CHAPTER V

DISCUSSION AND CONCLUSION

Determination of permeability and solubility of new chemical entity (NCE) is an initial step in development of orally taken drugs. In this study, Caco-2 cell monolayers was used as an in vitro model in the permeability study of phyllanthin. The Caco-2 cell 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 into enterocytes under the specific culture condition. After 21-days post seeding, the Caco-2 monolayers grow into absorptive barrier with the formation of tight junction and expression of important intestinal drug transporters (Ferrec E.L., Chesne C. et al. 2001, Taipalensuu J., Törnblom H. et al. 2001, Sun H., Chow E.C. et al. 2008). Hence, it 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). A good correlation between permeability measured across Caco-2 cell monolayers and fraction absorbed orally in human has been substantially demonstrated (Artursson P., Palm K. et al. 2001, Ungell A.L. and Karlsson J. 2003). High permeability drugs such as caffeine ($Papp_{AP-BL}$ 44.29 ± 5.12 × 10^{-6} cm/sec) and propranolol ($Papp_{AP-BL}$ 30.76 ± 1.91 × 10^{-6} cm/sec) were absorbed completely (100% f_a). The extent of absorption (f_a) markedly decreased in low permeability drug group such as hydrochlorothiazide

($Papp_{AP-BL}$ 2.24 ± 0.57 × 10^{-6} cm/sec, 70% f_a) and atenolol ($Papp_{AP-BL}$ 0.92 ± 0.36 × 10^{-6} cm/sec, 50%f_a) (Volpe D.A., Faustino P.J. et al. 2007).

Under the optimum cultured condition, the Caco-2 monolayers express a number of drug transporters in the ATP binding cassette superfamily including P-gp (Ferrec E.L., Chesne C. et al. 2001, Taipalensuu J., Törnblom H. et al. 2001). P-gp is a drug efflux pump localizing 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 across restrictive 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 2008, Staud F., Ceckova M. et al. 2010). Although the native Caco-2 cells are able to express P-gp at the appreciable extent, the amount of protein expression can be fluctuated. Several factors such as passage number and subculture process can cause the variation in the number of functional active protein (Sambuy Y., De Angelis I. et al. 2005). In the pilot study, the higher number of active P-gp in the Caco-2 were obtained by culturing the cells in the medium containing vinblastine (VBL; 10 nM) as described in the previously reported protocols (Shirasaka Y., Kawasaki M. et al. 2006, Siissalo S., Laitinen L. et al. 2007, Hellinger E., Bakk M.L. et al. 2012). These VBL-resistant Caco-2 cells steadily expressed high levels of functional active P-gp, even when the high passage numbers (96-112) of the cells were used. The extent of active P-gp in the VBL-resistant Caco-2 cells at the high passage number (96-112) and the native Caco-2 cells at the lower passage number (50-70) were not significantly

different. It was possible to use the VBL-resistant cells as an alternative for transport study. In this study, the native Caco-2 cells at the passage number between 50 to 70 were used to maintain the consistent level of P-gp expression.

It is crucial that the cultured monolayers can function as an intestinal absorptive barrier. Before the transport study, the integrity and function of the Caco-2 monolayers were assessed by measurement of transepithelial electrical resistance (TEER) and P-gp activity. In this study, the TEER values of Caco-2 monolayers at day 5 to day 21 after seeding increased in time dependent manner. The TEER values at day 21 measured before the transport experiments were 883.28 ± 18.11 Ω cm^2. In other laboratories, the TEER values of Caco-2 monolayers at day 21 have been reported in the range of 300-1400 Ω cm^2 (Wilson G., Hassan I.F. et al. 1990, Borchard G., Lueben H.L. et al. 1996, Wang Q., Strab R. et al. 2008). In addition, the TEER values of greater than 300 Ω cm^2 were generally accepted as an indicator of a good restrictive barrier for the transport study (Hunter J., Jepson M.A. et al. 1993, Troutman M.D. and Thakker D.R. 2003, van Breemen R.B. and Li Y. 2005). In this study, the function of P-gp was assessed by the differential amount of transported rhodamine 123 across the monolayers in the presence and absence of verapamil. The efflux ratio of rhodamine 123, a known P-gp substrate, was 6.73 ± 0.78. The presence of verapamil decreased the efflux ratio to 2.16 ± 0.24. Hence, verapamil, a known P-gp inhibitor, was shown to significantly inhibit the transport of rhodamine 123 from the basolateral side to the apical side. The USFDA suggests that (1) compounds being P-gp substrate should have the efflux ratio ≥ 2 and (2) the P-gp mediated transport can be significantly inhibited by one or more P-gp inhibitors (US Department of Health and Human Services Food and Drug Administration 2012).

Taken together, the Caco-2 monolayers in this study was suitable model of absorptive barrier with active P-gp at an appreciable level.

The effects of phyllanthin on the monolayer integrity were evaluated by measuring TEER values and leakage of lucifer yellow (LY) (Hubatsch I., Ragnarsson E.G. et al. 2007, Hellinger E., Bakk M.L. et al. 2010). In this study, the TEER values of Caco-2 monolayers before the transport experiments were in the range of 728.52-1092.78 Ω cm^2. These values did not change significantly after treatment with phyllanthin in the transport experiments. LY is a known paracellular transport marker (Bansal T., Singh M. et al. 2007, Wahlang B., Pawar Y.B. et al. 2011). This compound is hydrophilic and can be transported across the cell monolayers via only paracellular route (Bansal T., Singh M. et al. 2007, Wahlang B., Pawar Y.B. et al. 2011, Johannessen L.E., Spilsberg B. et al. 2013). The tight junctions can be viewed as a gate of the entrance to the paracellular route which also plays a role in limiting movement of chemical across the cell monolayers. Therefore, the opening of tight junction or the loss of tight junction integrity can lead to an increase of LY flux across the monolayer. Generally, the paracellular integrity of the monolayers remains intact if the transport rate of LY was less than 1% per hour (Bansal T., Singh M. et al. 2007). In this study, the LY flux measured at the end of each transport experiment was 0.05 $\pm$ 0.01% per hour. Hence, phyllanthin had no disruptive effect on the Caco-2 permeability model. It was unlikely that phyllanthin was able to induce an opening of tight junctions and crossed the epithelial monolayer via paracellular pathway.

Permeation of phyllanthin across the monolayers was investigated under pH-gradient condition (pH 6.5_{AP}-7.4_{BL}) at 37°C. This pH condition was similar to human intestinal conditions during drug absorption. It is known that the microenvironment

near the mucosal membrane in the drug absorptive area has lower pH than the luminal fluid (Fleisher D. 2000). In addition, the pH in the intestine is also an important factor influencing drug absorption particularly for ionizable drugs (Ungell A.L. and Karlsson J. 2003, El-Kattan A. and Varma M. 2012). The pH in the GI tract determines the degree of drug ionization. As known, drugs in ionized form hardly diffuse across the absorptive tissue. Moreover, the generated proton gradient across the epithelial membranes may serve as a driving force for some secondary active transporters (Yamashita S., Furubayashi T. et al. 2000, Ungell A.L. and Karlsson J. 2003). For example, weak acid drugs such as warfarin, furosemide, and ampicillin showed higher permeability under the pH-gradient condition (pH 6.0_{AP}-7.4_{BL}) than under the iso-pH condition (pH 7.4_{AP}-7.4_{BL}) (Yamashita S., Furubayashi T. et al. 2000). In contrast, weak basic drugs such as alprenolol, terbutaline, and atenolol showed lower permeability under the pH-gradient condition (pH 6.0_{AP}-7.4_{BL}) than under the iso-pH condition (pH 7.4_{AP}-7.4_{BL}) (Yamashita S., Furubayashi T. et al. 2000).

In order to facilitate the permeability study, the transport of phyllanthin was determined in concurrent with known permeability markers. This cocktail system provided an advantage in shortening the time course of experiment and sample analysis. This would be very useful for rapid screening of NCEs in candidate drug selection process. The cocktail mixture included phyllanthin and three permeability markers selected from the list of USFDA's recommendation (US Department of Health and Human Services Food and Drug Administration 2000). In this study, theophylline, antipyrine, and furosemide were chosen to represent the high and low permeability drug groups. The chemical interaction in the cocktail mixture was tested to ensure the sensitivity and precision of sample analysis. The validation of

HPLC method clearly showed the HPLC analysis system was able to effectively quantify each compound in the mixture. The accuracy and precision of the HPLC analysis were acceptable within the range of 100 ± 10% recovery and with less than 10% C.V., respectively. This study showed that compounds in this cocktail did not interact and did not change the sensitivity of analysis. Moreover, the permeability of each permeability marker in the mixture was comparable to its reported permeability when tested as a single compound (Yamashita S., Furubayashi T. et al. 2000, Koljonen M., Hakala K.S. et al. 2006, Volpe D.A., Faustino P.J. et al. 2007). One major concern when using the cocktail mixture in the transport study was the loss of sink condition for the high permeability compound due to long sampling time. The low permeability marker would need high number of sampling intervals in order to collect enough sample for permeability estimation. The prolonged period of sample collection might result in an underestimation of permeability of high permeable markers (Koljonen M., Hakala K.S. et al. 2006).

Permeability of phyllanthin in the cocktail mixture across Caco-2 cell monolayers in the absorptive direction (AP to BL) was slightly different from that being measured as a single compound. As shown in this study, the $Papp_{AP\,BL}$ of phyllanthin decreased from 40.09 ± 1.04 × 10^{-6} cms^{-1} (when measured as a single compound) to 34.90 ± 1.18 × 10^{-6} cms^{-1} (when measured in the cocktail mixture). This difference might be due to the non-sink condition in the receiver chamber, which possibly allowed phyllanthin to diffuse backwardly into the donor chamber. Despite the difference of Papp values, the permeability rank order of phyllanthin remained unchanged. Phyllanthin could be classified as a high permeable compound. Considering that permeability of each compound in the cocktail was

measured under the same condition, the $Papp_{AP-BL}$ of phyllanthin was in the same rank order with those of highly permeable drugs (antipyrine and theophylline). It has been reported that the $Papp_{AP-BL}$ of these compounds greater than 10×10^{-6} cms^{-1} correlated to substances with high absorption (70-100%) (Yee S. 1997). It was likely that phyllanthin could be highly absorbed in the intestine.

Permeability of phyllanthin determined under the pH gradient condition (pH 6.5_{AP}-7.4_{BL}) was similar to that measured under the iso-pH condition (pH 7.48_{AP}-7.48_{BL}) (Nguyen D.H., Sinchaipanid N. et al. 2013). This evidence indicated that phyllanthin was a non-ionizable compound under the pH range of 1.12-10.02 (Hanh N.D., Sinchaipanid N. et al. 2013). In this study, the concentration of phyllanthin at 75 μM was the maximal concentration that could be tested in the transport buffer system without its precipitation. At this concentration, phyllanthin did not cause cytotoxicity toward the cell monolayers. Being a good drug candidate with rapid absorption in the intestine, phyllanthin should be available as an oral drug.

In addition to permeability, solubility is also a critical factor for drug absorption. As known, chemical permeability and solubility were used to categorize drugs into four classes in the Biopharmaceutical Classification System (BCS) (US Department of Health and Human Services Food and Drug Administration 2000, Lindenberg M., Kopp S. et al. 2004, Wu C.Y. and Benet L.Z. 2005). The BCS applies the in vitro data for the prediction of bioavailability and drug absorption. The BCS is a tool in drug development to justify the need of in vivo bioavailability and bioequivalence studies for immediate-release solid oral dosage forms (US Department of Health and Human Services Food and Drug Administration 2000). Drugs in BCS class I and II are classified as high permeability drugs. These drugs are

good candidates for the oral route of drug delivery due to their high ability to diffuse across the intestinal absorptive barrier (Dahan A., Miller J.M. et al. 2009, Ungell A.-L. and Abrahamsson B. 2009). The difference between class I and class II is drug solubility. Drugs with high solubility belong to class I whereas the ones with low solubility are in class II. If the drug is a substrate of efflux transporters, solubility can be a significant limiting factor for the absorption. High solubilization allows the high amount of drug to be available in the solution. Consequently, the drug concentration at the enterocytes will be high enough to overcome the effects of transporters. Thus, transporter effects on drug transcellular transport will be minimal for the BCS class I compounds, but predominant for the BCS class II compounds (Wu and Benet, 2005). In this study, classification of phyllanthin according to the BCS would help to predict its absorption and oral bioavailability.

In this study, phyllanthin was practically insoluble or insoluble with an aqueous solubility of less than 5 µg/ml over the pH range of 1-7.5 at 37 ± 1°C. Hence, phyllanthin might be classified as a BCS class II (low solubility-high permeability) compound. The intestinal absorption of BCS class II compounds can be limited by solubility of those compounds in the absorption region of the intestine (Yu L.X., Amidon G.L. et al. 2002). In addition, drug efflux transporters in particular P-glycoprotein may hinder the absorption of BCS class II drugs if the drugs are their substrate (Wu C.Y. and Benet L.Z. 2005, Murakami T. and Takano M. 2008). The rate and extent of drug absorption can be enhanced by improving the solubility and dissolution through several techniques such as formulation design and particular size reduction (Sheng J.J. and Amidon G.L. 2010, Yasir M., Asif M. et al. 2010). It has been reported that the intestinal oral bioavailability of P-gp substrates in BCS class II could

be raised by increasing its aqueous solubility. The increase in solubility would allow more drug molecule at the absorptive area and the P-gp effect could be escaped (Murakami T. and Takano M. 2008).

Recently, it was reported that phyllanthin directly inhibited P-gp activity in Caco-2 cells (Sukhaphirom N., Vardhanabhuti N. et al. 2012). However, it was unclear whether phyllanthin-mediated interference on P-gp function significantly affected its transport across the absorptive barrier. In this study, the permeability ratio of the excretion to the absorption was close to unity, suggesting that P-gp had no influence on phyllanthin transcellular permeation across the Caco-2 monolayer. It was possible that the amount of phyllanthin at the absorptive area was able to saturate the drug efflux pump. Consequently, phyllanthin at the concentration of 75 μM could overcome the effects of efflux transporters. The major transport pathway of phyllanthin probable is transcellular pathway.

It might be noteworthy to mention that P-gp could be a significant limiting factor for the intestinal absorption of phyllanthin at the low concentration. It was reported that phyllanthin at a concentration of 18.46 μM (7.73 μg/ml) had an efflux ratio of 2.15 (Nguyen D.H., Sinchaipanid N. et al. 2013). The efflux ratio more than 2 was as an indicator of active efflux transport mechanism. Hence, active transport mechanism may be involved in the intestinal absorption of phyllanthin at the low concentration. Nevertheless, the concentration in this study was lower than the pharmacological active concentrations reported in vitro and vivo studies (Leite D.F., Kassuya C.A. et al. 2006, Islam A., Selvan T. et al. 2008). It can be assumed that the concentration of phyllanthin in oral dosage forms would be higher than the concentration in this study.

In conclusion, phyllanthin could be classified as a BCS class II compound with high permeable and low soluble properties. Phyllanthin was feasible to be developed into an oral drug. This lignan would be rapidly absorbed in the intestine with little hindrance from P-gp. Further pharmacokinetic studies should be carried out in order to evaluate its in vivo absorptive fraction and bioavailability for further drug development.