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

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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.

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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.

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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-

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