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Research Paper

Experimental and Computational Studies of Epithelial Transport of Mefenamic Acid Ester Prodrugs

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Purpose. A series of ester derivatives of mefenamic acid were synthesized with the aim of suppressing local gastrointestinal toxicity of mefenamic acid. A computational method was used to assist the design of the prodrug and to gain insights into the structure relationship of these compounds as P-glycoprotein (P-gp) substrates. The prodrugs were studied for their enzymatic stability, bidirectional permeability across Caco-2 monolayer, and their potential as transporter modulators

Methods. Bidirectional transport studies were performed using Caco-2 cells. Compounds exhibiting an efflux ratio of ≥ 2 were further examined for their potential interaction with P-gp and multidrug resistance–associated protein (MRP) using verapamil and indomethacin. Calcein efflux inhibition studies were conducted to investigate the efflux mechanism of these compounds. Geometry optimization of the esters was performed, and the spatial separation of two electron donor groups of each prodrug was measured.

Results. Morpholinoethyl ester (**3**) and pyrrolidinoethyl ester (**4**) of mefenamic acid showed evidence of efflux mechanism. Inhibition by verapamil had a pronounced effect on the transport of **3** and **4**. Indomethacin, however, completely inhibited the apical efflux of **3** but enhanced the efflux ratio of **4**. Both compounds increased the ratio of cellular calcein accumulation by 3- to 5-fold over control. Consistent with the experimental data, the computational results suggest the involvement of P-gp or its interaction in **3** and **4** transport.

Conclusions. Apical efflux of **3** is associated with P-gp and MRP, but the efflux of **4** involves P-gp and/or MRP. The computational approach used in this study provided the basis for P-gp substrates of compounds **3** and **4** from their electron donor subunits spatial separation.

KEY WORDS: Caco-2; MRP; P-gp; prodrugs; transport.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are generally used for the treatment of inflammatory diseases. Usage of NSAIDs, either short-term or chronic, is frequently associated with gastrointestinal (GI) adverse effects ranging from mild gastric upset to life-threatening ulceration and hemorrhage (1). These gastroenteropathies are generally believed to be caused by two different mechanisms. The first one involves a local irritant produced by acidic group of the NSAIDs. The second effect is attributed to blockage of prostaglandin biosynthesis in the GI tract, which inhibits its cytoprotective effect.

Prodrugs that temporarily mask the acidic group of NSAIDs have been reported to suppress GI toxicity due to local action (2). Mefenamic acid (**1**) is a widely used NSAID as a fever or pain reliever. In this study, various mefenamic acid ester prodrugs (**2**–**5**) were synthesized with the aim of decreasing the local irritation as shown in Fig. 1. Due to the existence of active transporters such as P-glycoprotein (P-gp) and multidrug resistance–associated proteins (MRPs) in the intestinal epithelium and the physicochemical consideration of the promoieties, a computational method was used to assist the design of prodrugs and to investigate whether the designed structures are P-gp substrates as described by Seelig (3) and Penzotti *et al.* (4). The physicochemical and transport properties of the prodrugs across Caco-2 monolayers were evaluated in both the absorption and the secretion direction. Two compounds (**3** and **4**) were shown to be substrates for an efflux mechanism. Subsequently, a calcein-AM assay was conducted to explore the potential of these two compounds as P-gp and/or MRP inhibitors.

MATERIALS AND METHODS

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 18. All

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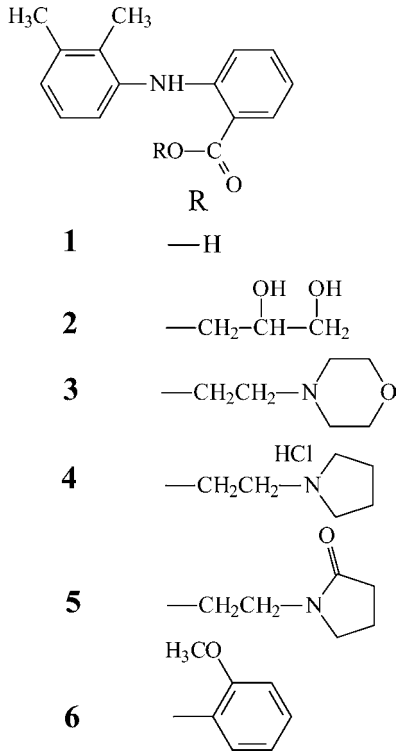

Fig. 1. Structure of mefenamic acid esters.

chemicals used for Caco-2 cell culture were obtained from Gibco BRL (Life Technologies, Grand Island, NY, USA). Phenylmethyl sulfonylfluoride (PMSF), *N,N′*-dimethylaminopyridine (DMAP), *N,N′*dicyclohexyl-carbodiimide (DCC), and *N*-hydroxysuccinimide were obtained from Sigma (St. Louis, MO, USA). Solketal, 4-(2-hydroxyethyl)morpholine, 1-(2-hydroxyethyl)-2-pyrrolidinone, and 1-(2-hydroxyethyl)-pyrrolidine were obtained from Fluka Chemie (Buchs, Switzerland). Other reagents and solvents were purchased from common suppliers and were used as received.

Partition coefficients were calculated using ClogP for Windows (Biobyte Corp., Claremont, CA, USA). The melting point was determined on a MEL-TEMP II capillary melting point apparatus and is uncorrected. Proton magnetic resonance spectra were recorded on a Varian Unity Inova (500 MHz) or a Jeol JNM-PMX 60SI (60 MHz). Chemical shifts are reported in parts per million (ppm, δ units) and peak multiplicities are expressed as follows: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; m, multiplet. The IR spectra were recorded on a Perkin Elmer model 1600 FT-IR spectrometer. Mass spectra (ESI) were measured on a Micromass Platform II mass spectrometer. Yields are of purified products and were not optimized. The purity of the esters was assessed by analytical HPLC.

Computational Method

The three-dimensional structures of compounds were built using Chem3D Ultra ver. 7 software (CambridgeSoft, Cambridge, MA, USA), and molecular calculation was directly performed using the Gaussian 03 W program (Gaussian, Inc., Pittsburgh, PA, USA) on the Chem3D window. Geometry optimization was performed initially by molecular mechanics force field and subsequently using the AM1 Hamiltonian of a semiempirical method. The obtained geometries were then optimized on the basis of the *ab initio* Hartree-Fock method at the 6-21G level. The spatial separation of two electron donor groups of each prodrug was subsequently measured.

Synthesis of Mefenamic Acid Prodrugs

2,3-Dihydroxypropyl 2-[(2,3-dimethylphenyl)amino]benzoate (**2**)

To a solution of mefenamic acid (5.00 g, 20.75 mmol) in dry CH_2Cl_2 (150 ml) was added a solution of solketal (5.42 g, 41.07 mmol) in dry CH_2Cl_2; the mixture was cooled at 0°C, followed by the addition of DMAP (0.10 g, 0.78 mmol) and DCC (5.20 g, 25.06 mmol). The reaction mixture was stirred at room temperature overnight. The precipitate of *N,N′*-dicyclohexylurea (DCU) was removed by filtration. The filtrate was concentrated *in vacuo*, and the residue was purified by column chromatography using ethylacetate:hexane (1:20) as the eluent and recrystallization from MeOH/H_2O to obtain 4.49 g (61%) of solketal ester of mefenamic acid as a white solid: mp 58.3–61.4°C; IR (KBr) 1689 cm^{-1}; ^1H NMR (60 MHz, $CDCl_3$,) δ 1.40 (s, 3H, CH_3), 1.47 (s, 3H, CH_3), 2.17 (s, 3H, $ArCH_3$), 2.29 (s, 3H, $ArCH_3$), 4.33–3.68 (m, 5H, CH_2CHCH_2), 6.56 (m, 2H, aromatic), 7.03–6.87 (m, 4H, aromatic), 7.90 (m, 1H, aromatic), 9.01 (s, 1H, NH, broad); ESI MS: *m/z* 378 $[M+Na]^+$, 356 $[M+H]^+$.

A mixture of solketal ester of mefenamic acid (0.99 g, 2.78 mmol) and 100 ml of 70% acetic acid was heated to 60°C for 1 h. The reaction mixture was cooled to ambient temperature and extracted with CH_2Cl_2 (3 × 20 ml); the CH_2Cl_2 extracts were washed in H_2O and brine. Precipitated solid was collected, washed with water, partially air dried, and dissolved in 50 ml CH_2Cl_2. The solution was dried with anhydrous sodium sulfate, and the solvent was removed by evaporation under reduced pressure. The residual was recrystallized from methanol to obtain 0.30 g (34%) of **2** as white crystals: >99% purity; mp 60.6–62.4°C; IR (KBr) 1680 cm^{-1}; ^1H NMR (500 MHz, DMSO-d6) δ 2.08 (s, 3H, CH_3), 2.27 (s, 3H, CH_3), 3.42–3.50 (m, 2H, CH_2OH) 3.79–3.84 (m, 1H, CH_2CH), 4.19 (dd, J = 0.02, 0.01 Hz, 1H, OCH_2), 4.33 (dd, J = 0.02, 0.01 Hz, 1H, OCH_2), 4.70 (t, J = 0.01 Hz, 1H, OH), 5.02 (d, J = 0.01 Hz, 1H, OH), 6.65–6.73 (m, 2H, aromatic), 7.03–7.13 (m, 3H, aromatic), 7.30–7.33 (m, 1H, aromatic), 7.94–7.96 (m, 1H, aromatic), 9.12 (s, 1H, NH, broad); ESI MS: *m/z* 316 $[M+H]^+$.

2-Morpholin-4-ylethyl 2-[(2,3-dimethylphenyl)amino]benzoate (**3**)

To a solution of **1** (5.00 g, 20.75 mmol) in CH_2Cl_2 (150 ml) was added 4-(2-hydroxyethyl)morpholine (2.86 g, 21.80 mmol) and DMAP (0.10 g, 0.83 mmol). The mixture was cooled to 0°C, followed by the addition of DCC (5.21 g, 25.11 mmol). The reaction was stirred for an additional 4 h at 0°C and stored overnight in the refrigerator. The precipitated DCU was removed by filtration. The filtrate was concentrated *in vacuo*, and the residue was purified by column chromatography using ethyl acetate:hexane (20:80) as the eluent. The product was recrystallized from hexane to afford 4.20 g (57%) of **3** as pale yellow crystals: >99% purity; mp 84.8°C; IR (KBr)

1678 cm-1; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.19 (s, 3H, CH_3), 2.34 (s, 3H, CH_3), 2.59 (m, 4H, $N(CH_2)_2$), 2.80 (t, J = 5.80 Hz, 2H, CH_2N), 3.72 (m, 4H, $O(CH_2)_2$), 4.47 (t, J = 5.80 Hz, 2H, OCH_2), 6.70–6.67 (m, 1H, aromatic), 6.76–6.75 (m, 1H, aromatic), 7.05–7.04 (m, 1H, aromatic) 7.18–7.10 (m, 2H, aromatic), 7.27–7.24 (m, 1H, aromatic), 7.98–7.96 (m, 1H, aromatic), 9.22 (s, 1H, NH, broad); ESI MS: *m/z* 355 $[M+H]^+$.

2-Pyrrolidin-1-ylethyl 2-[(2,3-dimethylphenyl)amino]benzoate HCl (**4**)

To a solution of mefenamic acid (3.00 g, 12.43 mmol) and *N*-hydroxysuccinimide (1.43 g, 12.43 mmol) in 1,2-dimethoxyethane (50 ml) was added DCC (2.56 g, 12.45 mmol). The mixture was stirred at 0°C for 1 h and stored overnight in the refrigerator. The precipitated DCU was removed by filtration. The filtrate was added to a solution of 1-(2-hydroxyethyl)-pyrrolidine (2.93 ml, 24.86 mmol) in 1,2-dimethoxyethane (15 ml). The mixture was stirred overnight at room temperature. The main product was purified by column chromatography using ethyl acetate:hexane (5:95) as the eluent, which was concentrated *in vacuo*. The crude product was diluted with absolute ethanol (50 ml) and treated with gaseous hydrogen chloride to give 0.47 g (10%) of **4** as a white solid: >99% purity; mp190–191°C; IR (KBr): 1681 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d6): δ 1.91 (m, 2H, CH_2CH_2N in pyrrolidine ring), 2.01 (m, 2H, CH_2CH_2N in pyrrolidone ring), 2.09 (s, 3H, CH_3), 2.28 (s, 3H, CH_3), 3.11 (m, 2H, CH_2N), 3.61 (m, 4H, CH_2NCH_2 in pyrrolidine ring) 4.62 (t, J = 5.03 Hz, 2H, OCH_2), 6.65–6.74 (m, 2H, aromatic), 7.05–7.15 (m, 3H, aromatic), 7.33–7.37 (m, 1H, aromatic), 8.06 (dd, J = 0.016, 0.003 Hz, 1H, aromatic), 9.14 (s, 1H, NH, broad); ESI MS: m/z 339 $[M+H]^+$.

2-(2-Oxopyrrolidin-1-yl)ethyl 2-[(2,3-dimethylphenyl)amino]benzoate (**5**)

This compound was prepared from **1** (5.00 g, 20.75 mmol) and 1-(2-hydroxyethyl)-2-pyrrolidinone (2.67 g, 20.67 mmol) as described for compound **3** and gave 4.67 g (64%) of **5** as white crystals: >99% purity; mp 66.7°C; IR (KBr): 1682 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.05 (m, 2H, $NC(O)CH_2CH_2$ in pyrrolidone ring), 2.18 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 2.40 (t, J = 8.00 Hz, 2H, $NC(O)CH_2$ in pyrrolidone ring), 3.56 (t, J = 7.50 Hz, 2H, CH_2N in pyrrolidone ring), 3.71 (t, J = 5.50 Hz, 2H, CH_2N), 4.45 (t, J = 5.50 Hz, 2H, OCH_2), 6.69–6.66 (m, 1H, aromatic), 6.76–6.73 (m, 1H, aromatic), 7.04–7.03 (m, 1H, aromatic), 7.16–7.09 (m, 2H, aromatic), 7.27–7.24 (m, 1H, aromatic), 7.94–7.92 (m, 1H, aromatic), 9.22 (s, 1H, NH, broad); ESI MS: *m/z* 353 $[M+H]^+$.

Aqueous Solubility

The aqueous solubility of mefenamic acid prodrugs was determined in water and acetate buffer pH 5.0 as well as phosphate buffer pH 7.4 at the buffer concentration of 0.05 M, ionic strength 0.1 M adjusted with NaCl. Excess amounts of each compound were added to 1–2 ml of buffer in screw-capped glass vials. Samples were then tumbled at 20 rpm at 25°C until a constant solubility value was obtained. The saturated solutions were filtered through 0.45-μm cellulose acetate membrane filters (Sartorius, Goettingen, Germany), quantitatively diluted with mobile phase and analyzed by HPLC as described in "Samples Analysis," below.

Chemical Stability

The stability of esters **2–5** was examined in 0.01 M HCl (pH 2.0), 0.05 M acetate buffer (pH 5.0), and 0.05 M phosphate buffer (pH 7.4). The ionic strength was maintained at 0.1 M by the addition of NaCl. Because the esters are mostly insoluble in aqueous solution, stock solutions of the esters were prepared by dissolving the compounds in methanol. The reaction samples were prepared by adding 20–100 μl of 1–2 mg/ml methanolic stock solution of the esters into prewarmed buffer solution, resulting in 5.0–25.0 μg/ml solutions with 1–2% v/v of methanol. The solutions were then placed into a thermostatically controlled water bath at 37°C. At appropriate times, 250–500 μl aliquots of samples were taken and stored at 4°C until assayed 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 *vs.* time. Triplicate samples were analyzed, and the mean value of the rate constant was reported. The corresponding half-life ($t_{1/2}$) was then obtained from the equation: $t_{1/2} = 0.693/k$.

Enzymatic Stability

Degradation kinetics of esters **2–5** were studied at 37°C in Caco-2 homogenate, human plasma, and rat liver homogenate preparations. Each ester was incubated at the concentration range of 10–100 μM (depending on the solubility of the ester) in prewarmed biological medium studied. Dimethyl sulfoxide (DMSO) at a final concentration of 1–2% v/v was added to assist in the dissolution. The mixtures were kept in a water bath at 37°C, and at appropriate intervals, aliquots (250–300 μl) of the reaction solution were withdrawn and analyzed by HPLC as described in "Samples Analysis." Reactions were followed for at least two half-lives. Apparent half-lives for the disappearance of ester and pseudo-first-order rate constants were calculated as previously described.

Caco-2 Homogenate Preparation

Caco-2 homogenate was prepared according to the method of Augustijns *et al.* (5) with slight modifications. Briefly, Caco-2 cells grown in 75-cm^2 culture flasks for 21–25 days were washed 1–2 times with ice-cold phosphate-buffered saline (PBS), pH 7.4. The cells were scraped off, collected in 4 ml ice-cold PBS, and homogenized using a 15-ml glass-Teflon polytron homogenizer (Thomas Scientific, Swedesboro, NJ, USA). Cell debris was removed by centrifugation at 12,000 rpm for 10 min at 40°C, the resultant supernatant was used as Caco-2 homogenate in stability studies (protein content = 0.63 mg/ml).

Rat Liver Homogenate Preparation

Rat livers were obtained from male 250–300 g Sprague-Dawley rats (Animal Care Units, Prince of Songkla University, Hat-Yai, Songkhla, Thailand). After blotting to dryness, tissue was sliced into small pieces and homogenized in ice-cold PBS (1 ml per g tissue). Aliquots (1.0 ml) of the tissue

homogenates were kept at –80°C until use. Before each experiment, the homogenates were quickly re-homogenized on ice using a 15-ml glass-Teflon polytron homogenizer. Cell debris was removed by centrifugation at 12,000 rpm for 10 min at 40°C, and the supernatant was used in stability studies. Liver homogenates were diluted to 50% (v/v) with phosphate buffer, pH 7.4, in stability studies yielding protein content of 47.3 mg/ml.

Human Plasma

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

Transport Studies

Cell Culture

Caco-2 cells were grown in a controlled atmosphere at 5% CO_2 and 90% relative humidity at 37°C. Culture media consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acid, 1% L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B. At 80% confluency, cells were detached from the plastic support by digestion using trypsin/EDTA solution and transferred to a new 75-cm^2 tissue culture flask. For transport studies, cells were seeded at a density of 60,000 cells/cm^2 on Transwell (Costar, Cambridge, MA, USA) polycarbonate membrane (24.5-mm diameter, 4.71 cm^2, 3.0-μm pore size). The culture medium was changed every other day. Cells from a passage number between 42 and 53 were used in transport experiments.

Caco-2 cells grown on Transwell for 21–23 days were used in the transport studies. The transport medium (TM) consisted of Hanks balanced salt solution (HBSS) supplemented with 25 mM glucose, adjusting to pH 6.5 for the apical (AP) compartment (1.5 ml) and 7.4 for the basolateral (BA) compartment (2.6 ml). To ensure monolayer integrity, the transepithelial electrical resistance (TER) was measured using the Millicell-ERS system (Millipore Co., Bedford, MA, USA). Monolayers with TER $\geq$ 250 $\Omega \cdot cm^2$ were used in the experiments. Cell monolayers were equilibrated in TM containing phenylmethyl sulfoxide (PMSF, 0.5 mM), an inhibitor of serine protease/mammalian esterase to prevent any possible ester degradation at the apical side, for 20 min at 37°C before the experiments.

Transport studies were performed in triplicate starting with the ester dissolved in HBSS (with 0.5–2% DMSO) yielding the final concentrations of 15, 12, 42, and 41 μM for esters **2**, **3**, **4**, and **5**, respectively. The concentration of each ester was chosen according to their solubility. Transepithelial transport of each ester was determined in both directions (AP-BL and BL-AP). Bidirectional transport was performed at 37°C in a shaking water bath (85 strokes min^{-1}). Aliquots of the receiver solution (0.2 ml) were taken from the receiver compartment at various time points and were replaced with equal volumes of fresh medium. Samples were kept at –80°C until analysis. Permeation, assessed when flux was linear related to time under sink condition, was expressed as apparent permeability coefficient, P_{app} (cm/s), and calculated according to the equation

$$P_{app} = \frac{1}{AC_0}\frac{dQ}{dt}$$

where dQ/dt is the rate of appearance of the drug in the acceptor side (μmol/s), C_0 is the initial drug concentration on the donor side (mM), and A is the surface area of the monolayer (cm^2).

Efflux Inhibition Studies

Inhibition of polarized drug transport across Caco-2 monolayer was performed to determine whether P-gp and/or MRPs are involved in the transepithelial transport of **3** and **4**. Verapamil, a P-gp modulator, and indomethacin, an MRP inhibitor, were used. Both compounds were applied at 100 μM to the AP side and included throughout the 3-h incubation period.

Statistical Analysis

Data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Scheffe's test. Values of $p < 0.05$ were considered significant.

Samples Analysis

The HPLC system consisted of a 600 Pump, a 717 plus Autosampler, a 486 Tunable Absorbance Detector, and a 746 Data Module (Waters, Milford, MA, USA). The analytical column was Rexchrom octyl, 15 cm × 4.6 mm, 5 μm (Regis Technologies, Morton Grove, IL, USA) equipped with a precolumn packed with Novapak RP-8, 3.9 × 20 mm, 5 μm (Waters). The mobile phase consisted of acetate buffer (0.05 M) and acetonitrile and/or methanol. The systems for analysis of **1**–**5** were acetate buffer (pH 5.0):methanol (40:60) for **2**; acetate buffer (pH 4.5):acetonitrile (57:43) for **1** and **3**; acetate buffer (pH 4.1):acetonitrile (50:50) for **4**; and acetate buffer (pH 4.1):acetonitrile (55:45) for **5**. The eluents were detected at 280 nm at a flow rate of 1.0 ml/min. Separation of the esters **2**–**5** from parent mefenamic acid was obtained with a total analysis time of less than 20 min.

For determination in biological media, samples were deproteinized by adding 3 volumes of acetonitrile. After mixing, and in the case of determining the accumulated amount of ester in Caco-2 cell monolayers, sonicating for 10 min, the samples were centrifuged for 10 min at 12,000 rpm at 4°C. Aliquots of clear supernatant were then injected into the HPLC system and analyzed as described above.

Protein content was determined using a Coomassie protein assay reagent (Pierce, Rockford, IL, USA) according to the method of Bradford. Bovine serum albumin (BSA) was used as a standard for protein content measurements.

Calcein-AM Assay

Caco-2 cells were seeded at 200,000 cells/well in 48-well plate (Costar) and were incubated for 48 h before the assay. Test compounds including prodrugs **3** and **4**, P-gp inhibitors verapamil and cyclosporin A, and an MRP inhibitor indomethacin were prepared at 50 μM in PBS pH 7.4. Caco-2 cell monolayers were washed twice with PBS before the experi-

ment. Ten microliters of the test compounds were added to each well, and the plate was subsequently incubated at 37°C for 30 min. Cells with the addition of PBS (containing 1% methanol) served as a control. One microliter of calcein AM (Molecular Probes, Eugene, OR, USA), prepared at 1.0 mM in DMSO, was subsequently added into each well to yield a final concentration of 2.0 μM. Fluorescence was immediately measured at 30-min intervals for 180 min, at wavelengths 485-nm excitation and 535-nm emission. The ratio of calcein accumulation was calculated based on the ratio of fluorescent intensity with P-gp or MRP inhibitors or prodrugs to the fluorescent intensity of control (medium only) at any time point as described by Olson *et al.* (6).

RESULTS AND DISCUSSION

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. However, to obtain the prodrug **2**, the solketal ester was subsequently hydrolyzed, and in the case of **4**, HCl was added to the ester to form HCl salt.

Solubility

Prodrug **4**, which was prepared as a hydrochloride salt, is highly soluble in water. Its solubility is about 15 times higher than mefenamic acid (**1**). The other prodrugs are less soluble than mefenamic acid. The solubilities of **2** and **5** in water are 6.0 and 2.6 μg/ml, respectively. The solubility of **3** and **4,** which are basic compounds, were shown to be pH dependent; the solubility was higher at the lower pH in which the compounds are protonated. The solubility of **3** was increased from 2.6 μg/ml at pH 7.4 to 6.2 μg/ml at pH 5, and that of **4** was enhanced from 109.3 μg/ml at pH 7.4 to 1045.6 μg/ml at pH 5.

Chemical and Enzymatic Stability

The hydrolysis of prodrugs was studied in aqueous buffers pH 2, 5, and 7.4 at 37°C. After the prodrugs were incubated in these buffer solutions up to 24 h, only **5** underwent chemical hydrolysis and released mefenamic acid with an apparent half-life of 5.2, 8.1, and 8.7 h at pH 2, 5, and 7.4, respectively. The others are stable to chemical hydrolysis in the aqueous buffer solutions; the release of mefenamic acid cannot be detected during 24-h incubation.

The stabilities of these prodrugs in various biological media including 80% human plasma, Caco-2 homogenate, and rat liver homogenate, were determined and their apparent half-lives are listed in Table I. All prodrugs were degraded in these biological media, especially in human plasma. The apparent half-life of prodrugs in human plasma ranged from 15.5 to 59.9 min. Prodrugs **2** and **5** underwent rapid hydrolysis in all biological media; however, **2** had a longer half-life.

These prodrugs have been shown to be resistant toward chemical hydrolysis in aqueous buffer solutions; however, they can undergo enzymatic cleavages in biological media and release the parent compound. This indicates that compounds **2**–**5** can be used as prodrugs.

Transport Studies

Because some prodrugs are degradable in Caco-2 homogenates, PMSF was used to inhibit the degradation process in all transport studies. Table II shows the apparent permeability coefficients across Caco-2 monolayers and the efflux ratio ($P_{app\ AP\text{-}to\text{-}BL}/P_{app\ BL\text{-}to\text{-}AP}$) for mefenamic acid (**1**) and its ester prodrugs (**2**–**5**). According to the efflux ratios, **1**, **2,** and **5** (Table II) did not prove to be substrates for efflux transporters, as their efflux ratios are close to 1. However, the apically polarized efflux mechanisms are demonstrated for compounds **3** and **4** with efflux ratios of 3.1 and 10.4, respectively. The efflux transport is probably a key factor limiting the permeation of these compounds, as indicated by their low $P_{app\ AP\text{-}to\text{-}BL}$ values.

Table I. Biological Stability of Esters of Mefenamic Acid[a]

Compounds	Half-lives in biological media (min) (mean ± SD)		
	Plasma	Caco-2 homogenate	Rat liver homogenate
2	46.1 ± 1.7	58.3 ± 1.0	28.7 ± 2.8
3	59.9 ± 7.2	736.0 ± 36.0	50.3 ± 6.1
4	37.2 ± 3.1	1274.0 ± 119.0	1362.0 ± 103.0
5	15.5 ± 1.2	5.3 ± 1.5	19.0 ± 1.3

[a] Each value represents the mean ± SD of three determinations.

The log p values for the prodrugs were estimated using the ClogP program, which calculates the value directly from its molecular structure. The calculated logP values for **1**, **2**, **3**, **4,** and **5** are 4.8, 3.5, 5.3, 6.0, and 4.8, respectively. It has been suggested that logP value may be used as a predictive tool for transepithelial transport of drugs (7). As shown in Table II, the P_{app} values in the AP-to-BL direction for **1**, **2,** and **5** are comparable but somewhat increase in the following order **1** ≃ **5** > **2**. The P_{app} in AP-to-BL direction for **1**, **2**, and **5** are positively correlated with their logP values. For prodrugs **3** and **4**, which are substrates for efflux transporters, logP value cannot be used for predicting their permeability. Therefore, it is not straightforward to use the relationship between lipophilicity and permeability when the active or efflux mechanism is involved in the transport system.

According to ANOVA analysis, the $P_{app\ AP\text{-}to\text{-}BL}$ for **1**

Table II. Permeability of Esters of Mefenamic Acid Across Caco-2 Cell Monolayers[a]

Compound	P_{app} ($\times 10^{-6}$ cm/s)		Efflux ratio	ClogP
	AP to BL	BL to AP		
1	$18.6 \pm 2.0^{*,NS}$	22.2 ± 2.1	1.2	4.8
2	12.7 ± 0.3	16.5 ± 0.3	1.3	3.5
3	2.0 ± 0.7	6.1 ± 1.9^{NS}	3.1	5.3
3 + verapamil	$6.5 \pm 1.8^{**,NS}$	7.9 ± 0.5^{NS}	1.3	
3 + indomethacin	$8.1 \pm 0.9^{**,NS}$	5.4 ± 0.8^{NS}	0.7	
4	2.3 ± 0.3^{NS}	24.1 ± 3.5^{NS}	10.4	6.0
4 + verapamil	3.4 ± 0.6**	28.7 ± 0.8^{NS}	8.5	
4 + indomethacin	2.8 ± 0.6^{NS}	41.7 ± 7.2**	14.7	
5	17.2 ± 5.0^{NS}	13.0 ± 2.6	0.8	4.8
6[b]	4.7 ± 0.1	5.3 ± 0.7	1.1	6.5

[a] Each value represents the mean ± SD of three independent determinations. One-way ANOVA and Scheffe's multiple comparison test. *$p < 0.05$ for comparison of **1, 2,** and **5.** **$p < 0.05$ within the same group. NS, not significant ($p > 0.05$ within the same group and for **1, 2,** and **5**).

[b] From Ref. 23.

and **5** as well as for **2** and **5** are not statistically different, but that for **1** is different from **2** ($p < 0.05$). This suggests that the prodrugs with the potential to decrease local toxicity of the drug exhibit comparable permeabilities to the parent compound.

Occasionally, substrates for efflux systems are identified by the efflux ratio and by inhibition of the efflux-mediated transporters. Because two efflux systems, P-gp and MRP, are expressed in Caco-2 cells (8,9), both verapamil and indomethacin were therefore used as inhibitors. The P-gp inhibitor verapamil caused an increase in the permeability in the absorptive (AP-to-BL) direction and an insignificant change in the secretory direction for both **3** and **4**. Accordingly, the efflux ratio was decreased for both compounds (from 3.1 to 1.3 for **3** and from 10.4 to 8.5 for **4**). The concentration of verapamil used (100 μM) may be sufficient to inhibit the efflux of **3**, but it partially inhibited the efflux of **4**. The discrepancy in efflux inhibition of the two compounds was probably due to the affinity of each compound to the efflux pumps and the concentration-dependent effect of the inhibitor on the permeability of each compound, as previously observed by Petri *et al.* (10). Because of the limited solubility of the ester and its mixture with verapamil as well as the detection limit in decreasing ester concentrations, the concentration of verapamil was not increased in this study. Consequently, the concentration-dependent permeability of each prodrug was not investigated. Nevertheless, these results could indicate that P-gp may be involved in the active transport of **3** and **4**.

The MRP inhibitor indomethacin increased the permeability of **3** in the absorptive direction but had an insignificant effect in the secretory direction. At the concentration of indomethacin used (100 μM), the efflux of **3** was completely inhibited, and the efflux ratio was reduced from 3.1 to 0.7. This data suggest 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** was increased in the presence of indomethacin. This MRP inhibitor did not statistically change the permeability in the absorptive direction but highly enhanced the permeability in the secretory direction. Various MRPs including an apically localized MRP2 and basolateral MRPs (MRP1, MRP3, MRP4, and MRP5) (9,11) probably influenced the efflux of **3** and **4**. Prodrug **3** may not be a good substrate for basolateral MRPs. The inhibition of an apical MRP2 thus reduces the efflux ratio of **3**. In contrast to **3**, **4** may be a better substrate for basolateral MRPs than apical MRP2. By inhibiting these MRPs, the permeability of **4** in the secretory direction is increased, resulting in a significant enhancement of the efflux of **4** over control (i.e., in the absence of inhibitor).

Calcein-AM Assay

Calcein-AM is a nonfluorescent substrate for both MRP and P-gp (11–13). It is cleaved in the cell to form a fluorescent product, calcein. Calcein is not a P-gp substrate, but being a negatively charged molecule, it can be a substrate for MRP (14–16). However, calcein is much less efficiently transported by MRP than calcein-AM (15). Essentially, the accumulation of calcein may be lower due to calcein-AM and calcein efflux. Nevertheless, the accumulation of the fluorescent calcein should be higher in the presence of P-gp and MRP modulators than in their absence. All tested compounds, both P-gp and MRP modulators as well as prodrugs **3** and **4**, increase the calcein fluorescence. The accumulation of calcein in the presence of all test compounds was about 3- to 5-fold higher than the control, as indicated by the calcein accumulation ratio (Table III). These results provide evidence for the inhibitory effect of prodrugs **3** and **4** on P-gp and/or MRP. In view of the fact that **3** can inhibit P-gp and/or MRP, the addition of verapamil or indomethacin together with **3** could result in a complete inhibition of the efflux of **3**, which was observed in this study (Table II).

Traditionally, P-gp inhibitors can either be transported or not transported by the efflux transporter (17). Those that can act as both inhibitor and substrate include verapamil and cyclosporin A, while progesterone represents a nontransported inhibitor (18–20). This information along with the observed bidirectional transport of the compounds suggests that **3** and **4** may belong to the type of P-gp inhibitors that can be transported. It has been reported that P-gp inhibitors do not inhibit MRP-dependent drug efflux, and MRP-mediated drug efflux cannot be inhibited by P-gp modulators (21). Therefore, the nonspecific reversal agents that can inhibit both protein functions may have an advantage over the specific inhibitors, and some of these agents have recently been investigated (21). These prodrugs may be useful as leads for designing novel nonspecific chemosensitizers. Further studies are required to investigate whether the prodrugs **3** and **4** can function as inhibitors of both P-gp and MRP.

Computational Method

It has been identified that the recognition elements for P-gp are formed by two (type I unit) or three electron donor groups (type II unit) with distinct spatial arrangements (3). For the type I pattern, two electron donors are separated by 2.5 ± 0.3 Å. The type II patterns contain either two electron donor groups separated by 4.6 ± 0.6 Å or three electron donors separated by 2.5 ± 0.3 Å with a 4.6 ± 0.6 Å separation of the outer two groups. A molecule with at least one type I or type II unit is predicted to be transported by P-gp.

As demonstrated in Fig. 2, the spatial separation of two electron donor groups, oxygen from C=O group of ester and nitrogen from morpholine ring (**3**) and nitrogen in pyrrolidine ring (**4**), is 4.9 Å, consistent with the type II pattern definition. Furthermore, this compound also contains hydrophobic groups (two aromatic rings) that have been recognized as an important descriptor for P-gp substrate (4,22). In agreement with the bidirectional transport studies, **3** and **4** are P-gp sub-

Table III. Ratio Accumulation of Calcein in Caco-2 Cell Monolayers in the Presence of Known P-gp and MRP Modulators and Prodrugs **3** and **4** at 50 μM of Each Compound

Compounds	Time (min)			
	30	60	90	120
Verapamil	4.9 ± 1.8	4.2 ± 1.0	3.6 ± 0.8	3.1 ± 0.6
Indomethacin	4.5 ± 1.6	4.0 ± 1.0	3.3 ± 0.8	2.9 ± 0.7
Cyclosporin A	4.2 ± 0.9	3.6 ± 0.6	3.3 ± 0.5	2.8 ± 0.6
3	5.8 ± 0.9	4.1 ± 0.4	3.6 ± 0.4	3.0 ± 0.2
4	4.6 ± 1.5	3.4 ± 1.4	3.1 ± 0.8	2.6 ± 0.6

Each value represents the mean ± SD of 3 determinations.

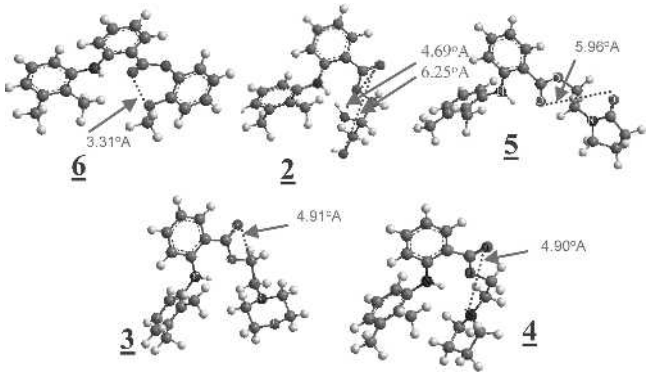

Fig. 2. Structure of mefenamic acid ester prodrugs (**2**–**6**) and calculated distance between two-electron donor groups for each prodrug.

strates; they exhibit apical efflux that is inhibited by P-gp inhibitor.

For prodrug **2**, even though the spatial separation of electron donor groups is 4.69 Å, this compound does not seem to be a substrate for efflux mechanism or P-gp according to the transport experiment. This compound contains an electron donor group (OH), which also has potential hydrogen bonding donor capacity. According to Seelig (3), these groups are not specifically favorable for an interaction with P-gp. Furthermore, the predictability of the compounds as non–P-gp substrates using the computational method was also demonstrated for prodrug **5** and mefenamic acid guaiacol ester **6** (23), whose spatial separation of electron donor was estimated to be 5.96 and 3.3 Å, respectively (Fig. 2). These compounds were experimentally shown to be non–P-gp substrates with an efflux ratio of 0.8 and 1.1, respectively (Table II). These results also confirm the concurrence of transport studies with this computational method.

In conclusion, ester prodrugs of mefenamic acid were synthesized for reduced gastric toxicity of this NSAID. A computational method was successfully used to design prodrugs that avoid the efflux process by P-gp. In addition, bidirectional transport studies indicated that two prodrugs were substrates of active efflux transporters. These prodrugs were able to increase cellular calcein accumulation, suggesting that they may function as P-gp and/or MRP inhibitors. These compounds may be useful as leads for designing new inhibitors of efflux transporters.

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