CHAPTER II

LITERATURE REVIEW

I *Phyllanthus amarus* and phyllanthin

Phyllanthus amarus Schum. et Thonn. (*P. amarus*) (Figure 1) is an herbal plant medicine belongs to the family Euphorbiaceae. This plant has been used in several countries for treatment of diarrhea, gastropathy, dysentery, ulcer, intermittent fevers, wounds, scabies, and ophthalmopathy (Calixto J.B., Santos A.R.S. et al. 1998, Patel J.R., Tripathi P. et al. 2011). *P. amarus* extracts showed several pharmacological activities such as anti-carcinogenic effect, anti-mutagenic effect, hepatoprotective effect, anti-microbial effect, hypoglycemic effect, anti-viral activity (Yeh S.F., Hong C.Y. et al. 1993, Lee C.D., Ott M. et al. 1996, Raphael K.R., Ajith T.A. et al. 2002, Sripanidkulchai B., Tattawasart U. et al. 2002, Raphael K.R. and R. 2003, Kumar K.B. and Kuttan R. 2005, Mazumder A., Mahato A. et al. 2006, Pramyothin P., Ngamtin C. et al. 2007, Singh G., Goyal R. et al. 2012). Examples of the pharmacological activities were listed as the following:

Chemoprotective effects

The extract of *P. amarus* extract was able to prevent cyclophosphamide (CTX) induced toxicity in mice after intraperitoneal treatment at 5 mg/kg for 14 days. In addition, the extract (250 and 750 mg/kg body weight) given orally to the mice for 35 days alleviated the effects of CTX on myelosuppression. The extract also enhances the WBC count and the number of maturing monocytes. Hence, the

extract was able to reduce the toxicity of CTX and was not interfering with the antitumor activity of CTX (Kumar K.B. and Kuttan R. 2005).

Anti-mutagenic effect

The aqueous extract of *P. amarus* was shown to prevent chemical-induced mutagenicity in *Salmonella typhimurium* strains TA1535, TA100, and TA102. At the concentration of 1-2 mg/plate, the extract was able to inhibit the mutagenic effect of sodium azide (NaN_3), N-methyl-N-nitro-N-nitrosoguanidine (MNNG), and 4-nitro-0-phenylenediamine (NPD) (Raphael K.R., Ajith T.A. et al. 2002). Moreover, treatment the rats with the extract (500 mg/kg) for 12 days was able to inhibit benzo[α] pyrene (10 mg/rat)-mediated urinary mutagenicity (Raphael K.R., Ajith T.A. et al. 2002).

Hepatoprotective effect

The hepatoprotective effect of *P. amarus* was widely studied. It has been shown that the aqueous extract (1-4 mg /ml) was able to suppress the ethanol-induced cytotoxicity in rat primary cultured hepatocytes (Pramyothin P., Ngamtin C. et al. 2007). In the rat model of ethanol-mediated hepatic injury, *P. amarus* extract was able to enhance the recovery process. The levels of serum transaminases (AST and ALT), hepatic triglyceride (HTG) and tumor necrosis factor alpha (TNF-α) decreased back to normal level in comparable to the effect of silymarin (5 mg/kg) (Pramyothin P., Ngamtin C. et al. 2007).

Figure 1. *Phyllanthus amarus* Schum. et Thonn. (India Biodiversity Portal , Vietnam plants and the USA plants 2010)

Phyllanthin

Phyllanthin is one of the major bioactive compounds found in *P.amarus* (Figure 2). Phyllanthin is a lignan compound. Several studies reported that phyllanthin exhibited a number of pharmacological activities similar to those of *P.amarus* (Leite D.F., Kassuya C.A. et al. 2006, Krithika R., Mohankumar R. et al. 2009). Examples of those activities included anti-carcinogenic, anti-hepatoprotective and anti-oxidative effects.

Anti-carcinogenic effect

Leite and co-workers (2006) investigated effects of the extract and lignans isolated from *P. amarus* on P-gp-mediated multidrug resistance in Lucina-1 cells. They reported that phyllanthin at a concentration of 102.74 μM (43 μg/ml) was able to potentiate anti-leukaemic activity of daunorubicin in human leukemic Lucena-1

cells (Leite D.F., Kassuya C.A. et al. 2006). Combined treatment between phyllanthin and daunorubicin at the non-toxic concentrations significantly reduced cell viability from 75.6% to 30.4%. In addition, phyllanthin increased an accumulation of rhodamine 123, a P-gp substrate, in vincristine-resistance Lucena-1 cell line (Leite D.F., Kassuya C.A. et al. 2006). These findings suggested that this lignan might be a good candidate for the combined treatment with the conventional cytotoxic drugs. Phyllanthin could be a multidrug resistance reversing agents via inhibition of P-gp function (Leite D.F., Kassuya C.A. et al. 2006).

Furthermore, in the Swiss albino mice model with Ehrlich Ascites Carcinoma, phyllanthin in combination with hypophyllanthin (in the ratio of 1:1) at the concentration of 50 and 100 mg/kg was able to inhibit the tumor volume, total cell count and packed cell volume (Islam A., Selvan T. et al. 2008). After the 14 day treatment period, the hematological parameters including hemoglobin, RBC, and WBC count were recovered, in comparable with those of the normal control group (Islam A., Selvan T. et al. 2008).

Hepatoprotective and anti-oxidative effects

Along with the *P. amarus* extracts, phyllanthin was able to prevent chemical-induced hepatotoxicity via its antioxidative activity (Krithika R., Mohankumar R. et al. 2009). For example, phyllanthin significantly increased cell survival against carbon tetrachloride (CCl_4)-induced toxicity in HepG2 cell (Krithika R., Mohankumar R. et al. 2009). This lignan at the concentration of 10-30 µmol/ml improved intracellular oxidative and glutathione status, and decrease lipid peroxidation. Thus, it could

prevent leakage of alanine transaminase (ALT) and lactage dehydrogenase (LDH) enzymes, and increased cell viability (Krithika R., Mohankumar R. et al. 2009).

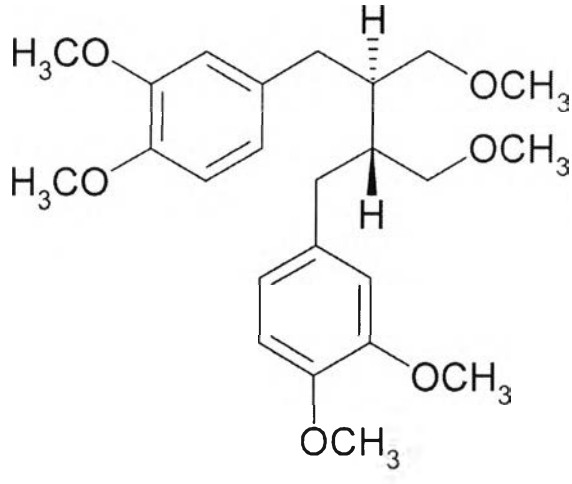

Figure 2. Chemical structure of phyllanthin.

II Intestinal absorption

The oral route is the most convenient and practical way for patient to take drugs. The solid dosage form such as tablet or capsule is the most preferable formulation. In this regard, oral bioavailability of a new chemical entity (NCE) should be considered in early phase of drug development process. A number of factors which can affect oral bioavailability should be explored in order to develop the biopharmaceutical support. Those factors include dissolution, solubility, stability in aqueous buffers and metabolism in the gastrointestinal tract. In addition, drug transporters can be the rate-limiting factor in the intestinal absorption for certain compounds (Figure 3) (Ungell A-L. and Abrahamsson B. 2009). Hence, the permeation of NCE should also be investigated.

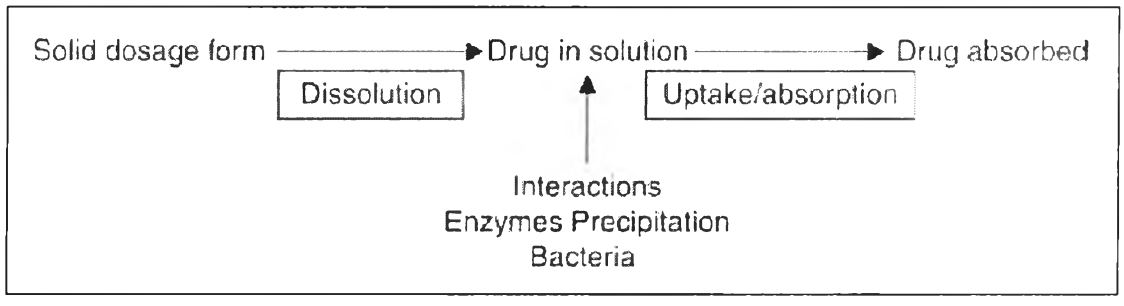

Figure 3. Various stages in the intestinal absorption including chemical dissolution from the solid dosage form, interactions with the dissolved material in the gastrointestinal lumen, and the uptake of the compound through the epithelial membrane. (Ungell A-L. and Abrahamsson B. 2009)

Factors affecting intestinal absorption

Solubility

Aqueous solubility of NCE is an important factor determining the mass available for intestinal absorption. The solubility of NCE can be used to predict the intestinal absorption profile. If NCE is a poorly soluble compound, its dissolution may be a rate-limiting step in the intestinal absorption. Highly soluble compounds are the compounds of which the highest dose strength is soluble in 250 ml or less of aqueous media across the pH range of 1.0-7.5 (Table 1) (US Department of Health and Human Services Food and Drug Administration 2000, Steele G. and Austin T. 2009). Poorly soluble compounds can be defined as the compound that has an aqueous solubility of less than 100 μg/ml over the pH range of 1.0-7.5 (Steele G. and Austin T. 2009).

Table 1. Solubility definitions (Steele G. and Austin T. 2009)

Descriptive Term	Parts of solvent required for 1 part of solute	Solubility range (mg/ml)	Solubility assigned (mg/ml)
Very soluble	< 1	> 1000	1000
Freely soluble	1 – 10	100 – 1000	100
Soluble	10 – 30	33 – 100	33
Sparingly soluble	30 – 100	10 – 33	10
Slightly soluble	100 – 1000	1 – 10	1
Very slightly soluble	1000 – 10,000	0.1 – 1	0.1
Practically insoluble or insoluble	≥ 10,000	< 0.1	0.01

Physicochemical properties of phyllanthin

Previous study reported that phyllanthin was non-ionizable with good stability in aqueous media over the pH range of 1.07-10.26 (Hanh N.D., Sinchaipanid N. et al. 2013). At pH 7.48, the log P_{ow} of phyllanthin was 3.30 ± 0.05 (El-Kattan A. and Varma M. 2012, Hanh N.D., Sinchaipanid N. et al. 2013). The n-octanol/water apparent partition coefficient (log P_{ow}) is a parameter to determine lipophilicity property of compounds. It was suggested that phyllanthin might be a good candidate for development into orally taken drug. However, phyllanthin showed low oral bioavailability of 0.62% (Murugaiyah V. and Chan K.L. 2007). Hence, the permeation across the intestinal absorptive barrier of phyllanthin should be further investigated.

III Drug transport in the gastrointestinal tract

Drug transport across the gastrointestinal tract can be divided into passive diffusion, carrier-mediated transport, active transport and pinocytosis or endocytosis (Figure 4).

1. Passive diffusion

Passive diffusion consists of two major pathways including transcellular pathway and paracellular pathway.

1.1 Transcellular passive diffusion

Transcellular diffusion is the process that compounds freely diffuse through lipid bilayers of epithelial cell membranes. This type of transport is the major pathway of intestinal absorption for mostly compounds due to the vast mucosal surface area of the intestinal epithelium. The mucosal surface area is more than the paracellular surface area by 1000 folds (Artursson P., Palm K. et al. 2001). Generally, factors influencing the transcellular diffusion include the physicochemical properties of drug molecules. The characteristics of compounds being transported across the transcellular pathway are unionizable, lipophilic (Log P > 0) with large molecular weight or size (more than 300 g/mole) (Ungell A.L. and Karlsson J. 2003, El-Kattan A. and Varma M. 2012).

1.2 Paracellular passive diffusion

The paracellular diffusion is the process that compounds diffuse across the water-filled pores of the paracellular space. This area is less than 0.1% of the total intestinal epithelial surface area (Fordtran J.S., Rector F.C. et al. 1965, Fleisher D. 2000, Artursson P., Palm K. et al. 2001). The paracellular route for absorption is very

restrictive due to the tight junction structure. Only small (molecular weight in the range of 300 – 400 g/mol) and hydrophilic molecules are able to cross the intestinal epithelium via paracellular pathway (Calixto J.B., Santos A.R.S. et al. 1998, Ungell A.L. and Karlsson J. 2003). In addition, the cation molecules can penetrate the tight junction more easily than the anion molecules (Ungell A.L. and Karlsson J. 2003).

2. Carrier-mediated transport

The carrier-mediated transport or facilitated transport is the transport process that involving a carrier protein in the transcellular transport process. This type of transport is not energy-driven and depends on concentration gradient. However, this process can be saturated especially when the concentration of substrate is high. This is due to a limited number of carrier protein on the membrane surface.

3. Active transport

The active transport needs the protein transporter to convey the molecule across the plasma membrane in similar to with the carrier-mediated transport. Unlike the carrier-mediated transport, the active transport process is energy (ATP)-driven process and independent of concentration gradient. Intestinal epithelial membrane expresses various influx and efflux transporters which can be grouped into two main superfamilies. They are adenosine triphosphate (ATP) binding cassette (ABC) superfamily and the solute carrier (SLC) superfamily (Murakami T. and Takano M. 2008, Sugano K., Kansy M. et al. 2010, El-Kattan A. and Varma M. 2012). These transporters are localized on the apical and basolateral sites of intestinal cells for facilitating movement of endogenous substances and xenobiotics across the

absorptive barrier (Murakami T. and Takano M. 2008, El-Kattan A. and Varma M. 2012). The ABC transporters are primary active transporters which utilize ATP to move its substrate across the plasma membrane. The ABC transporters that are expressed in the human intestine include P-glycoprotein (P-gp; MDR1; ABCB1), multidrug resistance proteins (MRP1 – 6; ABCC1 – 6), and breast cancer resistance protein (BCRP; ABCG 2) (El-Kattan A. and Varma M. 2012). Moreover, the SLC transporters use ion gradients such as H^+, Na^+, and Ca^{2+} which are generated from ATP dependent primary transporters such as Na^+/H^+-ATPase and Na^+/K^+-ATPase to move its substrates across the plasma membrane (Sugano K., Kansy M. et al. 2010, El-Kattan A. and Varma M. 2012).

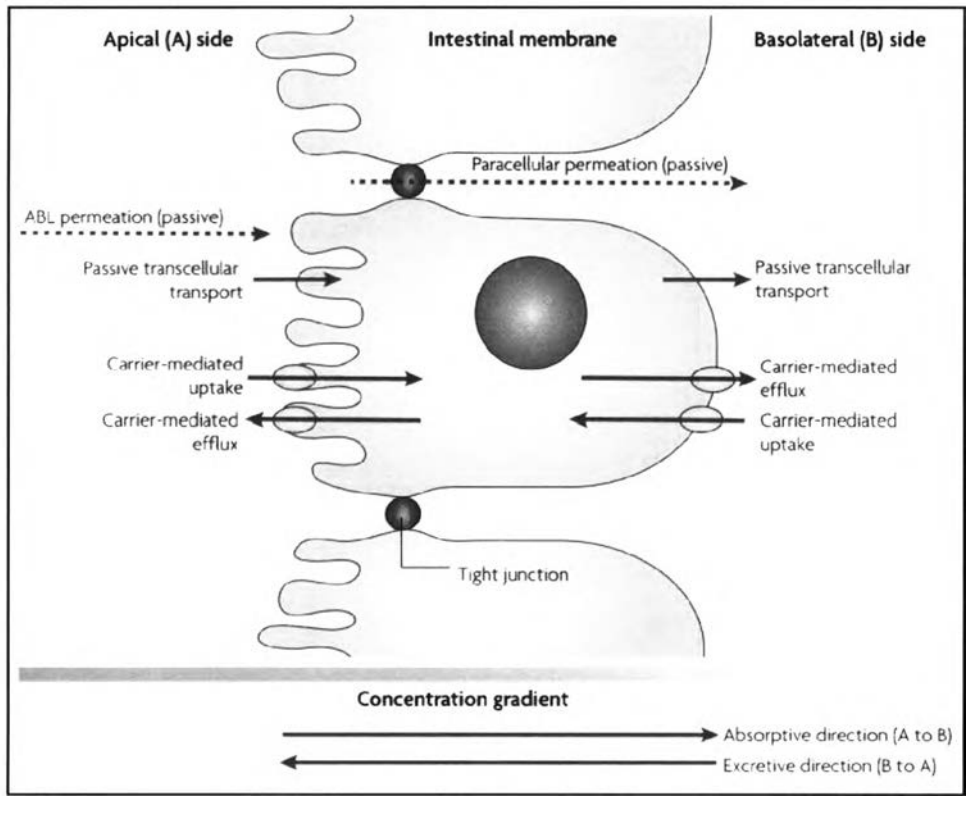

Figure 4. Transport processes for drugs and chemicals across in the intestinal cells. (Sugano K., Kansy M. et al. 2010)

ABL (aqueous boundary layer) is a permeation barrier in drug absorption.

IV The biopharmaceutics classification system (BCS)

The BCS classifies drugs into four classes based on the solubility and permeability (Figure 5) (US Department of Health and Human Services Food and Drug Administration 2000). The BCS is a scientific framework which is generally accepted to use for prediction of in vivo pharmacokinetic of drugs in drug discovery and development process (US Department of Health and Human Services Food and Drug Administration 2000, Wu C.Y. and Benet L.Z. 2005, Sheng J.J. and Amidon G.L. 2010).

Class I High Solubility High Permeability	**Class II** Low Solubility High Permeability
Class III High Solubility Low Permeability	**Class IV** Low Solubility Low Permeability

Figure 5. The biopharmaceutics classification system (BCS).

BCS class I drugs such as acetaminophen, antipyrine, caffeine, metoprolol, propranolol, theophylline, and verapamil are high solubility and high permeability. The rate limiting step of class I drugs is gastric emptying rate when the dissolution rate is very rapid (Table 2). Transporters have minimal effect on the absorption of these BCS class I compounds which may be due to their concentration in the intestinal lumen high enough to saturate the transporters (Wu C.Y. and Benet L.Z. 2005). Hence, it is unlikely that the efflux transporters such as P-gp have a significant

effect on absorption of their drug substrate (Wu C.Y. and Benet L.Z. 2005, Murakami T. and Takano M. 2008).

BCS class II drugs such as digoxin, indomethacin, ketoconazole, phenytoin, and carbamazepine are low solubility and high permeability. The rate limiting step of their absorption is the dissolution rate and extent, except the concentration of the drug is very high (Table 2). The oral bioavailability of this drug class varies greatly and can be improved by increase of dissolution rate. Unlike BCS class I drugs, transporters will have predominant effect on absorption of BCS class II compounds. If the drug is a substrate of efflux transporters, its dissolution can be a significant limiting factor for the absorption (Yasir M., Asif M. et al. 2010). High solubilization allows the high amount of drug to be available in the solution. Thus, the high drug concentration at the enterocytes will be high enough to overcome the effects of transporters (Wu C.Y. and Benet L.Z. 2005, Murakami T. and Takano M. 2008).

BCS class III drugs such as acyclovir, atenolol, cimetidine, captopril, and hydrochlorothiazide are high solubility and low permeability. The rate limiting step of intestinal absorption for BCS class III drugs is permeability (Table 2). The oral bioavailability of drugs in this class is highly variable. In order to enhance the bioavailability, the permeability of these compounds or the drug formulation should be improved (Wu C.Y. and Benet L.Z. 2005).

BCS class IV drugs such as furosemide, amphotericin B, and neomycin are low solubility and low permeability. The rate limiting factors of their absorption include gastric emptying, dissolution, and permeability (Table 2) (Yasir M., Asif M. et al. 2010). Drugs in this class differ greatly in their poor bioavailability (Wu C.Y. and Benet L.Z. 2005).

Table 2. The absorption rate control step for immediate release (IR) solid oral products based on the BCS class. (Yasir M., Asif M. et al. 2010)

Class	Solubility / Permeability	Absorption rate control step
Class I	High / High	Gastric emptying
Class II	Low / High	Dissolution
Class III	High / Low	Permeability
Class IV	Low / Low	Case by case

V The investigate drug-drug interaction in candidate drug selection process

Drug interaction is one of the concerns in candidate drug selection process. Generally, the drug interaction problems stem from interference either on drug efflux pumps or on drug metabolism.

1. Phyllanthin and efflux drug transporters

Because the roles of drug in modulating drug absorption, distribution, and elimination, the interference on drug transporters might affect plasma drug level (Zhang L., Strong J.M. et al. 2006, Zhou 2008, Staud F., Ceckova M. et al. 2010). P-gp is a drug efflux pump which is localized abundantly at the apical membrane of the epithelial cells in the intestine (Hunter J., Jepson M.A. et al. 1993, Anderle P., Niederer E. et al. 1998, del Amo E.M., Heikkinen A.T. et al. 2009). P-gp has a significant role in limiting the translocation of xenobiotics and drugs through restrictive intestinal barriers (Terao T., Hisanaga E. et al. 1996, Fromm M.F. 2004, Murakami T. and Takano M. 2008). Hence, the interference on intestinal P-gp function may affect absorption and excretion of its drug substrates, leading to

changes in drug bioavailability (Fromm M.F. 2003, Zhou S.F. 2008, Staud F., Ceckova M. et al. 2010). In this regard, the USFDA and the European Medicines Agency recommend that the investigation of drug-drug interaction should be performed in the early phase of candidate drug selection process (European Medicines Agency 2012, US Department of Health and Human Services Food and Drug Administration 2012).

The inhibitory effects of phyllanthin on multidrug resistance protein 2 (MRP 2) and P-glycoprotein (P-gp) functionality in Caco-2 cells were investigated (Sukhaphirom N., Vardhanabhuti N. et al. 2012). The result showed that phyllanthin at a concentration of 100 μM was able to inhibit P-gp function, but not the MRP2 activity. The inhibitory action of phyllanthin was reversible (Sukhaphirom N., Vardhanabhuti N. et al. 2012). However, it was unclear whether the interference of phyllanthin on P-gp fuction could significantly affect its transport through the absorptive barrier.

2. Phyllanthin and drug metabolism enzymes

As known, drug metabolizing enzymes, especially CYP3A can affect the pharmacokinetics and pharmacodynamics of drug candidates (US Department of Health and Human Services Food and Drug Administration 2000, European Medicines Agency 2012). Phyllanthin was a potent mechanism-based inhibitor of CYP3A4 with K_{inact}/K_I ratios higher than some therapeutic drugs such as erythromycin and clarithromycin (Taesotikol T., Dumrongsakulchai W. et al. 2011).

VI The Caco-2 cells as the model for intestinal absorption

The Caco-2 culture is a well characterized and accepted model for the prediction of intestinal drug permeability (US Department of Health and Human Services Food and Drug Administration 2000, Artursson P., Palm K. et al. 2001, Ungell A-L. and Abrahamsson B. 2009, Brouwers J., Deferme S. et al. 2010). The Caco-2 cell is a human epithelial colon adenocarcinoma cell line which can differentiate to enterocytes under the specific culture condition (Meunier V., Bourrié M. et al. 1995, Ungell A.L. and Karlsson J. 2003, Sambuy Y., De Angelis I. et al. 2005). After 21-days post seeding, the Caco-2 monolayers grow into an absorptive barrier with the formation of tight junction and the expression of important intestinal drug transporters such as H^+/di-tripeptide transporter (PEPT1), organic anion-transporting polypeptide 2B1 (OATP-B), monocarboxylic acid transporter 1 (MCT1), organic cation/carnitine transporter (OCTN2), P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), breast cancer resistance protein (BCRP) (Ferrec E.L., Chesne C. et al. 2001, Taipalensuu J., Törnblom H. et al. 2001, Xia C.Q., Liu N. et al. 2005, Sun H., Chow E.C. et al. 2008). Hence, the Caco-2 monolayer can be used for the study of drug permeability and drug transport mechanisms (van Breemen R.B. and Li Y. 2005, Hubatsch I., Ragnarsson E.G. et al. 2007). The presence of the influx and efflux transporters along with tight junction allows the Caco-2 model to be suitable for the study of passive and active transport mechanisms (Figure 6) (Hubatsch I., Ragnarsson E.G. et al. 2007). A good correlation between permeability measured across Caco-2 cell monolayers and fraction absorbed orally in human has been substantially demonstrated in Figure 7 (Artursson P., Palm K. et al. 2001, Ungell A.L. and Karlsson J. 2003).

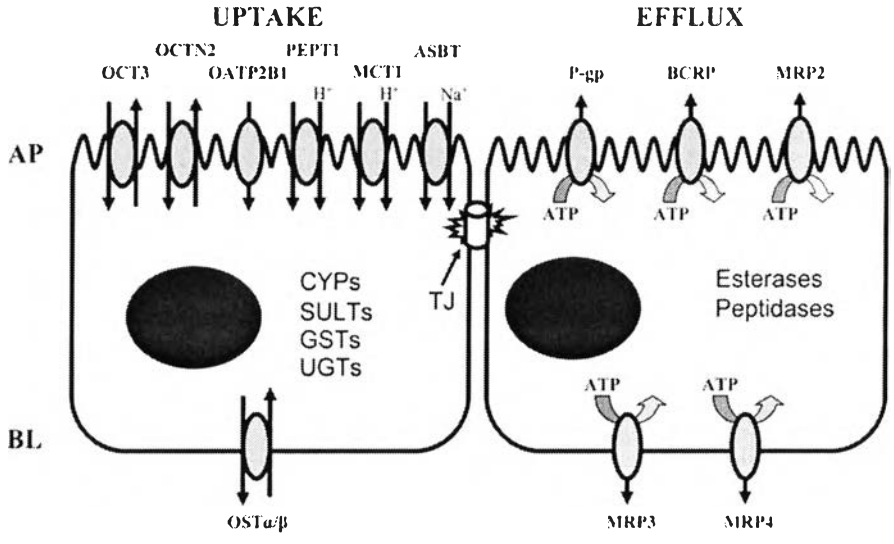

Figure 6. Apical and basolateral membrane transporters and metabolizing enzymes localized in Caco-2 cell monolayers. (Proctor W.R., Ming X. et al. 2007)

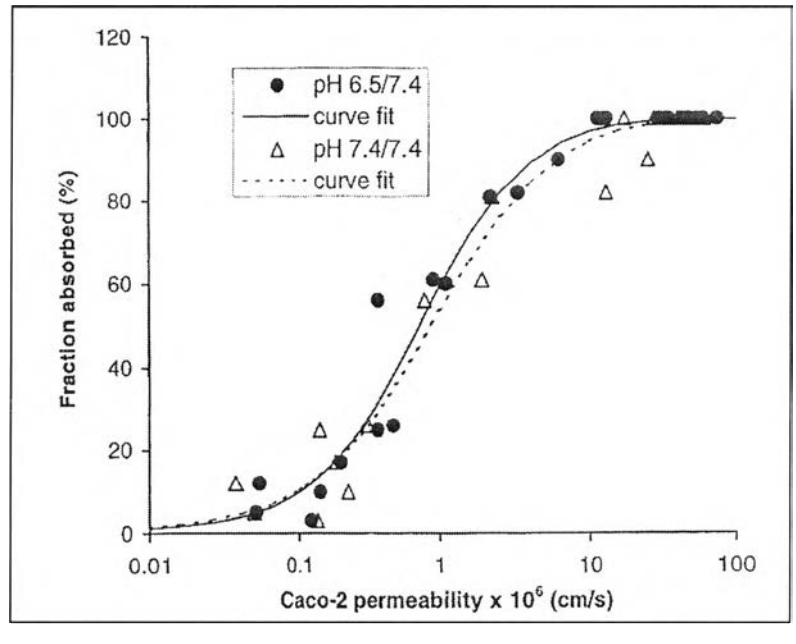

Figure 7. Relationship between the oral fraction absorbed in humans and apparent permeability coefficients obtained in Caco-2 cell monolayers at two different pH conditions. Mean apical to basolateral P_{app} values were determined at drug concentrations of 10 – 500 μM at pH 6.5/7.4 or 7.4/7.4 on the apical/basolateral sides of the cell monolayers. (Ungell A.L. and Karlsson J. 2003)