pgp (Chan et al., 2004) (Figure 7). Other

ABC transporter apically located is breast cancer resistance protein (BRCP, ABCG2), which is expressed in breast duct, and lobules, large and small intestinal epithelium, and the liver cannalicular membrane (Maliepaard et al., 2001). It is possible that more than one of these transporters expressed in polarized epithelial cells, for example Pgp, MRP2 and BRCP are co-localized at brush border of intestinal cells, while MRP1 and MRP3 are distributed to basolateral surface, as illustrated in Figure 7.

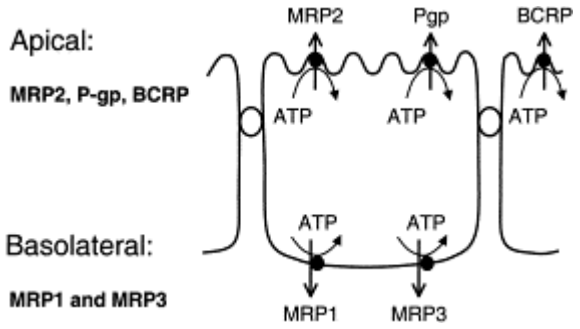


Figure 7 Asymmetric distribution of ABC transporters in polarized intestinal absorptive cells (Dantzig et al., 2003)

Table 2 ABC Transporters that confer multidrug resistance (Dantzig et al., 2003)

Name	Expression	Substrates	Examples
P-gp (ABCB1)	Intestine, kidney, lung, liver, blood brain barrier	Amphiphilic cationic and neutral	Vincas, taxanes, anthracycline
MRP1 (ABCC1)	Ubiquitous (mostly liver)	Anionic conjugates, LTC_4, GSSG, X+GSH	VCR, MXR, DOX, VP-16, MTX
MRP2 (ABCB2) cMOAT	Liver, duodenum, kidney	Anionic conjugates, LTC_4, bilirubin glucuronide	VCR,VLB,VP-16, DOX, MTX
MRP3 (ABCC3)	Liver, GI, kidney, placenta	Anionic conjugates, LTC_4, bile salts	VCR, VP-16, MTX
MRP4 (ABCB4) cMOAT	Prostate, lung, testis, ovary, bladder	Nucleoside, phosphates, antiviral drugs	Antivirals
MRP5 (ABCC5)	Ubiquitous	Base and nucleotide analogs, cAMP, cGMP	6-Mercaptopurine
BCRP (ABCG2)	Placenta, breast, liver. Intestine	Hydrophobic	MXR, MTX, Topotecan, DOX, SN-38

Abbreviation: DOX:Doxorubicin; VCR:Vincristine; VLB:Vinblastine; MXR: Mitoxantrone; MTX: Methotrexate; LTC_4 : Leukotriene-C_4

In contrast to Pgp which effluxes drugs without modification, MRPs transport either unmodified or conjugated drugs. MRP1 shares only 15% sequence similarity with Pgp, but still they have partly overlapping substrate specificities (Kruh et al., 2001), as shown in Table 2. A number of NSAIDS including indomethacin, sulindac, tolmetin, zomipirac, and mefenamic acid, was shown to significantly enhance cytotoxic effects of anticancer drugs (doxorubicin, daunorubicin, teniposide, VP-16, and vincristine) in MRP1 expressing cell line (Draper et al., 1997; Duffy et al., 1998; Roller et al., 1999). MRP2 shares some degree of overlapping substrate specificity with Pgp, as shown in Table 2, and was expressed at the highest levels in both human jejunum and Caco-2 cell (Taipalensuu et al., 2001). However, its contribution to drug disposition is still not as well characterized compared to Pgp (Schinkel et al., 1996;

Sparreboom et al., 1997; Terao et al., 1996). Preliminary *in vitro* screening for substrates/inhibitors of efflux transporters had been frequently demonstrated in Caco-2 cell monolayer. Expression levels of ABC transporters but BCRP in Caco-2 cells were similar to those in human jejunum, i.e. differed by only a factor of less than 2.5 (Taipalensuu et al., 2001). This might contribute partly to its prominence as a screening tool for roles of transporters on intestinal absorption of new candidates.

3. Functional activity assay for drug efflux transporters

Investigation if new drug candidate are substrates for Pgp and/or MRPs is important for pre-determining *in vivo* oral bioavailability of drug candidates. Several *in vitro* approaches have been currently used for evaluating drug efflux transporter activity, which can be grouped into 3 general categories: transport assay, accumulation and efflux assay, and ATPase assay. Principle and procedures of the methods have been reviewed by Zhang and coworkers (2003), and Varma and coworkers (2003) (Zhang et al., 2003 and Varma et al., 2003). In transport assay, efflux activity can be examined by the appearance of bidirectional differences in compound permeability, represented by the ratio of basolateral to apical (BL-AP) versus apical to basolateral permeability (AP-BL), i.e. efflux ratio. The influences of the test compound to efflux ratio then indicate potential inhibition of the transporter. This method is the most direct manner in identifying Pgp substrates/inhibitors and regarded as a standard measurement (Polli et al., 2001). However, monolayer efflux is quite labor-intensive due to cell culturing and analytical requirement, which can limit assay throughput. Fluorescence probes such as rhodamine-123 or calcein AM, normally used in accumulation and efflux assay, are particularly suited for rapid screening using fluorescence plate readers. Likewise, measurement of ATPase is typically performed in 96-well plates using colorimetric techniques. Calcein and ATPase assay offer higher throughput than monolayer transport assay (Polli et al., 2001), however, do not directly measure transport (Polli et al., 2001 and Laska et al., 2002). Among three in vitro assay methods for drug efflux activity, bidirectional transport assay is more reliable for compounds exhibiting low to moderate P_{app} (Polli et al., 2001). The transport study appears to be informative for determining functional

localization of drug efflux transporters. Moreover, it is predictive for the *in vivo* situation and becoming a method of choice for evaluating drug candidates in spite of its low throughput and complicated analytical methods (Zhang et al., 2003).

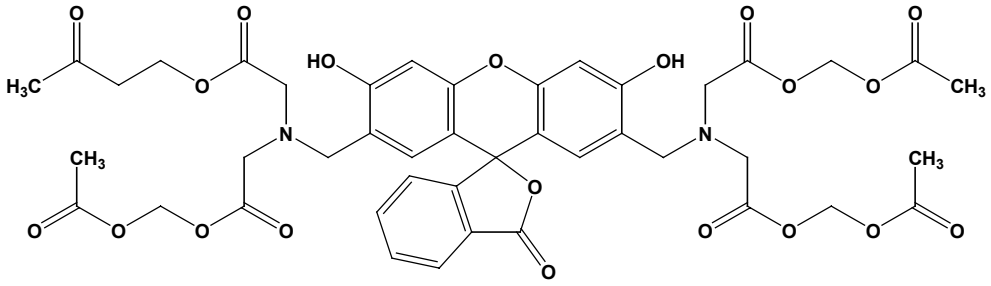
3.1 ATPase assay

ATPase activity can be determined using cell membranes, cultured cells and membrane vesicles. Cell membranes of Sf9 insect cells expressed with human MDR1 gene via a baculovirus vector is commercially available for screening of Pgp substrates and/or inhibitors (Gentest, Woburn, MA). Pgp utilizes two molecules of ATP to efflux a molecule of substrate (Scarborough 1995). The drug stimulated ATPase activity determined as the difference between amount of ATP consumed in the presence and absence of vanadate was used as the marker for complete Pgp inhibition. ATPase activity can be estimated by quantifying ATP, ADP released NADP (Zakaria 1985), or liberated inorganic phosphate (Rebbeor and Senior, 1998; Schmid et al., 1999). Ion-exchange chromatography is usually used for quantifying ATP and ADP, however, reversed phase HPLC with ion-pairing agents such as tetrabutylammonium dihydrogen phosphate is found to be advantageous with better reproducibility and faster retention time (Meyer 1999).

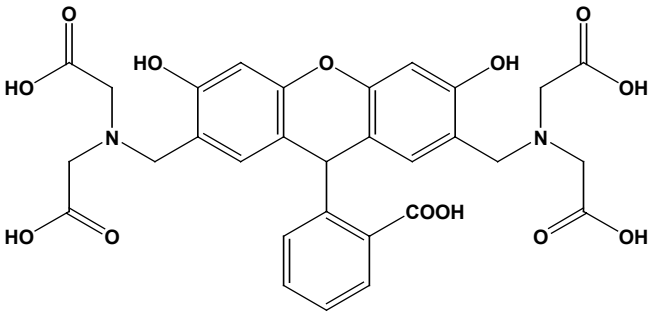
3.2 Calcein AM efflux inhibition Assay

This method has been accepted as a highly sensitive probe and high throughput screening of Pgp inhibitors (Varma et al., 2003). Several investigators have been used this method for functional assays of the Pgp and MRPs (Hollo et al., 1994; Terao et al., 1996; Fujita et al., 1997; Kool et al., 1997; Peng et al., 1999; Laska et al., 2002; Stephens et al., 2002; Hannah et al., 2004; Vellonen et al., 2004). Calcein acetoxymethylester (calcein AM, Figure 8), a nonfluorescent lipophilic compound, rapidly diffuses across cell membrane into the cell. In cytosol, calcein AM is cleaved by endogenous esterase yielding hydrophilic and highly fluorescent organic anion calcein. Due to its hydrophilicity, calcein cannot diffuse out of the cells. Calcein AM is a substrate of the efflux pumps Pgp and MRP1, but can also diffuse passively from

the cells (Essodaigui et al., 1998). Calcein, however, is a substrate for MRP1 and MRP2, but not for Pgp (Evers et al., 2000; Bauer et al., 2003), so the only route for calcein to escape from the cell is via MRP1 or MRP2-mediated efflux. With Pgp or MRPs inhibitors, calcein accumulation inside the cell is enhanced and thus the fluorescence would be increased. The activity of efflux proteins and their interaction with inhibitors can be quantified by measuring the increase in intracellular calcein fluorescence (Eneroth et al., 2001).



(A)



(B)

Figure 8 Chemical structures of (A) calcein acetoxymethyl ester (calcein AM) and (B) calcein.

OBJECTIVES OF THE RESEARCH

General objectives

1. To determine solubility, chemical and enzymatic stability profiles of the synthesized ester of mefenamic acid.
2. To examine transepithelial properties of mefenamic acid esters using Caco-2 monolayer as a model.
3. To investigate potential involvements of multidrug resistance transporters in apical efflux of some ester of mefenamic acid.

Specific Objectives

To systematically investigate esters of mefenamic acid regarding their epithelial permeability and physicochemical characteristics