CHAPTER II

EXPERIMENTALS

Research Outlines

In the present study, physicochemical properties of the ester of mefenamic acid were examined. Solubility in water as well as in buffer at pH 5 and 7.4 were determined. Lipophilicity was presented as clogP. Susceptibility toward chemical hydrolysis was determined at physiological pH and pH similar to those of GI environment, i.e. pH 2.0 and 5.0. The esters' susceptibility toward esterase cleavage was determined in selected biological media including human plasma, rat liver homogenate, and Caco-2 homogenate.

Permeation of the esters across intestinal epithelium was evaluated by transport studies using Caco-2 monolayer. Transports were performed in both directions, i.e. AP-BL and vice versa, to examine potential polarized transport of a compound. Efflux protein-mediated apical transport was additionally evaluated with the inclusion of either Pgp or MRPs inhibitors during the permeation studies. Furthermore, calcein AM efflux inhibition was employed to investigate the potential substrate/inhibitor characteristic of mefenamic acid esters.

1. Materials

1.1 Esters of mefenamic acid

Esters **1**-**7** (Figure 2) were synthesized at Department of Pharmaceutical Chemistry, Faculty of Pharmaceuical Sciences, Prince of Songkla University. Most ester prodrugs were synthesized in one step by directly coupling mefenamic acid with appropriate alcohol, and the acquired esters were purified using column chromatography (Wiwattanawongsa et al., 2005). To obtain the prodrug **2**, the solketal ester was subsequently hydrolyzed, and in the case of **4** and **5**, HCl was added to the ester to form HCl salt.

1.2 Chemicals

Chemicals obtained from Sigma (St. Louis, MO., U.S.A.) were diclofenac sodium (lot number 88F0203), dextromethorphan hydrobromide, monohydrate (lot number 85H 1384), and naproxen (lot number 79H3685), cyclosporin A (lot number 12K4021), and indomethacin (lot number 298134-590). Verapamil and mefenamic acid were obtained from local suppliers. Phosphate buffer saline (PBS, pH 7.2; lot number 609 A) was purchased from Biochrom KG (Berlin, Germany). Solvents for analytical works, i.e. acetonitrile and methanol, were HPLC grade and purchased from Mallinckrodt (Berlin, Germany).

1.3 Chemicals and reagents used for cells cultures

Caco-2 cells were obtained from the American Tissue Culture Collection (Rockville, MD, U.S.A.) at passage 18. Reagents and chemicals used for Caco-2 cells culturing were purchased from Gibco BRL (Life technologies, NY, U.S.A.). These included:

- Dulbecco's Modified Eagles Medium (DMEM with 4500 mg/L glucose), lot number 1090800 and 1115688)

- Fetal bovine serum (heat inactivated), lot number 40Q2015K and 40Q3534K.
- MEM Non essential amino acids (10 mM), lot number 1102965 and 1127865
- L-Glutamine (200 mM) solution, lot number 1175236
- Antibiotics solution (10,000 U/mL penicillin G, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B), lot number 1106811
- Trypsin-EDTA (0.05-0.02%) in HBSS, without Ca^{2+} and Mg^{2+}, lot number 1094614

Other reagents used in cell culture techniques obtaining from Sigma (St. Louis, MO., U.S.A.) included:

- Hanks Balanced Salt Solution (HBSS), lot number123K2382
- *N*-hydroxyethylpiperazine-*N*'-2-ethane sulfonate (HEPES), lot number 113K5401
- 2-(*N*-Morpholino)ethane sulfonic acid (MES), lot number 073K5425
- Dimethyl sulfoxide (DMSO) tissue culture tested grade, lot number 29H0004
- Phenylmethyl sulfonylfluoride (PMSF), lot number 60K0701
- 3-(4,5-dimethylthiazol-2-l)-2,5-diphenyl tetrazolium bromide (MTT), lot number 109H5077

2. Methods

2.1 Solubility Determination

The aqueous solubility of mefenamic acid esters **1**-**7** were determined in water, acetate buffer pH 5.0, and phosphate buffer pH 7.4. at the buffer concentration 0.05 M, ionic strength 0.1 M adjusted with NaCl. Excess amount of each compounds (~ 2-3 mg) was added to 1-2 ml buffer in screw-capped glass vials. Samples were then tumbled at 30 rpm at 25^{o}C until a constant concentration was obtained. Saturated solutions were subsequently filtered through a 0.45-μm cellulose acetate membrane filter (Sartorius, Germany). Aliquot of the filtrate was diluted with appropriate

volume of mobile phase and analyzed by HPLC as described in sample analysis section.

2.2 Chemical stability

Hydrolysis of ester prodrugs of mefenamic acid 1-7 were studied under near physiological conditions at pH 7.4 in 0.05 M phosphate buffer, pH 2.0 in 0.01M HCl, pH 5.0 in 0.05M acetate buffer, and 0.01 M NaOH (pH >10). The ionic strength was maintained at 0.1 M by the addition of NaCl. Since the esters are mostly insoluble in aqueous solution, stock solutions of the esters are prepared by dissolving the compounds in methanol. The reaction was initiated by adding 20-100 μL of esters stock solution (1-2 mg/ml in methanol) into pre-warmed buffer stock solution in screw-capped tube. The final concentration of the compounds was in the range of 5.0-25 μg/mL solutions with final concentration of 1-2% V/V of methanol. The solutions were then placed into a thermostatically controlled water bath at 37^{o}C. At appropriate times, 250-500 μl aliquots of samples were withdrawn, stored at 4^{o}C until assay by HPLC. Upon analysis, samples were thawed, added with 10 μL diclofenac solution (internal standard), diluted with mobile phase and analyzed by HPLC. Pseudo-first order rate constants (k) were determined from the slopes of linear plots of the logarithm of residual prodrug concentrations versus time. Triplicate samples were analyzed, rate constant was estimated and reported. The corresponding half-life ($t_{1/2}$) obtained from the equation: $t_{1/2} = 0.693/k$.

2.3 Enzymatic Stability

Esterase can be found in blood, liver and other organs and tissues. Human plasma, the main compartment for drug distribution, typically contains high activity of esterases (e.g. carboxylesterase) (Satoh et al., 1994), and was the source where rapid clearances of various ester molecules are frequently obtained (Smith et al., 2001). In mammals, the highest levels of non-specific esterases are found in liver and kidney (Smith et al., 2001). Liver microsomes contain a number of esterases that hydrolyze carboxyl ester or thioester of a variety of natural and xenobiotic substrates

(Mentlein and Heymann, 1984). Fresh or cryopreserved liver preparations, such as microsome, S9-homogenates, liver slices and hepatocytes, are important *in vitro* tools in drug metabolisms studies (De Graff et al., 2002). In addition to plasma and liver, intestinal epithelium possess a variety of enzymes distributing both at brush border membrane and in the cytosol. These include carboxylesterases, phase II drug metabolizing enzymes (i.e. enzymes responsible for glucuronidation and sulfation) (Narawanee and Lee, 1994), aminopeptidase, dipeptidyl aminopeptidase, carboxypeptidase P, and prolidase (Asgharnejad, 2000). Many brush border enzymes of small intestinal mucosa are found in Caco-2 cells in similar quantities to those *in vivo* (Artursson, 1990; Quaroni and Hochman, 1996), and esterase-mediated hydrolysis of ester compounds in Caco-2 homogenate were demonstrated by several investigators (Naesens et al., 1998; Van Gelder et al., 2000). Caco-2 cells was used as a model for assessing stability of the esters during intestinal uptake

Thus, hydrolysis of mefenamic acid este **1**-**7** were studied in selected biological media, including human plasma, Caco-2 homogenate, and rat liver homogenate preparations. The reaction was initiated by adding 20-80 μL of the stock solution (in methanol) of the esters to 2-5 ml of pre-warmed biological media, the final concentration of the compounds were in the ranges of 10-100 μM (depend on solubility of the ester. Dimethyl sulfoxide (DMSO) was added to aid in dissolution, yielding final organic solvent of 1-2% v/v. The mixtures were kept in water bath at 37^{o}C. At appropriate intervals, samples of 250-300 μL were withdrawn and added to 500-750 μL of acetonitrile or methanol in order to deproteinise protein in the biological media. After immediate mixing and centrifuge for 10 min at 3,000 rpm, 20-60 μL of clear supernatant was analyzed for remaining ester prodrugs and the respective parent mefenamic acid by HPLC, as described in sample analysis (section 2.5). Reactions were followed for at least two half-lives. In general, pseudo first order kinetics was applied for degradation half-lives of the esters in the biological media where rate constants (k) were estimated from the equation $t_{1/2} = 0.693/k$, as described in section 2.2. However, pseudo zero order kinetics was fitted for degradation profile of the ester exhibiting linear relationship between residual concentration and time.

In this case, apparent zero order rate constant was estimated from slope of plots of the residual prodrug concentrations versus time, and half-lives were calculated from the equation $t_{1/2} = C_0/2k_0$, where C_0 is initial concentration and k_0 is pseudo zero order rater constant.

2.3.1 Caco-2 homogenate Preparation

Caco-2 homogenate was prepared according to the method of previous published method (Augustijns et al., 1998) with slight modifications. Briefly, Caco-2 cells grown in 75-cm^2 culture flask for 21-25 days (see section 2.4 for cell culture techniques) were washed 1-2 times with ice-cold phosphate-buffered saline (PBS), pH 7.2. The cells were scraped off, collected in 4 ml ice-cold PBS, and homogenized using a 15-mL glass-teflon polytron homogenizer (Thomas Scientific, NJ, U.S.A). Cell debris were removed by centrifugation at 12,000 rpm for 10 min at 4^{o}C, the supernatant (corresponded to protein content = 0.63 mg/ml) was used as Caco-2 homogenate in stability studies.

2.3.2 Rat liver homogenate Preparation

Rat liver was obtained from male 250-300 g Sprague-Dawley rats (Animal care units, Prince of Songkla University, Hat-Yai, Songkhla). After blotting to dryness, tissue was sliced in small pieces and homogenized in ice-cold PBS (1ml per 1 gm tissue). Aliquots 1.0 ml of the tissue homogenates was kept at -80^{o}C until use. Before each experiment, the homogenate was quickly thawed and re-homogenized on ice using a 15-mL glass-teflon polytron homogenizer. Cell debris was finally removed by centrifugation at 12,000 rpm for 10 min at 4^{o}C, supernatant was used in stability studies. Liver homogenates was diluted to 50 % (v/v) with pH 7.4 phosphate buffer in stability studies yielding protein content = 47.3 mg/ ml.

2.3.3 Human Plasma

Human plasma was obtained from the Songklanakarind Hospital Blood Bank (Hat-Yai, Songkhla). For stability studies, human plasma was diluted to 80% (v/v) with phosphate buffer, pH 7.4 (protein content = 49.57 mg/ml).

2.3.4 Protein content determination

Protein content of the biological media was determined using a Coomassie® protein assay reagent (Pierce, Rockford, IL, U.S.A.) according to the method of Bradford (Bradford, 1976). Bovine serum albumin (BSA) was used as a standard for protein content measurement.

2.4 Cell culture

Caco-2 cell were grown in a 25-cm^2 tissue culture flask at 5% CO_2 and 90% relative humidity at 37^oC controlled atmosphere. Cells was supplied with Dulbecco's Modified Eagles Medium (DMEM), prepared from dry DMEM powder, and supplemented with 10% fetal bovine serum, 1% non-essential amino acid, 1% L-glutamine, 100 μg/mL streptomycin, 100U/mL penicillin, and 0.25 μg/mL amphotericin B. Normally, cell subculture was performed when reaching their 85% confluency, where detachment from the support was performed by trypsinization. Monolayer was washed once with PBS before 1-2 ml of trypsin EDTA was added. Incubate the flask until cell detachment was observed (this usually occurs after 10-15 min). Add 2.5-3.0 mL medium and disperse the cells by repeated pipetting cell suspension up and down for complete disaggregation. Count the cells using Hemocytometer (Sigma, MO., U.S.A.), and dilute the cell suspension to appropriate seeding concentration. Transfer the cells to a new culture flask at a split ratio of 1:6 to 1:10. For transport studies, cells were seeded at a density of 60,000 cells/cm^2 on polycarbonate membrane (24.5mm diameter, 4.71 cm^2, 3.0 μm pore size) Transwell® (Costar, Cambridge, MA, U.S.A.). Cells were normally grown for 21-25 days for transport experiments with medium changing every other day. Cells from passage numbers 35-55 were used in transport experiments.

2.4.1 Preparation of culture medium

Dulbecco's Modified Eagles Medium (DMEM), 1.0 L consists of

DMEM dry powder (high glucose)		1 pouch
Sodium pyruvate		0.11 gm
Sodium bicarbonate		3.70 gm
Sterile water	qs.	1,000 mL

Method of Preparations

Dissolve DMEM powder, sodium pyruvate, and sodium bicarbonate in ~ 900-950 mL sterile water. Adjust pH of the solution to 7.2-7.3 using 1.0 M HCl before adjusting the final volume to 1,000 mL. The medium was aseptically filtered through a 0.22 μm membrane filter (Sartorius, Germany). The filtered sterile medium was divided into sterile flasks using aseptic techniques. Small portion of the filtered medium was kept at 37^{o}C for 72 hrs to ensure no bacterial or fungal contaminations. Sterile DMEM should be used in 3 months and was normally kept at 4^{o}C.

Complete DMEM medium (~ 100 mL) consists of

DMEM sterile medium	100 mL
Fetal bovine serum	10 mL
L-Glutamine (200 mM)	1 mL
MEM Non essential amino acid (100x)	1 mL
Antibiotic solution (100x)	1 mL

Complete medium should be used in 2-3 weeks and was normally kept at 4^{o}C.

2.5 Samples Analysis

2.5.1 HPLC system

Isocratic HPLC system were developed using mobile phase consisting of 50 μM acetate buffer pH 4.1-5.0 and acetonitrile or methanol was used (Table 3). The system consisted of a 600 Pump, a 717 plus Autosampler, a 486 Tunable Absorbance Detector, and a 746 Data Module (Waters, MA, U.S.A.). The analytical column was Rexchrom octyl, 5μm, 15cm x 4.6 mm, (Regis Technologies, IL, U.S.A.) equipped with a precolumn packed with Novapak RP-8, 5 μm, 3.9 x 20 mm (Waters, MA, U.S.A.). The eluent were detected at 280 nm. Flow rate was set at 1.0 ml/min. Separation of the esters **1**-**7** from parent mefenamic acid was achieved with a total analysis time of less than 24 minutes, as shown in Table 3. In each analysis, diclofenac, an internal standard, was added to the analytical mixture with the final amount of 0.02-0.10 μg. In efflux inhibition studies, dextromethorphan (1.2 μg) and naproxen (0.25 μg) were used as internal standards for the assay of samples from transport of **3** with the inclusion of indomethacin, and verapamil, respectively. In transport inhibition studies of **4**, however, gemfibrosil (0.4 μg) normally use as an internal standard. These compounds were well separated from either indomethacin or verapamil, and the respective ester in the isocratic analysis system.

Table 3 Mobile phase used for HPLC analysis and retention time of each compound.

	Acetate buffer's pH and content		Retention times (min)	
Compound	pH	Content (%v/v)	Esters	Mefenamic acid
1	5.0	40	11.5	9.5
2	4.1	55	16.2	11.3
3	4.5	57	17.5	11.0
4	4.1	50	13.5	7.0
5	4.1	50	16.5	7.0
6	5.5	58	13.0	10.3
7	4.1*	47	11.0	14.8

* consists of 0.1%(w/v) heptanesulfonic acid

While octyl stationary phase was used for analysis of the samples from each experiments, pH and organic contents of the mobile phases used in the efflux inhibition studies were slightly modified. For example, ammonium acetate buffer pH 4.5 - acetonitrile (55-45%) was used for transport of **3** with indomethacin and KH_2PO_4 pH 4.35 - acetonitrile (55-45%) was used for **3** transport with verapamil.

2.5.2 Sample preparation

For determination of the esters and parent mefenamic acid in biological media, samples (plasma, liver homogenate, Caco-2 homogenate) were deproteinized with acetonitrile. After mixing with 3 volumes of acetonitrile, samples were centrifuged for 10 min at 4,000 rpm at 4°C. Aliquots of clear supernatant were then analyzed by the procedures described above.

Determination of intracellular contents of esters or mefenamic acid were performed by sonication for 10 min. Cell suspension (0.2 mL) was subsequently added with 0.4 mL methanol, after thorough mixing, cell debris was isolated by centrifugation at 4,000 rpm at 4°C. Supernatant was the subjected to HPLC analysis or properly diluted with mobile phase before injection into HPLC.

2.6 Transepithelial transport studies of mefenamic ester prodrugs across Caco-2 monolayer

In transport study, microporous membrane consisting of different materials with various pore sizes, surface area, and coating have been used to support the growth of Caco-2 monolayers. Materials such as polycarbonate, nitrocellulose or high-density polyethylene terephthalate (PETP) have been utilized as culture inserts. Study of Caco-2 cells grown on nitrocellulose showed that cells seeded onto non-collagen coated filter decreased attachment and failed to form monolayers. On the contrary, Hilgers et al (1990) demonstrated that collagen may not be necessary for the establishment of Caco-2 monolayers on the polycarbonate filter from a cell attachment and differentiation standpoint. This study, Caco-2 cells passage 35-55 were seeded on polycarbonate inserts at density of 60,000 cells/cm^2. Medium was changed every other days for 21-23 days. The last medium replacement occurs 24 hours before the start of the transport studies. The integrity of the monolayers were evaluated by measurement of transepithelial electrical resistance (TEER) using Millicell–ERS system (Millipore Co., Bedford, MA).

To initiate the experiments, the medium was removed from both sides of the membrane and replaced with apical (AP) and basolateral (BL) buffers, both warmed to 37^{o}C. Apical and BL buffers was adjusted to pH 6.5, and 7.4, respectively, according to the following formula :

Apical transport medium

Hank's Balanced Salt Solution (HBSS)	935 mL
1M D-glucose monohydrate	25
125 mM Calcium chloride	10
50 mM Magnesium chloride	10
1 M MES biological buffer	20
Adjusted with 1 M KOH to a pH of	6.5

Basolateral transport medium

Hank's Balanced Salt Solution (HBSS)	935 mL
1M D-glucose monohydrate	25
125 mM Calcium chloride	10
50 mM Magnesium chloride	10
1 M HEPES biological buffer	20
Adjusted with 1 M KOH to a pH of	7.4

After preincubation with transport medium for 15-20 min, the monolayer were equilibrated in transport medium containing phenylmethyl sulfonyl fluoride (PMSF, 0.5 mM), an inhibitor of serine protease/mammalian esterase, to prevent any possible ester degradation at 37°C.

Transport in both AP-BL (absorptive) and BL-AP (secretory) directions were performed after pretreatment with PMSF. Each ester dissolving in transport medium at the concentrations of 15-50 μM.(with 0.5-2.5% DMSO) was added to the donor compartment. Initial concentration of each ester was chosen according the esters' solubilities. Transwell plates were incubated at 37^{o}C in a shaking water bath (85 strokes/min) for at least 3 hours. At appropriate time points, solution (0.2-0.3 mL) was removed from the receiver compartment, then fresh pre-warmed transport medium was added at the equal volume to the receiver. Samples were kept at -80^{o}C until analysis for concentration of the ester and parent mefenamic acid in AP and BL buffers. At the end of the transport studies, the transport medium was removed and the cells were washed with PBS, pH 7.4. The cells were then scraped from the polycarbonate filters, collected in 200 μL PBS, and stored at -80°C until HPLC analysis.

Permeability determined from both apical to basolateral ($P_{app,\ A-B}$) and basolateral to apical ($P_{app,B-A}$) transport directions were employed to calculate 'efflux ratio'. Defined as the ratio of $P_{app,B-A}$ to $P_{app,\ A-B}$, the efflux ratio was used to assess the potential interaction of the ester with cellular efflux pumps. Compounds that are transported across Caco-2 monolayer only by passive diffusion should exhibit similar permeability values in either direction, so efflux ratio should be about 1. In contrast

compounds having affinity for cellular efflux pump and could be actively transported out of the cells, would show greater $P_{app,B-A}$ than $P_{app, A-B}$, then efflux ratio is greater than 1.

2.7 Data analysis

Permeability coefficients

Compound flux (J) was measured under sink condition (i.e. when less than 10% of the initial concentration appearing in the acceptor compartment per given time interval).

$$J = \frac{1}{A}\frac{dQ}{dt} \quad (1)$$

Where A is the surface area of the porous membrane in cm^2, and Q is the amount of compound transported over time t.

Since transport processes across the monolayer can occur by one or more routes than passive transcellular. Permeability, therefore, was expressed as an apparent value, which is the summation of permeabilities occurring by passive transcellular, passive paracellular, and carrier mediated, as shown in Equation 2:

$$P_{app} = P_{\ passive\ transcellular} + P_{paracellular} + P_{carrier} \quad (2)$$

Apparent permeability coefficient (P_{app}) is normally expressed as cm/sec.

Since flux (J) = P (C_0-C), P_{app} can be estimated from the flux, as shown in Equation 3:

$$P_{app} = \frac{J}{C_0} = \frac{1}{AC_0} \cdot \frac{dQ}{dt} \quad (3)$$

Where C_0 is the initial concentration of the test compound added to the donor compartment. C is the concentration of solute at receiver. *dQ/dt* is the slope of the plot of the cumulative receiver amount with time.

Efflux ratio (ER)

Estimation of ER was performed using Equation 4:

$$ER = \frac{P_{app,B-A}}{P_{app,A-B}} \quad (4)$$

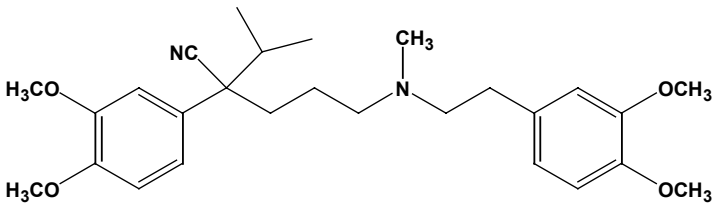
where $P_{app,B\text{-}A}$ and $P_{app,A\text{-}B}$ were apparent permeabilty coefficients from transport in the secretory and absorptive directions, respectively.

Statistical Analysis

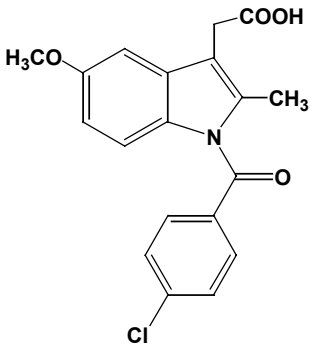
Statistical analysis for significant differences was performed using the two tailed Student's t-test and assuming equal variances. The criterion for significant differences in values was $p < 0.05$.

2.8 Efflux inhibition studies

Inhibition of polarized transport across Caco-2 monolayer was performed to determine whether Pgp and/or MRPs are involved in the transepithelial transport of the esters exhibiting ER > 2. Verapamil, a Pgp modulator, and indomethacin, an MRP inhibitor, structures shown in Figure 9, were used. Verapamil and indomethacin were prepared separately as 5 mg/ml stock solutions in methanol. Aliquots volume of the stock solution of each compounds was added to the AP incubation mixture to yield a final concentration of 100 μM of either verapamil or indomethacin. The inhibitors were included throughout the 3 hr-incubation period.



(A)



(B)

Figure 9 Chemical structures of verapamil (A) and indomethacin (B) used in efflux inhibition studies.

2.9 Calcein inhibition assay

Calcein AM efflux inhibition method was optimized from the published method (Feller et.al.,1995., Hollo et., 1994., Laska et al., 1994., Polli et.al., 2001) with slight modification in terms of cell culture conditions for plating (plating density and days in culture), calcein AM and test compounds concentrations, and time of assay. Caco-2 cells were seeded at 200,000 cells/well in 48 well plate (Costar, Cambridge, MA, U.S.A), and were incubated for 48 hours before the assay. Test compounds including

ester 3 and 4 of mefenamic acid, Pgp inhibitors verapamil and cyclosporin A, and a known MRP inhibitor, indomethacin, were prepared in methanol and diluted to final concentration 50 μM in pH 7.4 PBS (< 0.5 % v/v methanol). Caco-2 cell monolayers were washed twice with PBS before the experiment. Ten microliters of the test compounds (50 μM) were added to each well, and plate was sybsequently incubated at 37^{o}C for 30 min. Cells with the addition of PBS (containing 1.0% methanol) was served as a control. One microliter of calcein AM (Molecular Probe, OR, U.S.A.), prepared at 1.0 μM in DMSO, was subsequently added into each well to yield a final concentration of 2.0 μM. Fluorescences intensity was immediately measured at 30 min interval for 180 min, at 485-nm (excitation) and 535 nm (emission) using 7630 microplate fluorometer (Cambridge Technology, MA, U.S.A.). Calcein accumulation was expressed by fluoresecence intensity (F) with test compounds subtracted by F control (medium only).

2.10 Colorimetric MTT toxicity assay

Since PMSF was normally used in transport study to avoid degradation of the esters. To ensure that cell monolayer viability would not be affected by PMSF, the MTT assay was performed. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that is reduced by mitochondrial dehydrogenase in living cells to give a dark blue insoluble formazan (1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan), which can be analysed colorimetrically. In actively proliferating cells, an increase in MTT conversion is spectrophotometrically quantified. Conversely, in cells that are undergoing to cell death, MTT reduction decreases, reflecting the loss of cell viability.

In this study, confluent Caco-2 monolayer cultured on 96 well- plate were used. PMSF dissolved in DMSO was served as stock solution. With serial dilution in transport medium, PMSF at the concentration of 0.05-1.5 mM (corresponded to 0.05-1.20 % v/v DMSO in transport medium) were incubated with the monolayer for 1 h at 37^{o}C. Cells were then washed with PBS, and 0.1 mL of 5 mg/mL MTT stock solution (in transport medium) was added to each well and incubated for 1 hrs. MTT solution was aspirated and 0.2 mL of acidic isopropanol (isopropanol:1N HCl = 24:1)

was added into each well. Plate was then incubated for 4 hrs at 37^{o}C to solubilize the formazan product, which absorbance was subsequently measured at 550 nm. Untreated cell (cell with transport medium only) was used as a positive control and DMSO was used as a negative control. The absorbance was corrected with blank reading (MTT in acidic isopropanol). The levels of blue color development in untreated control wells were designated as 100% viability, cell survival was calculated as percentage of total cell survived, and compared to untreated control.