Biochemical Pharmacology 79 (2010) 455–462

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

# Role of basolateral efflux transporter MRP4 in the intestinal absorption of the antiviral drug adefovir dipivoxil

Xin Ming, Dhiren R. Thakker *

*Division of Molecular Pharmaceutics, UNC Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, USA*

ARTICLE INFO

*Article history:*
Received 10 July 2009
Accepted 28 August 2009

*Keywords:*
Intestinal absorption
Basolateral efflux
Caco-2 cells
MRP4
Adefovir dipivoxil

ABSTRACT

Adefovir dipivoxil is a diester prodrug of the antiviral drug adefovir, with much greater oral bioavailability than adefovir. Evidence shows that the prodrug is metabolized to adefovir in the enterocytes during intestinal absorption. However, it is unknown how the highly charged and hydrophilic adefovir crosses the basolateral membrane in the intestine. This study determines the role of specific basolateral transporter(s) in the egress of adefovir across the basolateral membrane when formed from adefovir dipivoxil in Caco-2 cells, a model for intestinal epithelium. Multidrug resistance-associated protein 4 (MRP4) plays an important role in renal secretion of adefovir. Immunofluorescence images showed that MRP4 is localized in the basolateral membrane of Caco-2 cells. This localization was further confirmed by Western blotting of the apical and basolateral membrane fractions that were isolated by a novel method involving biotinylation of respective membrane proteins and affinity enrichment. MRP4-knockdown Caco-2 cells were produced by stable transfection with MRP4-specific siRNA expression plasmid. These cells showed reduced MRP4 protein expression and corresponding reduction in the basolateral egress of adefovir when adefovir dipivoxil was dosed on the apical side. A comparison of these data with the reduction in the basolateral egress of adefovir by the general MRP inhibitor indomethacin established that MRP4, among MRPs, plays a predominant role in the basolateral egress of adefovir in Caco-2 cells. The results highlight the importance of MRP4 in oral absorption of adefovir dipivoxil, and suggest that significant drug–drug interactions can occur if an MRP4 inhibitor is co-administered with adefovir dipivoxil.



## 1. Introduction

Adefovir, an acyclic nucleoside phosphonate, is a reverse transcriptase inhibitor with antiviral activity against a wide range of viruses such as retroviruses, immuno-deficiency virus types 1 and 2 (HIV1 and HIV2), herpes viruses, and hepadnaviruses [1]. However, the permeability of adefovir across biological membranes is very low due to the presence of a phosphonate group with two negative charges (successive $pK_a$'s = 2.0 and 6.8). This also results in low oral bioavailability of adefovir in humans (less than 12%) [2]. Therefore, esters of adefovir have been synthesized in order to mask the negative charges and improve its membrane permeability. For example, bioavailability of adefovir in humans after oral administration of adefovir dipivoxil, a diester prodrug, is approximately 32–45% [3]. The active phosphonate form of adefovir was the only chemical entity that was detected in plasma after oral administration of adefovir dipivoxil in humans [4], indicating that adefovir dipivoxil is converted to the active drug by presystemic metabolism. Studies in the rat showed that upon oral administration, adefovir dipivoxil was not detected in the mesenteric vein [5], thus providing evidence that adefovir dipivoxil is metabolized completely in the intestine. Mechanistic studies with Caco-2 cell monolayers, a model for intestinal epithelium [6], confirmed this observation by establishing that adefovir was the major chemical entity in the basolateral compartment when adefovir dipivoxil was dosed in the apical compartment [7]. Accordingly, intestinal absorptive transport of adefovir dipivoxil involves diffusion of the prodrug across the apical membrane, followed by cellular metabolism into adefovir,

*Abbreviations:* ABC, ATP binding cassette; DMEM, Dulbecco's Modified Eagle Medium; EMEM, Eagle's minimum essential medium; FBS, Fetal bovine serum; HBSS, Hank's balanced salt solution; HEPES, N-hydroxyethylpiperazine-N′-2-ethanesulfonate; h, human; MDCKII, Madin–Darby Canine Kidney II; MRP, multidrug resistance-associated protein; NEAA, nonessential amino acids; P-gp, P-glycoprotein.

* Corresponding author at: Division of Molecular Pharmaceutics, UNC Eshelman School of Pharmacy, 100F Beard Hall, CB 7355, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7360, USA. Tel.: +1 919 9620092; fax: +1 919 9663525.
*E-mail address:* dhiren_thakker@unc.edu (D.R. Thakker).

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.08.029

and subsequent basolateral efflux of this active drug into the blood. It remains unclear as to how the negatively charged and hydrophilic adefovir crosses the basolateral membrane of the enterocytes. A previous study showed that the basolateral efflux of adefovir in Caco-2 cells may involve a carrier-mediated transport process, and that the efflux was not mediated by P-gp [8]. Whether multidrug resistance-associated proteins (MRPs, ABCC subfamily) are involved in the intestinal basolateral efflux of adefovir was not established.

MRP family transports anionic compounds. The "short" members of this family, such as MRP4 and MRP5, are distinguished by their ability to transport cyclic nucleotides and nucleoside-based agents [9]. MRP4 was initially identified as a homolog of MRP1 (ABCC1) by screening databases of human sequence tags, and was the first MRP isoform identified that does not have a third (N-terminal) membrane spanning domain [10]. MRP4 is up-regulated in adefovir-resistant cells, which suggests that the enhanced efflux of adefovir by MRP4 leads to this resistance [11]. The substrate specificity of MRP4 is quite broad; it transports adefovir, cAMP, cGMP, *p*-aminohippurate, urate, dehydroepiandrosterone sulfate, methotrexate, and estradiol-17β-D-glucuronide [11–14]. In human kidney, MRP4 is abundantly expressed [15] and localized in the apical membrane of the proximal tubules [12]. Adefovir is actively secreted in the urine, and most of the administered dose is recovered in the urine as intact drug [16]. It has been reported that the active renal secretion of adefovir involves uptake into tubular cells by the basolateral organic anion transporter 1 (*SLC22A6*) [17], and the apical efflux into urine by MRP4 [18]. Recent studies showed that MRR4 mRNA was detected in human jejunum and Caco-2 cells [15,19] and the Mrp4 protein was detected in mouse intestine [20]. In the present study, expression and cellular localization of the MRP4 protein in Caco-2 cells was investigated, and its role in the basolateral efflux of adefovir, formed inside Caco-2 cells during absorptive transport of adefovir dipivoxil, was examined by employing MRP4-knockdown Caco-2 cells.

## 2. Materials and methods

### 2.1. Materials

Caco-2 and MDCKII cells were obtained from the American Type Culture Collection (Manassas, VA). Eagle's minimum essential medium (EMEM) with Earle's salts and L-glutamate, Dulbecco's Modified Eagle Medium (DMEM), nonessential amino acids (NEAA, 100×), N-hydroxyethylpiperazine-N′-2-ethanesulfonate (HEPES, 1 M), and penicillin–streptomycin–amphotericin B solution (100×) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) and trypsin–EDTA solution (1×) were obtained from Sigma Chemical Co. (St. Louis, MO). Hank's balanced salt solution (HBSS) was obtained from Mediatech, Inc. (Herndon, VA). Geneticin was obtained from Invitrogen Co. (Carlsbad, CA). [$^3$H]adefovir dipivoxil (11 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Adefovir dipivoxil, adefovir, and indomethacin were purchased from Sigma (St. Louis, MO).

### 2.2. Cell culture

#### 2.2.1. MRP3- and MRP4-expressing MDCKII cells

Human MRP3-transfected MDCKII cells were provided by Professor Piet Borst (The Netherlands Cancer Institute, The Netherlands) and the presence of MRP3 was confirmed by RT-PCR. Human MRP4 cDNA in pcDNA3.1/hygro vector was provided by Professor Dietrich Keppler (German Cancer Research Center, Germany).

MDCKII cells were transfected with pcDNA3.1/hygro empty vector or the vector containing the full-length MRP4 cDNA using the Nucleofector® System (Amaxa, Gaithersburg, MD) according to the manufacturer's protocol. Transfectants were selected with 0.2 mg/ml hygromycin B for 12 days. A clone with the highest expression level of MRP4, screened by Western blot for MRP4 expression, was chosen as a stably transfected cell line for further studies. The stably transfected MDCKII cells were cultured in DMEM with 10% FBS, 10% NEAA, 100 unit/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B plus 0.2 mg/ml hygromycin B.

#### 2.2.2. Caco-2 cell monolayers

Caco-2 cells were cultured in EMEM, supplemented with 10% FBS, 1% NEAA, 1% penicillin–streptomycin–amphotericin B solution at 37 °C in a humidified atmosphere with 5% $CO_2$. The cells were passaged every 4 days using trypsin–EDTA (1×), and plated at densities of 1:5 in 75 cm$^2$ T-flasks. Caco-2 cells were seeded at a density of 60,000 cells/cm$^2$ on Transwell™ filters (Corning Inc., Lowell, MA). Medium was changed the day after seeding, and every other day thereafter. The cells were cultured for 21–25 days before use. Transepithelial electrical resistance was measured to ensure cell monolayer integrity. Measurements were obtained using an EVOM Epithelial Tissue Voltammeter and an Endohm-12 electrode (World Precision Instruments, Sarasota, FL). Cell monolayers with the resistance values greater than 300 Ω cm$^2$ were used in transport experiments.

### 2.3. Transport studies

Caco-2 cell monolayers were incubated for 30 min with transport buffer in the absence or presence of 30 μM indomethacin in both apical and basolateral sides. Transport studies were initiated by replacing the donor chamber solution with 10 μM [$^3$H]adefovir dipivoxil with or without 30 μM indomethacin. The receiver chamber was sampled at selected times and the samples were analyzed by HPLC (Agilent Technologies, 1050 Series, Palo Alto, CA) using a 100× 3 mm C18 Aquasil column (5 μm; Keystone Scientific, Inc., Bellefonte, PA) according to a previously published method [7]. Fractions were collected, and the amount of radioactivity was determined by scintillation spectrometry. The identity of adefovir and adefovir dipivoxil was confirmed by comparison of their retention times with those of their respective authentic standards.

### 2.4. Accumulation study

MDCKII or Caco-2 cells were grown as monolayers in 24-well plates. Medium was changed every other day. The cells were used 5–7 days post-seeding. Cells were incubated with 10 μM [$^3$H]adefovir dipivoxil in the absence or presence of 30 μM indomethacin for 2 h. Then the dose solution was aspirated and cells were washed three times with 4 °C transport buffer. Cells were dissolved in 500 μl 0.1 N NaOH/0.1% SDS for 4 h with shaking. Radioactivity was determined by scintillation spectrometry. Protein content was determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard.

### 2.5. Immunoblot analysis

Caco-2 cells, cultured for 4 days in flasks or for 25 days on Transwell™ inserts, were lysed in a solution containing 1% SDS, 1 mM EDTA, and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The protein concentration of the clear cell lysate was determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard. Proteins (50 μg per lane) was resolved by electrophoresis on NuPAGE 4–12% Bis–Tris gel (Invitrogen, Carlsbad, CA) and were transferred onto polyvinylidene difluoride membranes (Invitro-

gen, Carlsbad, CA). The membrane was blocked and incubated with the M4I-10 antibody (0.3 μg/ml) (Axxora, San Diego, CA) and then with an anti-rat IgG, horseradish peroxidase-linked antibody (Pierce, Rockford, IL). Signal was developed using the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL). Human embryonic kidney (HEK) cell lysate was used as a positive control for MRP4 expression and GAPDH protein served as a loading marker.

### 2.6. Immunofluorescent staining

Caco-2 cells were grown for 25 days on 6.5-mm diameter Transwell™ inserts (Corning Inc., Lowell, MA) for the purpose of immunostaining. Inserts were washed three times in phosphate-buffered saline (PBS); similar washes were included between each of the following stages. To obtain X–Z images in immunostaining Caco-2 cells, inserts were frozen in Tissue-Tek Cryo-OCT Compound (Fisher Scientific, Pittsburgh, PA) and sectioned in longitudinal direction. Samples were fixed in 1% paraformaldehyde for 15 min and then permeabilized with 0.1% Triton X-100 for 10 min. Nonspecific binding sites were blocked by incubation for 30 min with 5% normal sheep serum. Sections were incubated with primary antibody M4I-10 (Axxora, San Diego, CA), and diluted to 5 μg/ml with PBS for 60 min. Primary antibody was detected by incubation with Alexa Flour 568 goat anti-rat antibody for 60 min. Cell nuclei were stained with DAPI (Invitrogen, Carlsbad, CA) for 5 min. Inserts were washed and mounted in Permount Mounting Medium (Andwin Scientific, Addison, IL). Staining was viewed using a Zeiss Axiovert 200M microscope (Carl Zeiss Inc., Thornwood, NY).

### 2.7. Immunoblot analysis of enriched proteins from apical or basolateral membrane of Caco-2 cells

Caco-2 cells were grown for 25 days on 6-well Transwell™ filters (Corning Inc., Lowell, MA) with the method described above. Biotinylation experiments were conducted using the Cell Surface Protein Isolation Kit (Pierce, Rockford, IL) according to the manufacturer's protocol with modifications (Fig. 4A). After washing with ice-cold PBS, sulfo-N-hydroxysuccinimide-SS-biotin (1.5 mg/ml in PBS) was added to the apical chambers of three Transwell™ filters or the basolateral chambers of the other three Transwell™ filters, and incubated for 30 min at 4 °C. Cells were washed three times with PBS, containing 100 mM glycine, and incubated for 20 min at 4 °C with the same buffer. Cells were washed with PBS, scraped into 1 ml of PBS, and pelleted by centrifugation at 800 × *g* for 2 min. The pellet was treated with 700 μl of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100, pH 7.4, and complete protease inhibitor cocktail) for 1 h at 4 °C with shaking. Cell lysates were spun at 16,000 × *g* for 10 min and the supernatant was added to the column with NeutrAvidin™ Gel. The column was incubated for 60 min at room temperature with end-over-end mixing and then was washed (4×) with the washing buffer. The biotinylated proteins were released by incubation of the gel with 2× Laemmli buffer (Invitrogen, Carlsbad, CA) for 30 min at room temperature. Samples of the biotinylated fractions (25 μl) were subjected to Western blot analysis for detection of MRP4 with M4I-10 antibody (Axxora, San Diego, CA) after they were diluted to 0.3 μg/ml with PBS, and for detection of the basolateral marker protein $Na^+$–$K^+$ ATPase with the ATPase antibody (Abcam, Cambridge, MA) after dilution to 0.4 μg/ml.

### 2.8. Generation of MRP4-knockdown Caco-2 cells with MRP4-specific siRNA

An siRNA sequence was designed to silence the MRP4 gene expression based on a published report [21]. Chemically synthesized oligonucleotide encoding the siRNA sense strand, 5′-gatcccaatcctgcacatgcaccatcttgatatccggatggtgcatgtgcaggatttttttttc-caaa-3′, was annealed with the complementary DNA oligonucleotide, 5′-agcttttggaaaaaaaatcctgcacatgcaccatccggatatcaagatggtg-catgtgcaggattgg-3′. The resulting double-stranded DNA was inserted into the BamHI and HindIII sites of the pRNATin-H1.2/Hygro vector (GenScript, Piscataway, NJ). Caco-2 cells at 90% confluence were transfected with the MRP4 siRNA plasmid, using the Nucleofector® System (Amaxa, Gaithersburg, MD) according to the manufacturer's protocol specific for Caco-2 cells. Transfectants were selected by growing the cells with 0.2 mg/ml hygromycin B for 3 weeks, and screened by Western blot a for MRP4 expression. A clone with the lowest MRP4 expression was chosen as a stably transfected cell line for further functional study.

### 2.9. Data analysis

Data are expressed as mean ± S.D. from three measurements unless otherwise noted. Statistical significance was evaluated using *t*-test or ANOVA followed by Dunnet's test for multiple comparisons. The data were analyzed with SigmaStat 2.0 (Systat Software, Inc., San Jose, CA).

Apparent permeability ($P_{app}$) was defined as a parameter that quantifies the appearance of adefovir in the basolateral (receptor) compartment after dosing adefovir dipivoxil in the apical (donor) compartment, and was determined using Eq. (1):

$$P_{app} = \frac{dQ/dt}{A \cdot C_0} \quad (1)$$

where $dQ/dt$ is determined from the amount of adefovir appearing in the basolateral compartment ($Q$) over time ($t$) during the experiment, $A$ is the surface area of the porous membrane, and $C_0$ is the initial concentration of adefovir dipivoxil in the donor side.

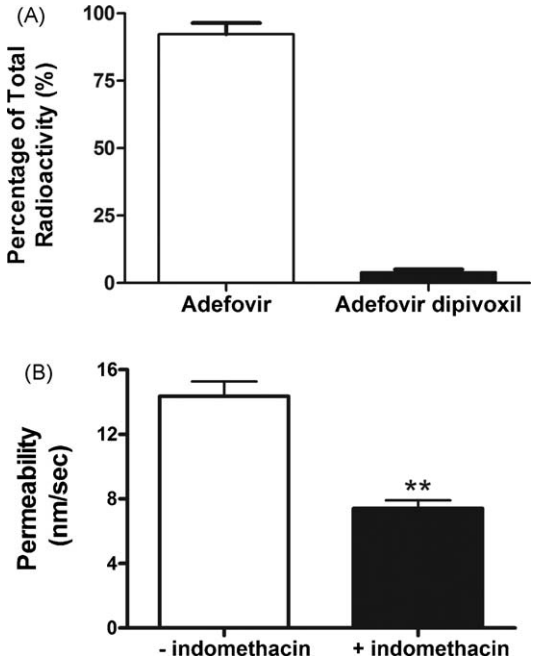


**Fig. 1.** Transport and metabolism of [$^3$H]adefovir dipivoxil in Caco-2 cells. Transport of 10 μM [$^3$H]adefovir dipivoxil across Caco-2 cells was determined with or without 30 μM indomethacin. Samples in the receptor chamber were analyzed by HPLC and scintillation spectrometry (A), and the apparent permeability ($P_{app}$) to represent the availability of adefovir in the receptor compartment was calculated (B). Data represent mean ± S.D. of a representative experiment in triplicate. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with the control.

## 3. Results

### 3.1. Transport and metabolism of adefovir dipivoxil in Caco-2 cells

When the absorptive transport of [$^3$H]adefovir dipivoxil across Caco-2 cell monolayers was investigated, little prodrug was detected in the basolateral compartment and [$^3$H]adefovir accounted for 93.6% of the total radioactivity (Fig. 1A); the apparent permeability ($P_{app}$) calculated by measuring adefovir concentrations in the basolateral compartment represented the availability of adefovir in the receiver compartment after dosing adefovir dipivoxil in the apical (donor) compartment. Treatment of cell monolayers with a general MRP inhibitor, indomethacin (30 μM), on both apical and basolateral sides reduced the apparent permeability of adefovir by 48% (Fig. 1B). These results indicated that (i) the dipivoxil prodrug was hydrolyzed almost completely in the cells, (ii) adefovir appeared in the basolateral compartment by egress across the basolateral membrane, and (iii) MRP transporter(s) may contribute to the basolateral efflux of adefovir. Inhibition of apical MRP would have increased, not decreased apparent permeability of adefovir; further, adefovir is not substrate for MRP2 [18], the major apical MRP isoform in the intestine and Caco-2 cells.

### 3.2. Substrate activity of adefovir toward MRP3 or MRP4

As discussed later, MRP3 and MRP4 are the major MRP isoforms in the basolateral membrane of intestinal epithelial cells and Caco-2 cells. Substrate activity of adefovir toward MRP3 and MRP4 was determined by measuring accumulation of [$^3$H]adefovir in MRP3- or MRP4-expressing MDCKII cells in the presence or absence of MRP inhibitor indomethacin after the cells were incubated with [$^3$H]adefovir dipivoxil. This lipophilic prodrug of adefovir diffuses through the cell membrane and is metabolized to adefovir by intracellular esterases, after which an efflux transporter like MRP is required to pump the anionic adefovir out of cells. In the presence of the MRP inhibitor, indomethacin, the MRP pump is inhibited, and adefovir is accumulated in the cells. The ratio of [$^3$H]adefovir accumulation in the absence vs. presence of indomethacin increased from 1.5 for mock MDCKII cells to 4.6 for MRP4-transfected cells (Fig. 2B). In contrast, the ratio of [$^3$H]adefovir accumulation in the absence vs. presence of indomethacin for MRP3-transfected cells (1.7) was close to that in the mock cells (Fig. 2B). These results indicated that adefovir is a substrate for MRP4 but not for MRP3.

### 3.3. Expression and localization of MRP4 protein in Caco-2 cells

Because adefovir was found to be a substrate for MRP4, it was a good candidate for a transporter that would mediate the egress of adefovir across the basolateral membrane of Caco-2 cells during absorptive transport of adefovir dipivoxil across the cell monolayers. Hence, Western blot analysis was performed to measure the expression of MRP4 protein in Caco-2 cells. As shown in Fig. 3A, the M4I-10 antibody for human MRP4 labeled a protein in the lysates of Caco-2 cells, either cultured for 4 days in flasks or for 25 days on Transwell™ inserts, and of HEK cells (positive control) with an apparent molecular weight around

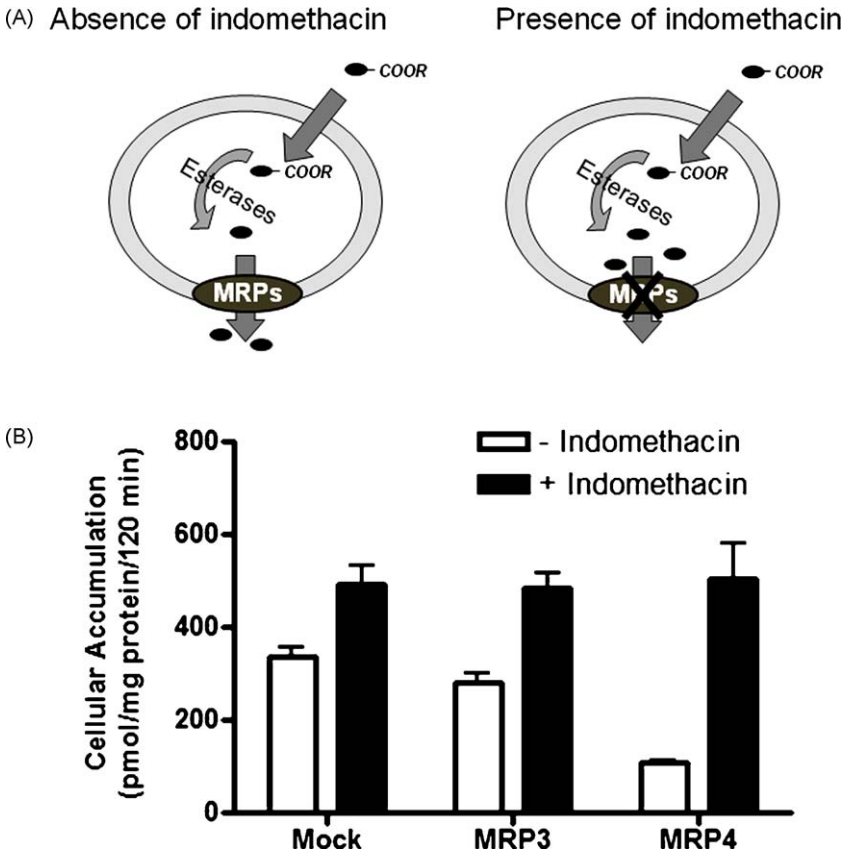


**Fig. 2.** Accumulation of adefovir in MDCKII cells expressing MRP3 or MRP4 upon treatement with adefovir dipivoxil. Functional activity of MRP3 and MRP4 in efflux of adefovir was examined by measuring accumulation of [$^3$H]adefovir in MRP3- or MRP4-transfected MDCKII cells that were treated with [$^3$H]adefovir dipivoxil in the presence or absence of the MRP inhibitor indomethacin (A). Small increase in adefovir accumulation by indomethacin treatment of the mock cells is likely due to background expression of Mrp transporters in MDCKII cells. The ratio of [$^3$H]adefovir accumulation in the absence vs. presence of indomethacin is 1.5, 1.7, and 4.6 for the mock, MRP3- and MRP4-transfected MDCK cells, respectively (B). Data represent mean ± S.D. of a representative experiment in triplicate.

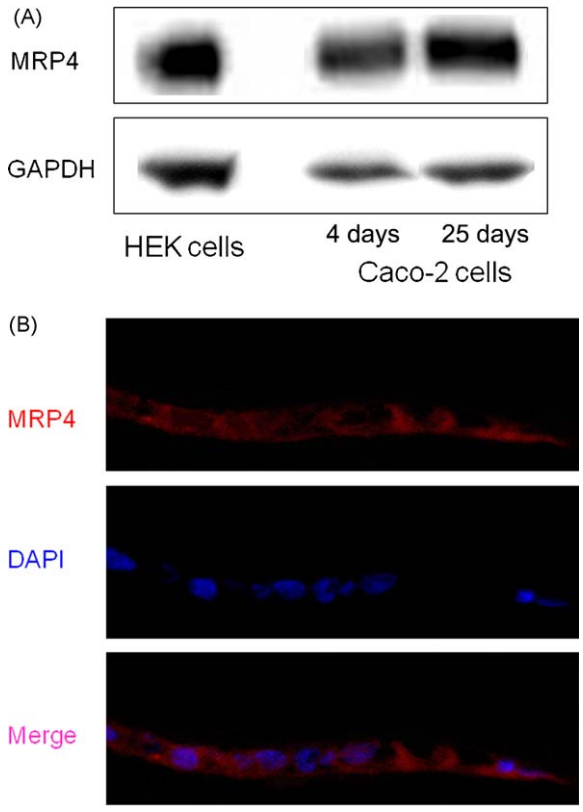


**Fig. 3.** Immunoblot analysis (A) and immunolocalization (B) of MRP4 in Caco-2 cells. Caco-2 cells, cultured either for 4 days in flasks or for 25 days on Transwell™ inserts, were lysed and total protein was subjected to Western blot analysis. The M4I-10 antibody (Axxora, San Diego, CA) was used to stain the membrane for detection of MRP4. HEK 293 cell lysate was used as a positive control and GAPDH protein served as a loading marker (A). Caco-2 cell monolayers on the transwell were frozen and sectioned in longitudinal direction. Indirect immunofluorescent staining was performed with the M4I-10 antibody (Axxora, San Diego, CA) and viewed via fluorescence microscopy. MRP4 staining is shown in red and DAPI staining in blue (B).

200 kDa. These results clearly demonstrate that human MRP4 protein is expressed in Caco-2 cells and that culturing the cells for 25 days on Transwell™ inserts does not change its basal expression level. Fluorescence microscopy images of the Caco-2 cells, immunostained with MRP4 antibody, showed its localization in the basal and lateral cell membrane under or surrounding the nuclear staining (Fig. 3B).

That MRP4 is localized in the basolateral membrane of Caco-2 cells, was further confirmed by fractionating the basolateral and apical membrane proteins, and analyzing the fractions enriched in either apical or basolateral membrane proteins for the presence of MRP4 protein. Isolation of the apical and the basolateral membranes was accomplished by a novel approach (see Fig. 4A), in which the surface proteins on either the basolateral or the apical membrane were biotinylated in two separate experiments by treating the cell monolayers with a biotin-linked electrophile on the respective side. Subsequent affinity enrichment using avidin gel allowed the isolation of the apical and basolateral membrane fractions. Enrichment of the basolateral proteins in the isolated basolateral membrane fraction was established by Western blot analysis indicating the presence of significantly greater amount of the basolateral marker $Na^+$–$K^+$ ATPase (Fig. 4B). The MRP4 protein was enriched in the same membrane fraction as the one in which $Na^+$–$K^+$ ATPase was enriched (Fig. 4B), confirming that MRP4 is expressed in the basolateral membrane of Caco-2 cell monolayers.

### *3.4. Transport of adefovir dipivoxil in MRP4-knockdown and wild-type Caco-2 cells*

Caco-2 cells with diminished MRP4 activity were prepared transfecting MRP4-specific siRNA to determine the role of MRP4 in the egress of adefovir (generated from its prodrug) across the basolateral membrane. Caco-2 cells were transfected with the MRP4 siRNA plasmid. After selection by hygromycin B, 32 clones were obtained and evaluated by Western blotting. As shown in Fig. 5A, MRP4 expression in Caco-2 clones 5, 16 and 21 was suppressed significantly compared to the wild-type Caco-2 cells. The MRP functional activity was examined in the wild-type cells and in clones showing decreased MRP4 expression by measuring accumulation of [$^3$H]adefovir after treatment of cells with [$^3$H]adefovir dipivoxil with or without the MRP inhibitor indomethacin (Fig. 5B). Accumulation of adefovir increased from 311 pmol/mg protein/120 min for wild-type cells to 597 pmol/mg protein/120 min for clone 16 with decreased MRP4 expression. This result provided evidence for suppression of an efflux transporter (e.g. MRP4) in clone 16. Consistent with the reduced MRP4 expression, the ratio of the adefovir accumulation in control (untreated) cells vs. indomethacin-treated cells decreased from 3.6 for wild-type Caco-2 cells to 1.7 for clone 16 (Fig. 5B). Therefore, one of these clones, i.e. clone 16, was used for adefovir transport studies. Fig. 6 depicts the apparent permeability of adefovir in wild-type Caco-2 cells and in cells derived from clone 16 with reduced expression of MRP4. The apparent permeability of adefovir was approximately 40% lower in the MRP4-knockdown Caco-2 cells than in the wild-type cells. For comparison, treatment of the wild-type Caco-2 cells with the general MRP inhibitor, indomethacin (30 μM), reduced the apparent permeability of adefovir by 48%. Taken together, these results suggest that MRP4 is the major MRP isoform contributing to the absorptive transport of adefovir dipivoxil across Caco-2 cell monolayers.

## 4. Discussion

Results presented in this study show that the anionic nucleoside phosphonate, adefovir, is formed from its ester prodrug, adefovir dipivoxil, inside Caco-2 cells, and that MRP4 plays an important role in mediating adefovir egress across the basolateral membrane. This is the first report of a basolateral efflux transporter in the MRP family mediating absorptive transport of an anionic drug in Caco-2 cell monolayers, a well-established model for human intestinal epithelium, therefore suggesting a role of basolateral MRP transporters in assisting intestinal absorption of hydrophilic anionic drugs. Our understanding about the role of transporters that either facilitate or attenuate intestinal absorption of drugs has grown rapidly over the past few years [22–24]. However, most of the work has focused on the apical transporters involved in trafficking of compounds between intestinal lumen and the cellular compartment of enterocytes. Although presence of a basolateral transporter is implicated in the intestinal absorption of compounds, for example peptides [25], our knowledge about transport of hydrophilic and/or charged compounds from the enterocytes into the mesenteric blood circulation is relatively poor.

Among the MRP family transporters that are expressed in the human intestine (MRP1–6) [19], MRP1 does not transport adefovir [26] and is not found localized on either the apical or basolateral membrane [27] although the protein is detected in Caco-2 cells [27,28]. MRP2 is localized in the apical membrane [27,29] and does not transport adefovir [18]. These reports rule out a role of MRP1 or MRP2 in basolateral efflux of adefovir. Further, literature reports rule out MRP6 as likely transporters of a nucleotide [30]. Among the remaining MRP transporters likely involved in the basolateral efflux of adefovir, MRP5 mRNA is minimally expressed in Caco-2

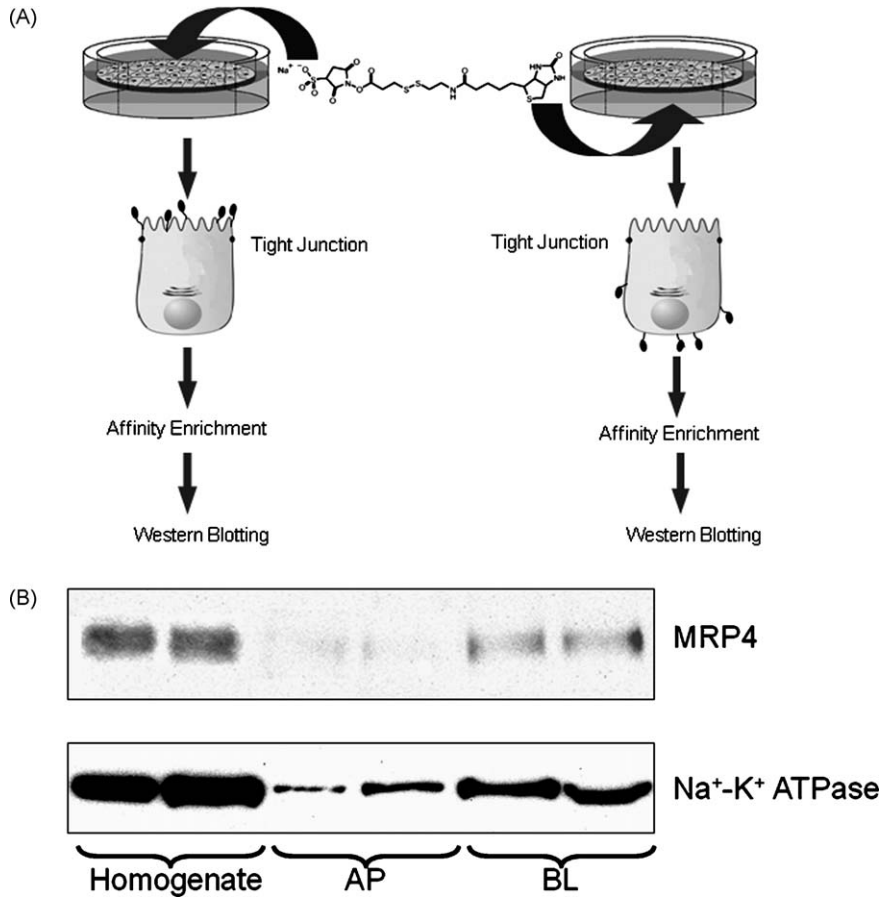


**Fig. 4.** Immunoblot analysis of MRP4 in enriched proteins from either apical or basolateral membrane of Caco-2 cells. Proteins from apical and basolateral membranes of Caco-2 cells were enriched by biotinylation of plasma membrane proteins followed by affinity enrichment of the membrane fractions as described in Section 2 (A). Samples were analyzed by Western blot for MRP4 expression. $Na^+$–$K^+$ ATPase protein served as a basolateral membrane marker (B).

cells and immunostaining of these cells with MRP5 antibody failed to show the protein expression [15,19,27]. Hence, MRP3 and MRP4 were considered as the most likely transporters of adefovir in the MRP family. The studies with MRP3- and MRP4-expressing MDCKII cells (Fig. 2) clearly established that MRP4, but not MRP3, transports adefovir efficiently. Therefore, localization of MRP4 in Caco-2 cell monolayers was examined in order to investigate its role in the absorptive transport of adefovir. Immunostaining followed by fluorescence microscopy provided evidence that MRP4 is localized on the basolateral membrane of Caco-2 cells grown as monolayers. To confirm this finding, MRP4 localization was further investigated by isolation of apical and basolateral membrane proteins and subjecting them to Western blot analysis. A novel approach was used to isolate the apical and basolateral membrane fractions derived from Caco-2 cells. In this approach, the surface proteins on either the apical or the basolateral membrane of the polarized cells, grown on the porous membrane of the Transwell™ apparatus, were selectively modified with a biotin-linked electrophile as shown in Fig. 4A. The biotinated apical and basolateral membrane fragments were isolated by affinity enrichment using avidin gel. Western blot analysis of the apical and basolateral membrane fractions confirmed that the same membrane fragment that was enriched in the basolateral membrane marker $Na^+$–$K^+$ ATPase, was also enriched in MRP4. This novel method has a broad applicability to determine localization of proteins on apical or basolateral membrane of polarized cells.

To determine the role played by MRP4 in the egress of adefovir across the basolateral membrane of Caco-2 cells, a MRP4-knockdown cell model was created by stably expressing a MRP4-specific siRNA. [$^3$H]Adefovir dipivoxil was placed in the apical compartment of wild-type or MRP4-knockdown Caco-2 cell monolayers grown in a Transwell™ apparatus, and the basolateral compartment was sampled for [$^3$H]adefovir as a function of time. The results in Fig. 1 show that adefovir dipivoxil can enter Caco-2 cells across the apical membrane, and is hydrolyzed in the cell so that adefovir is mainly detected in the basolateral compartment. A significantly reduced adefovir permeability in the MRP4-knockdown Caco-2 cells (Fig. 6) established that MRP4 plays an important role in the egress of adefovir formed in Caco-2 cells from adefovir dipivoxil.

Breast cancer resistance protein (BCRP, ABCG2) is an apical transporter of adefovir, and it can compensate for the low expression of MRP4 in MRP4-knockdown mice by up-regulation [31], possibly accounting for reduced efflux of adefovir in MRP4 siRNA transfected Caco-2 cells. However, in both MRP4 siRNA transfected and wild-type Caco-2 cells, Western blot analysis did not reveal the presence of BCRP protein (data not shown). Furthermore, the accumulation of adefovir after treatment with adefovir dipivoxil for 2 h increased in MRP4 siRNA transfected Caco-2 cell clone (Fig. 5B), which indicated a significant suppression, not induction, of efflux transporters. Further, in the presence of indomethacin, an inhibitor for MRPs but not BCRP, adefovir accumulation decreased significantly (3.6-fold) in the wild-type cells but only slightly (1.7-fold) in MRP4 siRNA transfected cells. These result confirmed that reduction of MRP4 expression, and not induction of BCRP, diminished basolateral egress of adefovir in MRP4 siRNA transfected Caco-2 cells. However, it will be interesting to study the role of BCRP in intestinal absorption of adefovir after administration of adefovir dipivoxil using an intestinal model with significant BCRP expression.

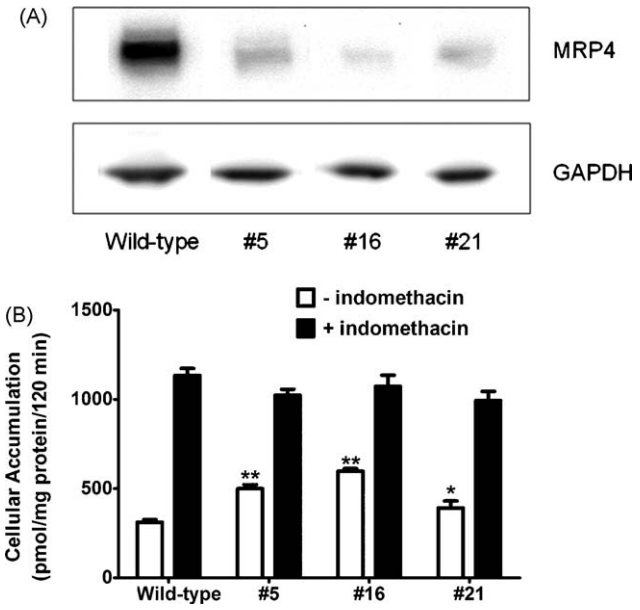


**Fig. 5.** Effect of MRP4-specific siRNA on MRP4 expression and [$^3$H]adefovir accumulation in Caco-2 cells. The MRP4-specific siRNA plasmid was transfected into Caco-2 cells using the Nucleofector® System (Amaxa, Gaithersburg, MD) and transfectants were selected with 0.2 mg/ml hygromycin B treatment for 3 weeks. (A) Total proteins of the selected Caco-2 clones were analyzed by Western blot for MRP4 expression; GAPDH protein served as an internal control. (B) Accumulation of [$^3$H]adefovir after treatment of wild-type Caco-2 cells and MRP4-knockdown clones with [$^3$H]adefovir dipivoxil (10 μM) in the presence or absence of indomethacin (30 μM). Data represent mean ± S.D. of a representative experiment in triplicate. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with the wild-type cells.

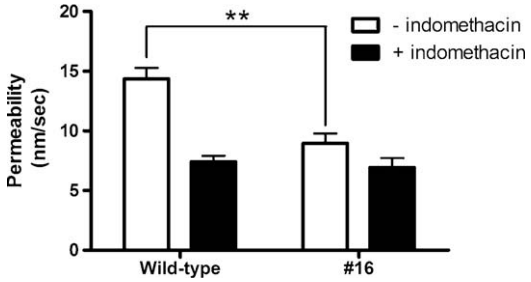


**Fig. 6.** Apparent permeability of adefovir in wild-type or MRP4-knockdown Caco-2 cells. Transport of 10 μM [$^3$H]adefovir dipivoxil was determined in the absence or presence of 30 μM indomethacin across MRP4-knockdown or wild-type Caco-2 cell monolayers. [$^3$H]Adefovir was measured in the basolateral compartment. Data represent mean ± S.D. of a representative experiment in triplicate. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with the wild-type cells.

Recent studies with Mrp4-knockout (*Mrp4*$^{-/-}$) mice suggested that Mrp4 is expressed basolaterally in the mouse intestine [20]. *Mrp4*$^{-/-}$ mice were significantly more sensitive to adefovir administered intravenously, as indicated by a clear separation of the dose–response curves for knockout and wild-type mice, and an increased gastrointestinal toxicity in the knockout mice as compared to the wild-type mice [20]. This result supported the notion that Mrp4, present in the basolateral cell membrane, reduces the accumulation of adefovir entering into the enterocytes from the circulation, and protects the enterocytes from adefovir cytotoxicity. These results provide support to the basolateral secretory role of MRP4 in the intestinal epithelium. However, direct extrapolation of the results from *in vitro* or animal models to clinical outcomes should be attempted with extreme caution. Although a human-derived cell line, Caco-2 cells are indeed colonic cancer cells. In addition, there are also differences in the expression and localization of transporters between mouse and human. Studying the expression and functional activity of MRP4 in human intestine will provide additional confirmation that this transporter plays a role in the intestinal absorption of adefovir.

The physiological functions of MRPs in ABCC subfamily in the intestine are unknown despite their relatively high expression levels. Detoxifying the enterocytes by pumping out drugs and metabolites of the intestine was proposed [9]; however, definitive evidence is still missing. This study suggests that the basolateral MRP4 may play an important role in the oral absorption of adefovir dipivoxil, an ester prodrug of the anionic drug adefovir, by mediating basolateral egress of the anionic drug formed inside the enterocytes. The study thus provides a new insight about the role of MRPs in the intestinal absorption of hydrophilic anionic drugs that are taken up into the enterocytes via apical uptake transporters or produced inside the cells from their prodrugs. Design of ester prodrugs is a major strategy used to improve the permeability of drugs that are charged and/or hydrophilic [32]. However, due to the presence of esterases in the enterocytes, many prodrugs are hydrolyzed in the intestinal epithelium. The advantage of presystemic activation is that only the active drug would enter systemic circulation, minimizing or eliminating the exposure of most organs and tissues to the prodrug or intermediate metabolites. However, for such a prodrug to be effective, the parent drug generated in the enterocytes must be transported out of the cells across the basolateral membrane by transporters. Such a prodrug will yield not only good oral bioavailability of the parent drug but also minimal systemic exposure to chemical entities other than the active drug. Finally, results presented in this study also suggest that transporter-based drug–drug interactions can occur involving a transporter like MRP4 that is present on the basolateral membrane of enterocytes. In the reports on drug–drug interactions involving intestinal transporters, the focus thus far has been entirely on apical transporters (see the US Food and Drug Administration guidance for assessing P-gp inhibition: http://www.fda.gov/RegulatoryInformation/Guidances/default.htm). However, the present study highlights the importance of expanding such considerations to include likely drug–drug interactions involving basolateral transporters.

## Acknowledgements

We gratefully acknowledge Professor Piet Borst (The Netherlands Cancer Institute, The Netherlands) for providing MRP3 transfected MDCKII cells and Professor Dietrich Keppler (German Cancer Research Center, Germany) for providing the MRP4 plasmid pcDNA3.1/hygro/MRP4.

## References

[1] De Clercq E. Clinical potential of the acyclic nucleoside phosphonates cidofovir, adefovir, and tenofovir in treatment of DNA virus and retrovirus infections. Clin Microbiol Rev 2003;16:569–96.
[2] Cundy KC, Barditch-Crovo P, Walker RE, Collier AC, Ebeling D, Toole J, et al. Clinical pharmacokinetics of adefovir in human immunodeficiency virus type 1-infected patients. Antimicrob Agents Chemother 1995;39:2401–5.
[3] Barditch-Crovo P, Toole J, Hendrix CW, Cundy KC, Ebeling D, Jaffe HS, et al. Anti-human immunodeficiency virus (HIV) activity, safety, and pharmacokinetics of adefovir dipivoxil (9-[2-(bis-pivaloyloxymethyl)-phosphonylmethoxyethyl]adenine) in HIV-infected patients. J Infect Dis 1997;176:406–13.
[4] Noble S, Goa KL. Adefovir dipivoxil. Drugs 1999;58:479–87. discussion 88-9.
[5] Annaert P, Tukker JJ, van Gelder J, Naesens L, de Clercq E, van Den Mooter G, et al. In vitro, ex vivo, and in situ intestinal absorption characteristics of the antiviral ester prodrug adefovir dipivoxil. J Pharm Sci 2000;89:1054–62.
[6] Gan LSL, Thakker DR. Applications of the Caco-2 model in the design and development of orally active drugs: elucidation of biochemical and physical barriers posed by the intestinal epithelium. Adv Drug Deliv Rev 1997;23:77–98.
[7] Annaert P, Kinget R, Naesens L, de Clercq E, Augustijns P. Transport, uptake, and metabolism of the bis(pivaloyloxymethyl)-ester prodrug of 9-(2-phosphonylmethoxyethyl)adenine in an in vitro cell culture system of the intestinal mucosa (Caco-2). Pharm Res 1997;14:492–6.

[8] Annaert P, Van Gelder J, Naesens L, De Clercq E, Van den Mooter G, Kinget R, et al. Carrier mechanisms involved in the transepithelial transport of bis(-POM)-PMEA and its metabolites across Caco-2 monolayers. Pharm Res 1998;15:1168–73.
[9] Kruh GD, Belinsky MG, Gallo JM, Lee K. Physiological and pharmacological functions of Mrp2, Mrp3 and Mrp4 as determined from recent studies on gene-disrupted mice. Cancer Metastasis Rev 2007;26:5–14.
[10] Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, et al. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res 1997;57:3537–47.
[11] Schuetz JD, Connelly MC, Sun D, Paibir SG, Flynn PM, Srinivas RV, et al. MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. Nat Med 1999;5:1048–51.
[12] van Aubel RA, Smeets PH, Peters JG, Bindels RJ, Russel FG. The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. J Am Soc Nephrol 2002;13:595–603.
[13] Zelcer N, Reid G, Wielinga P, Kuil A, van der Heijden I, Schuetz JD, et al. Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP) 4 (ATP-binding cassette C4). Biochem J 2003;371:361–7.
[14] van Aubel RA, Smeets PH, van den Heuvel JJ, Russel FG. Human organic anion transporter MRP4 (ABCC4) is an efflux pump for the purine end metabolite urate with multiple allosteric substrate binding sites. Am J Physiol Renal Physiol 2005;288:F327–33.
[15] Hilgendorf C, Ahlin G, Seithel A, Artursson P, Ungell AL, Karlsson J. Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines. Drug Metab Dispos 2007;35:1333–40.
[16] Cundy KC. Clinical pharmacokinetics of the antiviral nucleotide analogues cidofovir and adefovir. Clin Pharmacokinet 1999;36:127–43.
[17] Cihlar T, Lin DC, Pritchard JB, Fuller MD, Mendel DB, Sweet DH. The antiviral nucleotide analogs cidofovir and adefovir are novel substrates for human and rat renal organic anion transporter 1. Mol Pharmacol 1999;56:570–80.
[18] Imaoka T, Kusuhara H, Adachi M, Schuetz JD, Takeuchi K, Sugiyama Y. Functional involvement of multidrug resistance-associated protein 4 (MRP4/ABCC4) in the renal elimination of the antiviral drugs adefovir and tenofovir. Mol Pharmacol 2007;71:619–27.
[19] Taipalensuu J, Tornblom H, Lindberg G, Einarsson C, Sjoqvist F, Melhus H, et al. Correlation of gene expression of ten drug efflux proteins of the ATP-binding cassette transporter family in normal human jejunum and in human intestinal epithelial Caco-2 cell monolayers. J Pharmacol Exp Ther 2001;299:164–70.
[20] Belinsky MG, Guo P, Lee K, Zhou F, Kotova E, Grinberg A, et al. Multidrug resistance protein 4 protects bone marrow, thymus, spleen, and intestine from nucleotide analogue-induced damage. Cancer Res 2007;67:262–8.
[21] Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci USA 2003;100:9244–9.
[22] Dobson PD, Kell DB. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? Nat Rev Drug Discov 2008;7:205–20.
[23] Thwaites DT, Anderson CM. $H^+$-coupled nutrient, micronutrient and drug transporters in the mammalian small intestine. Exp Physiol 2007;92:603–19.
[24] Kunta JR, Sinko PJ. Intestinal drug transporters: in vivo function and clinical importance. Curr Drug Metab 2004;5:109–24.
[25] Irie M, Terada T, Okuda M, Inui K. Efflux properties of basolateral peptide transporter in human intestinal cell line Caco-2. Pflugers Arch 2004;449:186–94.
[26] Dallas S, Schlichter L, Bendayan R. Multidrug resistance protein (MRP) 4- and MRP 5-mediated efflux of 9-(2-phosphonylmethoxyethyl)adenine by microglia. J Pharmacol Exp Ther 2004;309:1221–9.
[27] Prime-Chapman HM, Fearn RA, Cooper AE, Moore V, Hirst BH. Differential multidrug resistance-associated protein 1 through 6 isoform expression and function in human intestinal epithelial Caco-2 cells. J Pharmacol Exp Ther 2004;311:476–84.
[28] Lemos C, Kathmann I, Giovannetti E, Dekker H, Scheffer GL, Calhau C, et al. Folate deprivation induces BCRP (ABCG2) expression and mitoxantrone resistance in Caco-2 cells. Int J Cancer 2008;123:1712–20.
[29] Mottino AD, Hoffman T, Jennes L, Vore M. Expression and localization of multidrug resistant protein mrp2 in rat small intestine. J Pharmacol Exp Ther 2000;293:717–23.
[30] Kruh GD, Guo Y, Hopper-Borge E, Belinsky MG, Chen ZS. ABCC10, ABCC11, and ABCC12. Pflugers Arch 2007;453:675–84.
[31] Takenaka K, Morgan JA, Scheffer GL, Adachi M, Stewart CF, Sun D, et al. Substrate overlap between Mrp4 and Abcg2/Bcrp affects purine analogue drug cytotoxicity and tissue distribution. Cancer Res 2007;67:6965–72.
[32] Beaumont K, Webster R, Gardner I, Dack K. Design of ester prodrugs to enhance oral absorption of poorly permeable compounds: challenges to the discovery scientist. Curr Drug Metab 2003;4:461–85.