CHAPTER III

RESULTS AND DISCUSSIONS

1. Solubility

The aqueous solubilities and calculated partition coefficient (clogP) of mefenamic acid esters **1**-**7** are summarized in Table 5. Since the esters promoieties are very lipophilic, partition coefficients determined from the measured ester concentration in octanol and buffer phases would be hardly obtained with reliability (Bruggeman et al., 1982). Partition coefficients of the esters **3**-**6** and all other esters were therefore estimated using clogP program (Biobyte, CA, U.S.A), which calculate the value from its molecular structures. Most of the esters, except **1**, **2**, and **7**, showed increased lipophilicity compared to the parent mefenamic acid (Table 5). Aqueous solubility of the esters is generally lower than that of parent mefenamic acid, which is related to the increased lipophilicity of the esters. Aqueous solubility of the esters **1**-**3** and **6** were less than 10 μg/mL which is 10-100 folds less soluble than the parent mefenamic acid. Increased molecular size by introducing a bulky group through ester linkage, as well as masking the hydrophilic carboxylic group of mefenamic acid, can lead to poor solubility of the esters. Increased aqueous solubility, however, was observed for compound **4** and **5** which are prepared as HCl salt, as well as **7**, which contains hydrophilic arginine promoiety. As shown in Table 4, linkage to hydrophilic groups, such as glycerol (**1**) and pyrrolidinone (**2**) promoieties, yielded less lipophilic compounds compared to the parent mefenamic acid, though no enhanced aqueous solubility was attained.

In spite of their poor aqueous solubilities, the esters are very soluble in organic solvents, as determined for **2** and **3** (Appendix one). For most esters (**1**, **2**, and **6**), solubilities at pH 5.0 and 7.4 are similar to those in water (pH 6.8). These esters are structurally neutral and their solubilities are independent of pH. In contrast, compounds **3** and **4** which contain basic amine functional groups showed several folds decreased in solubility at higher pH (pH 7.4). This is due to the basic properties of **3** and **4** undergoing higher degree of ionization at lower pH. Solubility in pure water

and in phosphate buffer was similar for most of the esters except **4** and **5**, which solubility in phosphate buffer is dramatically decreased. Approximately 40 (**4**) and 100 (**5**) folds less solubility in buffer pH 7.4 than in pure water were observed for both esters. The discrepancy of the values obtained from two media may be due to the indirect effect from chloride present in the buffer, which can depress the solubility of HCl salt by the common ion effect (Lipinski et al., 2001). Minimal solubility was observed for **6**, possessing a sparingly soluble neutral paracetamol moiety with high lipophilicity. At pH 7.4, solubility of the esters (regardless of **4**, **5**, **7**) decreased in the order **1** > **2** > **3** > **6** which is correlated with lipophilicity (c log P) of the esters.

Table 4 Solubility of mefenamic acid esters in water and in 0.05 M buffer solutions at pH 5.0 and 7.4 at 37°C

		Solubility (μg/mL)		
Compound	clogP	pH 5.0	pH 7.4	H_2O
Mefenamic acid	5.12*	-	33.6	67.0
1	3.50	-	5.02	6.02
2	4.80	4.89	3.72	2.59
3	5.28	6.28	2.61	4.40
4	5.99	1.05×10^3	109	4.49×10^3
5	6.54	-	15.8	1.96×10^3
6	5.73	-	3.34×10^{-1}	6.52×10^{-1}
7	-0.085	102	95.3	94.1

The solubility values are determined in duplicate, except for **3** and **4**,which were determined in triplicate.

* Experimental logP

Aqueous solubility is a physicochemical property dictating the sufficiency of the molecule to be solubilized prior to absorption. Lipophilicity governs the interaction of the molecule with intestinal membrane. Molecules intended for oral administration must be sufficiently hydrophilic to dissolve in GI fluids; yet, they must also possess hydrophobic character to be passively transported across intestinal epithelia. Mefenamic acid, a weak organic acid with pKa 4.2, is sparingly soluble in water, and was categorized as poorly soluble but highly permeable compound representative of Biopharmaceutics Classification System (BCS) class II (Galia et al., 1998; Taub et al., 2002; TenHoor et al., 1991; Yazdanian et al., 2004). Aqueous solubility of mefenamic acid at 37°C obtained in this study (67 μg/mL) is consistent with the value reported of 80 μg/ml (TenHoor et al., 1991). The esters **1**-**7** used in this study, though containing both hydrophobic and hydrophilic promoieties, exhibit lower solubility than that of parent mefenamic acid. Their permeabilities, however, were studied and discussed in section 3.4 . Solubility determination demonstrated in the current study provides a basic understanding of a compound's solubility at the loaded concentration and pH used in permeability and stability studies.

2. Chemical stability

Chemical hydrolysis of the esters was performed to evaluate stability of the molecule so that they can be formulated in a stable dosage form. Buffers employed in chemical hydrolysis study covered pH of microclimate of stomach (pH 1-2), duodenum (pH 4.0-5.5), jejunum (5.5-7.0) and systemic circulation (pH~7.4) (Daugherty and Mrsny, 1999). Results of hydrolysis of prodrugs **1**-**7** in aqueous buffer pH 2, 5, and 7.4, expressed as half-lives of the esters, are summarized in Table 5 (see Table 10 in Appendix one for rate constant of degradation). Esters **2** and **6** were hydrolyzed at all pH's studied whereas **1**, **4**, **5**,and **7** are stable at all studied pH's. At pH 2, compounds **2** and **6** were hydrolyzed with apparent half-lives of 5.2 and 19.4 hrs, respectively (Table 5). Marginal hydrolysis, however, was observed at pH 7.4 for esters **2**, **3**, and **6**, yielding apparent half-lives 8.7, 5.0, and 4.2 hrs, respectively, while others are stable at this pH. Since most esters are stable at pH ≤ 7.4, these data indicated that the esters **1**-**7** are resistant toward hydrolysis at pH of

stomach acidic microenvironment, pH of the small intestinal mucosa, and at physiological pH. When degraded, each ester quantitatively yielded mefenamic acid (Appendix one).

Table 5 Apparent half-lives ($t_{1/2}$) of mefenamic acid esters **1**-**7** in 0.05 M buffer solution at pH 2.0,5.0, and 7.4 at 37°C *

Compounds	Half-lives (hrs)**		
	pH 2	pH 5	pH 7.4
1	-	-	-
2	5.20 ± 0.73	5.29 ± 0.99	8.71 ± 1.77
3	-	-	4.97 ± 1.71
4	-	-	-
5	-	-	-
6	19.4 ± 1.72	31.1 ± 3.94	4.19 ± 0.78
7	-	-	-

* Rate constants and correlation coefficient of the fitting were shown in Appendix one
** Each value represents mean ± S.D. of three determinations

3. Enzymatic Stability

Degradation of the esters by enzymes from biological media including human plasma, Caco-2 homogenate, and rat liver homogenate, were determined and their apparent half-lives are listed in Table 6. All esters except **7**, which posseses stable amide linkage, degraded in biological media. Quantitative releases of parent mefenamic acid from these esters upon hydrolysis were obtained, i.e. hydrolysis profiles of **1** in human plasma (Figure 10).

In human plasma, facile cleavage of the esters **1**-**4** was attained with apparent half-lives range from 15 to 60 min observed. However, prolonged degradation in human plasma with half-lives of 300-1770 min, were observed for esters **5** and **6**. In Caco-2 homogenate, **3**, **4**, and **5** are relatively resistant to enzymatic cleavages, while esters **2** and **6** were readily hydrolyzed with apparent half-lives of 5.3 and 9.8 min, respectively. In rat liver homogenate, rapid degradation of esters **1**-**3** with half-lives

less than 60 min was observed, while **4** and **5** were more resistant toward hydrolysis, exhibiting apparent half-lives greater than 7 hrs (Table 6)(see Table 11 in Appendix one for rate constant of degradation).

Table 6 Apparent half-lives ($t_{1/2}$) of mefenamic acid esters **1**-**7** in various biological media

Compound	Half-lives (min)[a]		
	Plasma	Caco-2 homogenate	Rat liver homogenate
1	46.1 ± 1.69	58.3 ± 0.98	28.7 ± 2.82
2	15.5 ± 1.17	5.31 ± 1.47	19.0 ± 1.26
3	57.6 ± 19.5	738 ± 36	50.3 ± 6.06
4	37.2 ± 3.09	1277 ± 159	1472 ± 271
5	299 ± 14.4	1773 ± 320	466 ± 50.4
6	1770 ± 293 [b]	9.75 ± 1.05	*c*
7	*	*	*

* No degradation observed was observed during 24 hr incubation
[a] Each value represent mean ± S.D. of three determinations
[b] Zero order
c Not determined

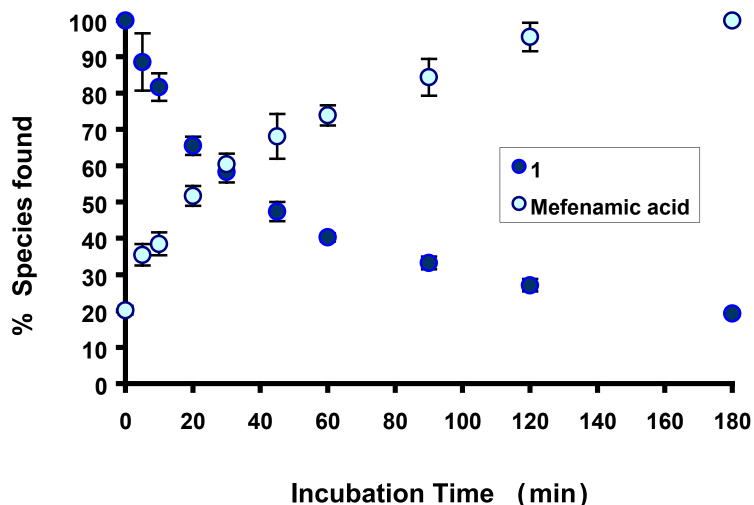

Figure 10 Degradation profile of **1** in human plasma (pH 7.4, 37°C). Each value represented mean ± S.D. of triplicate studies.

Bioconversion of the esters **1**-**7** in the biological media used in this study occurred with variable rates. Contents and types of the esterases presented in the biological media might be different depending on the source of the media and this could affect the overall catalysis. For example non-specifiic esterase activity was high in human plasma and liver whereas peptidases and protease was found in GI tract in addition to esterases. Brush border membrane of Caco-2 cell presents typical enzymes, e.g. aminopeptidase, endopeptidase and phosphatase (Quaroni and Hochman, 1996), which may influence hydrolysis of the ester. However, phenyl-methylsulfonylfluoride (PMSF), a serine protease inhibitor, can suppress these enzymatic hydrolysis as demonstrated in Figure 11.

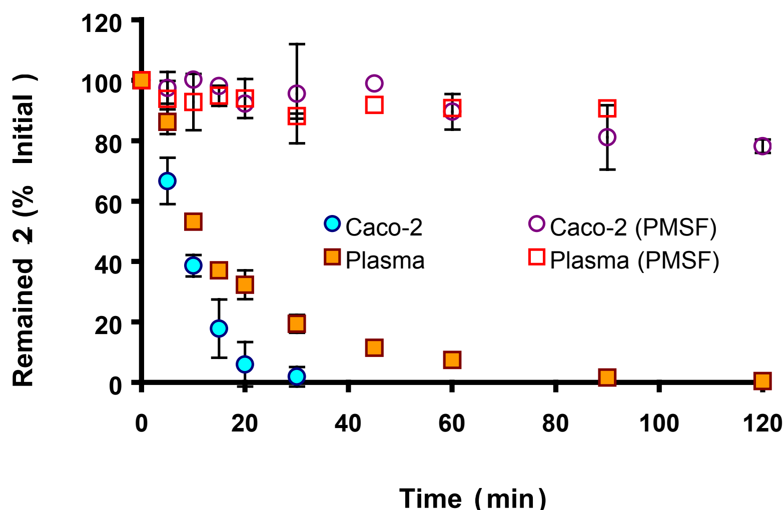

Figure 11 Degradation profile of **2** in human plasma and Caco-2 homogenate (pH 7.4, 37°C), with and without protease inhibitor, PMSF (0.5 mM). Each value represented mean ± S.D. of triplicate studies.

Hydrolysis of **6** in plasma was found to follow pseudo zero order kinetic although degradation of **6** in Caco-2 homogenate as well as other esters in all biological media was well described by pseudo first order rate kinetic. This could be attributed to saturated incubation mixture of **6** used in the hydrolysis studies. Among all esters studied, compound **6** showed minimal aqueous solubility (~ 0.652 µg/mL, Table 5) whereas initial concentration employed (0.552 µg/mL) was approximately its aqueous solubility. Kinetic of hydrolysis in plasma therefore followed to those normally observed in suspension system, whose degradation profile is well described by pseudo zero order kinetic. Lowering substrate concentration (to ~ 50% of the concentration used) has led to undetectable signal when analyzing plasma samples by HPLC method. Moreover, analysis of **6** in rat liver homogenate was severely interfered by endogenous background. Analytical method of **6** in rat liver homogenate, therefore, would require intensive sample clean up procedures other than a simple deproteinization step as employed in this study.

The hydrolysis profiles of all esters were illustrated in Appendix One, whereas representative chromatograms from hydrolysis studies are depicted in Appendix Two.

For oral delivery approach, the ester prodrugs of mefenamic acid should be absorbed across the intestinal epithelium in intact form, and cleaved when exposing to blood circulation. Half-lives of degradation in Caco-2 homogenate can serve as a predictive tool for stability of the esters in the GI tract. Accordingly, esters **1**, **3**, **4**, and **5** with the half-lives ranged from 1.0 - 29 hrs in Caco-2 homogenate, were expected to be adequately stable for absorption *in vivo* since the residence time of normal human duodenum and jejunum were 0.5-0.75 hr and 1.5-2.0 hr, respectively (Daugherty and Mrsny, 1999). Hydrolysis in blood circulation after traversing the intestinal cells would be expected for these esters, except **5,** which exhibits half-life in plasma of approximately 5 hrs. Prolonged half-lives in plasma encountered by **5**-**7** precluded their potential uses as prodrugs and thus were not included in transport studies. Therefore, esters **1**-**4** shown for their optimal bioconversion, i.e. chemically stable but highly enzymatic susceptibility were selected as prodrug candidates for permeability investigation.

4. Transport Study

Apical or intracellular hydrolysis of the ester prodrugs, caused by a number of brush border enzymes, can be prevented by PMSF. Hence, in transport study, PMSF was applied to Caco-2 monolayer prior to the experiments to minimize esterase-mediated degradation during transepithelial transport. To investigate cytotoxicity of PMSF to monolayer viability, MTT assay was thus performed. Caco-2 cells treated with PMSF at the concentration of 0.05-0.10 M in the presence of 0.5-1.0% DMSO yielded comparable results to that of untreated cell ($p = 0.47$). These results suggested that PMSF is not toxic to cells.

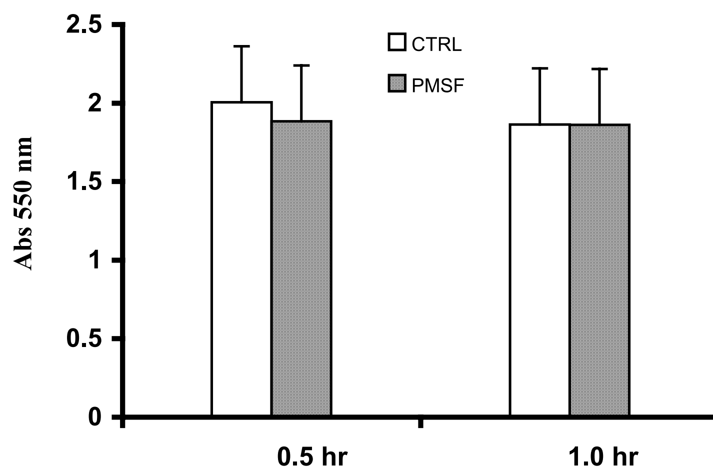

Figure 12 Cytotoxicity of phenylmethyl sulfonyl fluoride (PMSF) to Caco-2 monolayer using MTT assay. Untreated cells (control) was regarded as 100 % viability. Absorbance was measured after 4 hrs of formazan formation.
Each bar indicates mean ± S.D. for triplicate studies

Permeabilty, expressed by apparent permeability coefficient (P_{app}), and the efflux ratio ($P_{app,\ B\text{-}A}$/ $P_{app,\ A\text{-}B}$) for ester **1**-**4** were summarized in Table 7 and depicted in Figure 12. The efflux ratio ≥ 2 is considered as an evidence for apical efflux. According to absorptive permeability coefficient ($P_{app,\ A\text{-}B}$), mefenamic acid, **1**, and **2**, demonstrated high permeability with $P_{app,A\text{-}B}$ of 12.3-18.6 x 10^{-6} cm/s, whereas **3** and **4** represented low permeability category with $P_{app,A\text{-}B}$ only 10% of the values of **1** and **2**. In general, factors responsible for low permeability found both *in vitro* and *in vivo* could be physicochemical properties, e.g. zwitterions form, degree of ionization, molecular weight, polar surface area (Winiwarter et al., 1998). Additionally, intestinal efflux could be another process limiting intestinal permeation of the compound. In this study, the efflux ratio of 3.06 and 10.4 observed for **3** and **4**, respectively (Table 7), suggested potential affinity for cellular efflux pumps of the compounds. No clear evidences for polarized transport for **1**, and **2**, as the efflux ratio of these compounds were approximately 1. Larger value of efflux ratio observed for **4** than of **3** in spite of their comparable absorptive permeabilities was resulting from greater value of secretory P_{app} of **4**.

It has been demonstrated that mefenamic acid is a highly permeable compound with no apparent interactions with cellular efflux pumps (Yazdanian et al., 2004). Absorptive P_{app} of mefenamic acid obtained in our study (18.6 ± 2.02 x 10^{-6} cm/s) was consistent to that described by Yazdanian (2004) of 17.9 ± 0.4 x 10^{-6} cm/s. Among compounds with high permeability, their lipophilicities (clogP) followed the order of mefenamic acid > **2** > **1**. Likewise, $P_{app,A-B}$ of **1**, **2**, and mefenamic acid followed the order mefenamic acid > **2** > **1**. Thus, P_{app} in AP-BL direction for **1**, **2**, and mefenamic acid are well correlated with their logP values.

Table 7 Bidirectional permeability, expressed as apparent permeability coefficient (P_{app}), and efflux ratio of mefenamic acid esters across Caco-2 cell monolayer.

Compound	c log P	P_{app} (x10^{-6} cm/s) (mean ±S.D.)		Efflux Ratio
		AP-BL	BL-AP	
Mefenamic acid	5.12*	18.6 ± 2.02	22.2 ± 2.10[a]	1.19
1	3.50	12.3 ± 0.45	16.5 ± 2.59	1.30
2	4.80	17.2 ± 4.98	13.0 ± 2.60	0.76
3	5.28	1.98 ± 0.66	6.05 ± 1.92	3.06
4	5.99	2.33 ± 0.25	24.1 ± 3.53	10.4

a: from Yazdanian et al., 2004
* Experimental value

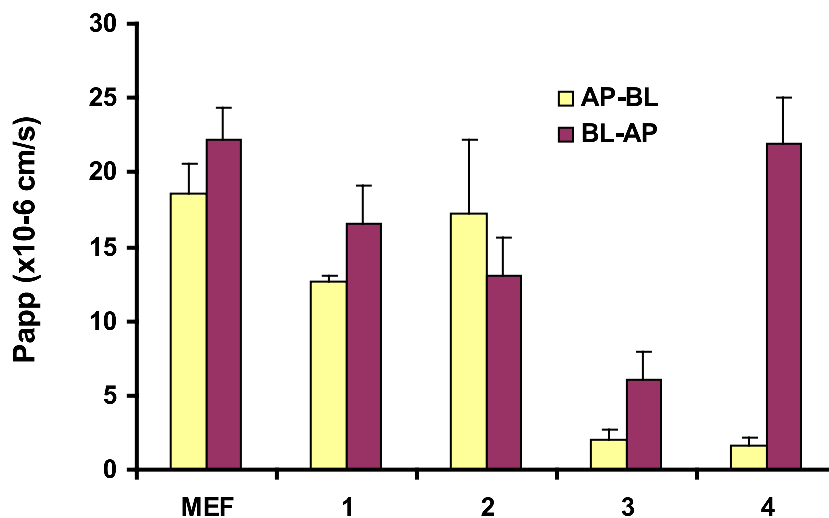

Figure 13 Bidirectional transport across Caco-2 monolayers of four ester derivatives of mefenamic acid. Each bar indicates mean ± S.D. for triplicate studies

4.1 Efflux Inhibition Study

Inhibitors of efflux transporters has been widely employed in identifying the transporter responsible for secreting of compounds out of the cells. Evaluating the transport of a drug in the presence of specific inhibitors can provide useful information on the role of various transporters on drug permeability. Two most characterized efflux systems, Pgp and MRPs (specifically MRP-2), are expressed at the apical surface of Caco-2 cells (Chan et al., 2004; Prime-Chapman et al., 2004). Thus, a classic Pgp inhibitor, verapamil, and an MRP inhibitor, indomethacin (Figure 9) were used in this study.

Bidirectional transport results, in the presence and absence of verapamil and indomethacin, were summarized in Table 8. As shown in Table 8, a Pgp inhibitor verapamil caused a statistically increase in absorptive permeability but insignificant changes in secretory permeability of **3**. Neither absorptive nor secretory permeability of **4** was significantly changed by verapamil though some increments in permeability of both directions were observed. The enhancement in P_{app} was higher in the absorptive than secretory direction leading to decreases in efflux ratio of both **3** and **4**

with verapamil inclusion (Table 8). Efflux ratio of **3** and **4** was decreased to 1.3 for **3**, and 8.5 for **4**, suggesting almost complete inhibition of efflux of **3** by verapamil but not for **4**. Increase in $P_{app,A\text{-}B}$ by verapamil was more pronounced for **3**, 1.98 $x10^{-6}$ cm/s (control) *vs.* 6.64 $x10^{-6}$ cm/s with verapamil, than for **4** , 2.33 x 10^{-6} cm/s (control) *vs.* 3.37 $x10^{-6}$ cm/s with verapamil. The discrepancy in inhibition the efflux of two compounds by verapamil was probably due to the different affinity of each compound to Pgp binding sites. At 100 μM used in this study, verapamil may competitively inhibit the transport of **3**, but less efficient in inhibiting the efflux of **4**. Because of the limit solubility of the esters, particularly in the solution with verapamil, examination of concentration dependent of verapamil by increasing concentration was not feasible. Nevertheless, these results could indicate the potential involvement of Pgp in the active transport of **3** and **4**.

Table 8 Effects of verapamil and indomethacin on apparent permeability of esters **3** and **4** across Caco-2 monolayer

Compounds	P_{app} ($x10^{-6}$ cm/s) (mean ± S.D.)		Efflux Ratio
	AP-BL	BL-AP	
3	$1.98 \pm 0.66^{**}$	$6.05 \pm 1.93^{**}$	3.06
3 + Verapamil	$6.64 \pm 1.88^{*, NS}$	8.72 ± 0.64^{NS}	1.31
3 + Indomethacin	$8.06 \pm 0.85^{*,**}$	$5.40 \pm 0.81^{**}$	0.67
4	2.33 ± 0.25	24.1 ± 3.53	10.4
4 + Verapamil	3.37 ± 0.65	28.7 ± 0.76	8.51
4 + Indomethacin	2.83 ± 0.55	$41.7 \pm 7.22^{*}$	14.7

* $p < 0.05$ compared to control of the respective transport direction
** $p < 0.05$ of mean values of AP-BL and BL-AP experiments, using paired t-test
NS, not significant ($p > 0.05$) of mean values of AP-BL and BL-AP experiments, using paired t-test

The MRP inhibitor indomethacin at the concentration of 100 μM, significantly increased ($p < 0.05$) absorptive permeability, and in the same time decreased secretory permeability of **3** (though insignificant, $p = 0.62$). This resulted in reduction of efflux

ratio from 3.06 to 0.67, suggesting the involvement of MRP in the transport of **3**. In contrast to the effect of indomethacin on the efflux of **3**, the efflux ratio of **4** increased in the presence of indomethacin. Obviously, indomethacin did not significantly alter absorptive permeability of **4**, but dramatically enhanced secretory permeability (p = 0.006) resulting in elevated efflux ratio compared to control, efflux ratio of 10.4 (control) *vs.* 14.7 with indomethacin.

Although indomethacin is thought to be rather unspecific as transport inhibitor, it has been reported that it does not affect the Pgp-mediated activity (Draper et al., 1997; Evers et al., 1996). Indomethacin treatment in **3** resulted in significant larger absorption than secretory permeability (p =0.017) leading to the efflux ratio reduction to approximately unity, while these observations were not obtained with verapamil treatment. These results might indicate that blockage of the efflux by indomethacin were more effective than those of verapamil. Otherwise, it is possible that the inhibitions are merely due to the blockage of MRP at the AP side of Caco-2 cell. Due to the noticeable effects of indomethacin on transport of **3**, it is very likely that MRP (mostly MRP2) primarily regulate transport of **3**. The effects of verapamil on transport of **3** could be explained by the fact that verapamil, in addition to be a strong modulator for Pgp, is also a moderate inhibitor of MRP1 (Goh et al., 2002; Walgren et al., 2000), the partial inhibition of MRP1-mediated efflux by verapamil were therefore observed.

The net effects of indomethacin on permeability **4** are more complicated than those of verapamil. Since at least 7 isoforms of MRPs were characterized and noticed for their differential localization and expression levels (Chan et al., 2004; Prime-Chapman et al., 2004). MRP2 or cMOAT is the only isoform located at the apical membrane of as intestine, kidney, liver, and Caco-2 cell, such where it is believed to mediate drug secretion and efflux in intestine and liver (Chan et al., 2004; Prime-Chapman et al., 2004). Other isoforms of MRPs (e..g. MRP1,MRP3, MPR 4, and MRP 5) was expressed at basolateral (BL) membrane of polarized tissues (Chan et al., 2004., Prime-Chapman et al., 2004). In contrast to Pgp or MRP2 which extrude intracellular substances to the apical membrane (i.e. luminal side of the intestinal tract), the basolateral orientation of MRP1, MRP3, MRP4, MRP5 suggest that they protect cells by extruding their substrates into blood (Figure 7) (Chan et al., 2004).

Transport mediated by these MRPs, oriented as absorptive transporters, would facilitate a flux toward the basolateral side (Lalloo et al., 2004). Inhibition of these absorptive transporters will result in limited AP-BL flux.

Since dramatic enhancement of BL-AP flux of **4** was observed with the presence of indomethacin, inhibition of absorptive transporters (i.e. MRP1 and/or MRP3) rather than secretory transporters (i.e. Pgp or MRP2) was speculated. The fact that these MRP isoforms exhibit a certain degree of overlapped substrate specificity (Table 2), ester **4** could be substrate for MRP 1-5. Although mRNA expression levels of MRP2 were highest in Caco-2 cells, the contribution of MRP 1, MRP3, MRP4 and MRP5 were indispensable (Prime-Chapman et al., 2004). Various MRPs including an apically localized MRP2 and basolateral MRPs probably influences the efflux of **3** and **4**. In contrast to **3**, ester **4** may be a better substrate for basolateral MRPs than apical MRP2. Inhibition of these absorptive MRPs would result in dramatic increases in secretory permeability of **4** over control.

4.2 Calcein AM inhibition assay

The primary *in vitro* methods used to study Pgp substrate activity of compounds include: (i) bidirectional transport assays across polarized cell monolayers grown on Transwell® (Bakos et al., 1998; Polli et al., 1999); (ii) calcein AM fluorescence uptake assays in cell monolayer (Hamilton et al., 2001); and (iii) ATPase activity assays using microsome (Sharom et al., 1999). Each method has its advantages and disadvantages. Although ATPase and calcein AM assays offer high throughput determination and are easily automated, but these assays cannot distinguish Pgp substrates from inhibitors (Hamilton et al., 2001; Kondratov et al., 2001). Kinetics of drug transport and/or inhibitions cannot be obtained from these assays. Moreover, Pgp displays constitutive ATPase activity even in the absence of substrate, which may be due to the transport of endogenous chemicals and lipids. Thus, enhancement in ATPase activity is not true for all substrates as observed with cyclosporin A, daunorubicin and colchicins, which maintain the basal levels of ATPase activity (Polli et al., 2001). It has also been reported that ATPase activity may not be well correlated with rhodamine 123 efflux inhibition activity (Tantishiyakul and Wongpuwarak, 2005). In general, drugs produce a bimodal ATPase activity, with stimulation at low

concentrations and inhibition at higher concentrations. These complications in ATPase activity quantification may lead to false negatives during screening (Varma et al., 2003).

Bidirectional transport assays are labor intensive because of cell culturing and analytical requirements. However, these assays provide both the Pgp substrate characteristics and biomembrane permeability characteristics of drug; therefore, they are widely used as a screening tool for drug disposition in pharmaceutical industry. Polli and co-workers have evaluated structurally diverse compounds using these 3 methods and suggested that the bidirectional transport assay should be used as the primary Pgp substrate screening method (Polli et al., 2001). Therefore, we performed bidirectional transport study to preliminarily characterize compounds as the substrate for efflux transporters. Calcein AM assays were subsequently utilized to identify functional activity of the transporters.

Calcein AM possess planar aromatic domains, which are characteristics feature of many Pgp interacting agents (Pearce et al., 1990). The molecule contains a basic nitrogen atom and extended carbon side chain, which are additional structural requirements associated with Pgp blocking efficacy (Figure 8) (Pearce et al., 1990). Calcein, structurally a fluorescein derivative, possesses a high molar emission coefficient and was insensitive to changes in pH within physiological levels (pH 6.0 - 8.5), as well as cytoplasmic Ca^{2+} and Mg^{2+}contents, and apparently non-cytotoxic (Hollo et al., 1994; Liminga et al., 1994). Calcein fluorescence excitation and emission maxima of 485-496 and 517-540 nm, respectively, lending it as a suitable for both conventional fluorescence/flow cytometric and laser-scanning microscopic application.

Free calcein is well retained inside the cell and transported only by MRP. Half-life of calcein leakage is about 3 hrs at 37°C (Hollo et al., 1994). Cellular efflux of free calcein via MRP, however, is rather slow and inefficient compared to calcein AM loading. Analysis of MRP-mediated efflux of calcein AM has showed about 5,000 fold lower K_m than that of free calcein (i.e. K_m‘s for calcein AM and calcein are 0.05 and 268 μM, respectively) (Essodaigui et al., 1998). Transport of calcein AM by Pgp, however, was comparable to MRP (transport K_m= 0.12 and 0.05 μM for Pgp and MRP, respectively).

Results have shown that calcein accumulation was 3-5 fold increased in the presence of all inhibitors used, as well as the ester **3** and **4**, as shown in Figure 14. Among the known efflux transporters, verapamil, a classic Pgp inhibitor, showed the maximum fluorescence intensity. Indomethacin, an MRP inhibitor, showed approximately 90-95% of the response by verapamil (Figure 15). Another specific Pgp inhibitor, cyclosporin A, yielded almost the same degree of inhibition as **3**, i.e. 70-80% of that of verapamil. As illustrated in Figure 15, **4** gave lowest response of only 50-60% of the maximal response. Highest calcein retention levels observed for verapamil might indicate a strong interaction with calcein AM binding sites on Pgp. Lowest calcein retention observed by **4** reflected weak affinity or different binding site on Pgp and/or MRP. Increasing F overtime in control can be attributed to intracellular esterase hydrolysis (Figure 14). This non-specific hydrolysis at each time point was subtracted from treatment response. Without calcain AM, none of the inhibitors gives fluorescence; therefore, interferences from each compound were not observed.

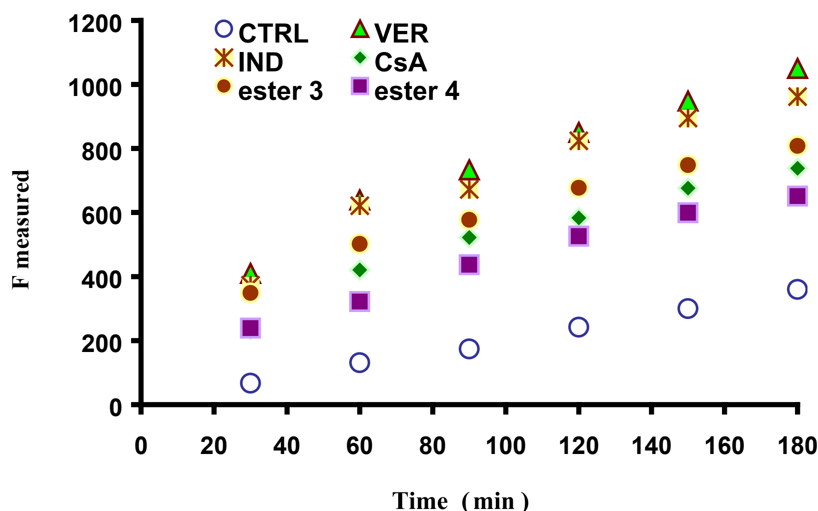

Figure 14 Inhibition effects on calcein efflux in Caco-2 cells. Each inhibitor was used at 50 μM and fluorescence intensity (F) was measured at 30 min interval for 180 min after adding calcein AM. Each point represented averaged F readings. Abbreviation: CTRL: control; VER: Verapamil; IND: Indomethacin; CsA: Cyclosporin A.

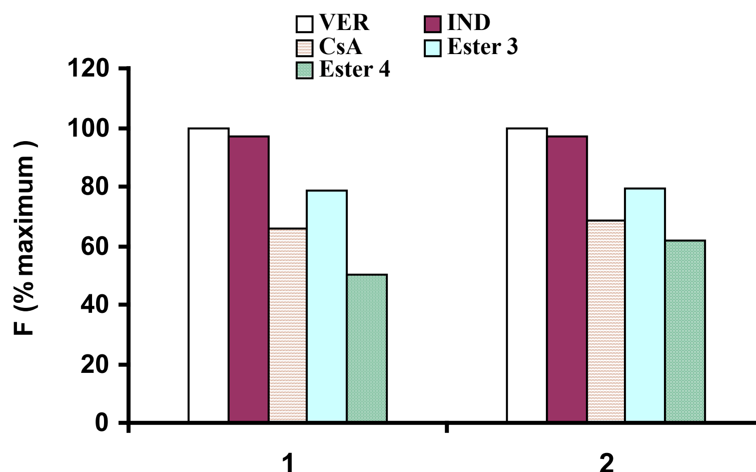

Figure 15 Effect of inhibitors on calcein accumulation in Caco-2 cells. Concentration of each inhibitor was 50 μM, each bar represented the percentage of the maximum response, obtained from verapamil. **1** = F measured at 60 min; **2** = F measured at 120 min. Abbreviation: CTRL: control; VER: Verapamil; IND: Indomethacin; Cs A: Cyclosporin A.

Since Caco-2 cell expressed both Pgp and MRPs, decreased accumulation of calcein could be due to active transport of calcein by MRP and/or calcein AM by Pgp. Calcein accumulation defect also could be partly due to actively transported calcein AM by MRPs. Inhibition of cellular efflux of calcein AM was efficiently obtained by verapamil and indomethacin. Cyclosprin A, a specific modulator of Pgp, unexpectedly showed weaker inhibition than those by verapamil and indomethacin. Cyclosporin A, a hydrophobic peptide, slowly diffused across the epithelium due to its bulkiness, and has great affinity to Pgp due to large interaction surface (Litman et al., 1997). Cyclosprin A exerts inhibitory effects but not completely block Pgp functions by being an 'obstructive substrate' and slowing down Pgp transport rate (Litman et al., 2001). Magnitude of calcein restoration by cyclosporin A was similar to those of **3**, both showed greater inhibition potency than **4**. Since more than one binding sites on Pgp were reported (Safa, 1998; Shapiro et al., 1999), kinetic analysis

of calcein efflux inhibition might be useful in explaining the reason attributing to low inhibitory effects of **4**.

Enhancement in calcein retention by **3** and **4** provide an additional evidence that the esters **3** and **4** can inhibit Pgp and/or MRP in Caco-2 cell. It is recognized that Pgp and MRPs exhibit wide overlapping inhibitors and substrate specificity. Data from calcein efflux inhibition along with the observed bidirectional transports of the compounds suggests that **3** and **4** may belong to the type of Pgp inhibitors that can be transported.

We focused on roles of Pgp and MRP2 in transport of the esters since these two membrane transporters are well-characterized, highly expressed in confluent Caco-2 monolayer (Prime-Chapman et al., 2004), and evidenced for contribution to low intestinal absorption in human. A recent member of ABC drug efflux proteins, breast cancer resistant protein (BCRP, ABCG2), a half transporter, was identified for its expression in the placenta, the cannalicular membranes of the liver, the apical region of epithelium of small and large intestine and ovaries. BCRP showed overlapping substrate specificity with Pgp suggesting that BCRP could play a similar role to Pgp in regulating drug absorption and disposition (Allen et al., 1999). However, with 100 fold lower expression level in Caco-2 cell compared with human jejunum (Taipalensu et al., 2001) and inefficiency transport of calcein AM and calcein by BCRP (Litman et al., 2000), it is less likely for the involvement of BCRP to transport of these esters across Caco-2 monolayer.

Concordance was found between data from transport studies and calcein efflux inhibition assay, indicating apical efflux of esters **3** and **4** were mediated by Pgp and/or MRP in Caco-2 monolayer. Moreover, esters **3** and **4** may function as Pgp and/or MRP inhibitors. However, identification of definite transporters responsible for the apical efflux of these esters cannot be obtained, due to the lack of specific inhibitors. Pathway mediating the efflux can be elucidated using transfected cell line or selected cell line expressing particular membrane transporters in combination with specific transporters.

Among the compounds studied, ester **1** and **2** exhibited high permeabilities, however, **1** is the most promising compound in term of permeabilities and bioconversion. Low permeabilities of **3** and **4** due to apical efflux could prevent the

compounds development as prodrugs of mefenamic acid. However, negatively charged compounds do not readily enter cells, therefore, they are less likely to be lead compounds for drug development. Ester **3** and **4** whose carboxyl group was shielded can serve as lead compounds for designing inhibitors for the efflux transporters.