Functional Involvement of Multidrug Resistance-Associated Protein 4 (MRP4/ABCC4) in the Renal Elimination of the Antiviral Drugs Adefovir and Tenofovir

Tomoki Imaoka, Hiroyuki Kusuhara, Masashi Adachi, John D. Schuetz, Kenji Takeuchi, and Yuichi Sugiyama

Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan (T.I., H.K., K.T., Y.S.); and Department of Pharmaceutical Sciences, St. Jude Children’s Research Hospital, Memphis, Tennessee (M.A., J.S.)

Received June 22, 2006; accepted November 15, 2006

ABSTRACT

Acyclic nucleotide phosphonates (adefovir, cidofovir, and tenofovir) are eliminated predominantly into the urine, and renal failure is their dose-limiting toxicity, particularly for adefovir and cidofovir. In this study, we examined the involvement of multidrug resistance-associated protein (MRP4) (ABCC4) in their luminal efflux in the kidney. ATP-dependent uptake of adefovir and tenofovir but not cidofovir was observed only in the membrane vesicles expressing MRP4. The ATP-dependent uptake of adefovir and tenofovir by MRP4 was not saturated at 1 mM. The ATP-dependent uptake of adefovir by membrane vesicles expressing MRP4 was osmotic-sensitive. No ATP-dependent uptake of either agent was observed in the membrane vesicles expressing human MRP2 or breast cancer resistance protein. These nucleotide analogs were given to mice by constant intravenous infusion, and the plasma, urine, and tissue concentrations were determined. The kidney accumulation of adefovir and tenofovir was significantly greater in Mrp4 knockout mice (130 versus 66 and 191 versus 87 pmol/g tissue, respectively); thus, the renal luminal efflux clearance was estimated to be 37 and 46%, respectively, of the control. There was no difference in the fraction of mono- and diphosphorylated forms of adefovir in the kidney between wild-type and Mrp4 knockout mice. In mice, cidofovir was also eliminated via the urine by tubular secretion as well as glomerular filtration. There was no change in the kinetic parameters of cidofovir in Mrp4 knockout mice. Our results suggest that MRP4 is involved in the luminal efflux of both adefovir and tenofovir, but it makes only a limited contribution to the urinary excretion of cidofovir.

Acyclic nucleotide phosphonates adefovir, cidofovir, and tenofovir (Fig. 1) have been developed as antiviral agents with strong and selective activity against a wide range of viruses such as retroviruses, including human immunodeficiency virus type 1 and 2 (HIV1 and HIV2), herpes viruses, and hepadnaviruses (De Clercq, 2003; Izzedine et al., 2005). These nucleotide analogs are phosphorylated inside the cells to their active form, and the diphosphorylated metabolite is responsible for their antiviral activity (Balzarini et al., 1991; Ho et al., 1992; Eisenberg et al., 2001). These drugs are actively secreted in the urine, and most of the administered dose is recovered in the urine in intact form (Cundy, 1999). Accumulation in the kidney has been suggested to be associated with the dose-limiting toxicity of adefovir and cidofovir (Naesens et al., 1992; Roy et al., 2003). Indeed, proximal tubular toxicity has been reported during cidofovir cytomegalovirus therapy and high-dose adefovir HIV therapy (Lalezari et al., 1997; Kahn et al., 1999). Although tenofovir is an analog of adefovir (Fig. 1), patients treated with tenofovir exhibit a lower incidence of renal dysfunction at doses used to treat HIV (Gallant et al., 2005; Roling et al., 2006). Tubular secretion of drugs in the kidney has been characterized by...

This study was supported by Grant-in-Aid for Scientific Research (B) KAKENHI 17209005 from the Japan Society for the Promotion of Science (to Y.S.); by Grant-in-Aid for Scientific Research on Priority Areas KAKENHI 18059007 (to H.K.); a grant for the 21st Century COE program, “Strategic Approach to Drug Discovery and Development in Pharmaceutical Sciences,” from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to T.I.); by National Institutes of Health grant GM60904 (to J.D.S.); by Cancer Center Support grant P30 CA21745 (to J.D.S.); and by the American Lebanese Syrian Associated Charities (to J.D.S.). Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org. doi:10.1124/mol.106.028233.

ABBREVIATIONS: HIV, human immunodeficiency virus; OAT, organic anion transporter; MRP, multidrug resistance-associated protein; BBM, brush-border membrane; BCRP, breast cancer resistance protein; HEK, human embryonic kidney; DHEAS, dehydroepiandrosterone sulfate; h, human; GFP, green fluorescent protein; GFR, glomerular filtration rate; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.
multispecific organic anion and cation transport systems (Inui et al., 2000; Koepsell and Endou, 2004; Lee and Kim, 2004; Sweet, 2005; Wright, 2005; Sekine et al., 2006). It has been demonstrated that acyclic nucleotide phosphonates are substrates of one of the basolateral organic anion transporters, OAT1 (SLC22A6) (Cihlar et al., 1999). Furthermore, Chinese hamster ovary cells expressing human OAT1 exhibit enhanced toxicity to adefovir and cidofovir (500-fold) in comparison with parental cells (Ho et al., 2000), presumably, due to increased cellular accumulation. Unlike adefovir and cidofovir, tenofovir is less nephrotoxic to human renal tubule epithelial cells and OAT1-expressing Chinese hamster ovary cells (Cihlar et al., 2001). Currently, OAT1 is considered to be responsible for the renal uptake of acyclic nucleotide phosphonates; therefore, OAT1 inhibitors, such as nonsteroidal anti-inflammatory drugs, are expected to protect the kidney from adefovir- and cidofovir-induced cytotoxicity by preventing their cellular accumulation (Mulato et al., 2000). In addition to the uptake process, subsequent luminal efflux could be another crucial factor governing the tissue accumulation of these nucleotide analogs, and, ultimately, the incidence of nephrotoxicity. The transporter responsible for the luminal efflux remains unknown. In the present study, multidrug resistance-associated protein (MRP)4 (ABCC4) is hypothesized to account for the urinary efflux across the brush-border membrane (BBM).

MRP4, the fourth member of the ABCC family, was initially identified as a homolog of MRP1 (ABCC1) by screening databases of human expressed sequence tags (Kool et al., 1997). It was found that MRP4 was overexpressed by gene duplication in the adefovir-resistant human T-lymphoblast CEM cell line and that the enhanced efflux of adefovir by MRP4 accounts for this resistance (Schuetz et al., 1999). Overexpression of MRP4 in HEK293 cells could attenuate the cytotoxicity of adefovir but not cidofovir (Reid et al., 2003). The broad substrate specificity of MRP4 has been demonstrated in subsequent analyses, and MRP4 substrates include cAMP, cGMP, p-aminobiphenur, urate, dehydroepiandrosterone sulfate, methotrexate, and estradiol-17β-glucuronide as well as adefovir (Schuetz et al., 1999; van Aubel et al., 2002; Zelcer et al., 2003; Van Aubel et al., 2005). In addition, glutathione modulates the transport activity of MRP4, and the ATP-dependent uptake of taurocholate by MRP4 is stimulated by cotransport of reduced glutathione or its S-methyl derivative, lacking reducing activity (Rius et al., 2003). In the rodent and human kidney, where MRP4 is abundantly expressed (Maher et al., 2005; Nishimura and Naito, 2005), MRP4 is localized on the BBM of the proximal tubules (van Aubel et al., 2002), indicating its involvement in the luminal efflux of drugs. Recently, Assem et al. (2004) produced Mrp4 knockout mice, which are fertile and do not exhibit any physiological abnormalities. Furthermore, Leggas et al. (2004) demonstrated the importance of MRP4 as a xenobiotic pump in the barriers of the central nervous system. In the present study, an in vivo pharmacokinetic analysis was performed to obtain the kinetic parameters for the renal elimination of adefovir and cidofovir using Mrp4 knockout mice to estimate the contribution of Mrp4 to the tubular secretion. Furthermore, an in vitro transport investigation using MRP4-expressing membrane vesicles was performed to directly demonstrate the ATP-dependent transport of the acyclic nucleotide phosphonates by MRP4.

### Materials and Methods

**Materials.** [3H]Adefovir (10 Ci/mmol), [3H]cidofovir (9 Ci/mmol), and [3H]tenofovir (10 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). [14C]Inulin (8 mCi/mmol) and [3H](dehydroepiandrosterone sulfate (DHEAS) (74 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Nonradioactive adefovir and cidofovir were kindly supplied by Gilead Sciences (Forest City, CA). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

**Animals.** Female Mrp4 knockout and wild-type mice (12–15 weeks old) were used in the present study. Mrp4 knockout mice had been established previously (Leggas et al., 2004). All animals were maintained under standard conditions with a reverse dark-light cycle and were treated humanely. Food and water were available ad libitum. The studies were carried out in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, the University of Tokyo, Tokyo, Japan).

**Construction and Infection of Recombinant Adenovirus and the Membrane Vesicle Preparation.** Recombinant adenovirus harboring human MRP4 (hMRP4) gene (NM_005845) was produced according to the manufacturer’s protocol. Recombinant adenovirus harboring the human MRP2 and BCRP gene had been previously established by Hirouchi et al. (2004) and Kondo et al. (2004). For the preparation of the isolated membrane vesicles, HEK293 cells cultured in a 15-cm dish were infected by recombinant adenovirus containing human MRP4, MRP2, and BCRP transporter cDNA. GFP was used as a negative control. Cells were harvested 48 h after infection, and then the membrane vesicles were isolated from −1 to 2 × 10⁶ cells using a standard method described previously. Briefly, cells were diluted 40-fold with hypotonic buffer (1 mM Tris-HCl and 0.1 mM EDTA, pH 7.4, at 37°C) and stirred gently for 1 h on ice in the presence of 2 mM phenylmethylsulfonyl fluoride and 5 μg/ml aprotinin. The cell lysate was centrifuged at 100,000g for 30 min at 4°C, and the resulting pellet was suspended in 10 mM isotonic TS buffer (10 mM Tris-HCl and 250 mM sucrose, pH 7.4, at 4°C) and homogenized using a Dounce B homogenizer (glass/glass; tight pestle; 30 strokes). The crude membrane fraction was layered on top of a 38% (w/v) sucrose solution in 5 mM Tris-HEPES, pH 7.4, at 4°C, and centrifuged in a Beckman SW 41 rotor centrifuge at 70,000 g for 30 min at 4°C. The turbid layer at the interface was collected, diluted to 23 ml with TS buffer, and centrifuged at 100,000g for 30 min at 4°C. The resulting pellet was suspended in 400 μl of TS buffer. Vesicles were formed by passing the suspension 30 times through a 27-gauge needle using a syringe. The membrane vesicles were finally frozen in liquid nitrogen and stored at −80°C until use. Protein concentrations were determined by the Lowry method, and bovine serum albumin was used as a standard.

**Transport Studies with Membrane Vesicles.** The transport studies were performed using a rapid filtration technique. In brief, 15 μl of transport medium (10 mM Tris-HCl, 250 mM sucrose, and 10 mM MgCl₂, pH 7.4) containing radiolabeled compounds, with or without unlabeled substrate, was preincubated at 37°C for 3 min and then rapidly mixed with 5 μl of a membrane vesicle suspension (5 μg

---

**Fig. 1.** Structures of adefovir, tenofovir, and cidofovir.
of protein). The reaction mixture contained 5 mM ATP or AMP along with the ATP-regenerating system (10 mM creatine phosphate and 100 μg/μl creatine phosphokinase). The transport reaction was terminated by the addition of 1 ml of ice-cold buffer containing 10 mM Tris-HCl, 250 mM sucrose, and 0.1 M NaCl, pH 7.4. The stopped reaction mixture was filtered through a 0.45-μm hemagglutinin filter (Millipore Corporation, Billerica, MA) and then washed twice with 5 ml of stop solution. The radioactivity retained on the filter was determined in a liquid scintillation counter (LS6000SE, Beckman Coulter, Fullerton, CA) after the addition of scintillation cocktail (Clear-sol I; Nacalai Tesque, Tokyo, Japan).

**In Vivo Infusion Study in Mice.** Female BL6–129 and Mrp4 knockout mice weighing approximately 20–30 g were used for these experiments. Under pentobarbital anesthesia (30 mg/kg), the kidney was weighed, flash-frozen in liquid nitrogen, and subsequently homogenized. A suitable volume of homogenate was incubated at 55°C for 4 to 6 h after the addition of 2 ml of Soluene-350 (PerkinElmer Life and Analytical Sciences) to dissolve the tissues. The kidney homogenate was treated with ice-cold methanol followed by centrifugation at 10,000 × g for 30 min at 4°C. The supernatant was analyzed by HPLC.

The kidney was killed after 90 min, and the entire kidney was excised immediately. The kidney was weighed, flash-frozen in liquid nitrogen, and subsequently homogenized. A suitable volume of homogenate was incubated at 37°C in the presence of ATP or AMP was 865 ± 106 and 231 ± 14 μl/mg protein, respectively. The time-dependent uptake of [3H]adefovir and [3H]tenofovir by hMRP4-expressing membrane vesicles is shown in Fig. 2, A and D. The uptake of [3H]adefovir and [3H]tenofovir was markedly stimulated by ATP in membrane vesicles prepared from hMRP4-expressing HEK293 cells but not in those from HEK293 cells infected with GFP containing recombinant adenovirus.

The concentration-dependent uptake of adefovir and tenofovir is shown in Fig. 2, B and E. The uptake was not saturated even at the maximum concentration examined (1 mM). The inhibitory effect of DHEAS on the MRP4-mediated transport of adefovir was examined to show the specific uptake (Fig. 2, C and F). DHEAS inhibited the ATP-dependent uptake of [3H]adefovir and [3H]tenofovir by hMRP4-expressing membrane vesicles in a concentration-dependent manner.

**Osmotic Sensitivity of the Uptake of [3H]Adefovir into Membrane Vesicles Expressing hMRP4.** Osmotic sensitivity was studied by examining the uptake of [3H]adefovir into membrane vesicles expressing hMRP4 in the presence of several concentrations of sucrose in the medium to confirm that a major part of the accumulation can be accounted for by transport into the intravesicular space, but not by binding to the vesicle surface. As shown in Fig. 3, the uptake of [3H]adefovir was reduced as the sucrose concentration medium increased. The y-intercept for the relationship between the amount of [3H]adefovir associated with the vesicles versus the reciprocal of the sucrose concentration in the medium was 0.19 μl/mg protein/10 min (Fig. 3). The amount of adefovir bound to the vesicle surface was less than 10% of
the total vesicle uptake at 10 min, if the transport experiment was performed in isotonic medium.

**Uptake of [3H]Adefovir and [3H]Cidofovir via hBCRP- and hMRP2-Expressing Vesicles.** Both MRP2 and BCRP are expressed on the lumen of the kidney; therefore, we investigated whether either of these transporters transported adefovir. Membrane vesicles were prepared from HEK293 cells expressing MRP2 or BCRP as described previously (Hirouchi et al., 2004; Kondo et al., 2004). The uptake of [3H]adefovir by hBCRP-and hMRP2-expressing membrane vesicles was evaluated (Fig. 4, A and C, respectively). No significant ATP-dependent uptake of [3H]adefovir was observed in either hBCRP- or hMRP2-expressing vesicles. The transport activities associated with hBCRP and hMRP2 were confirmed by the uptake of typical substrates, [3H]estrone sulfate (Fig. 4B) and [3H]estradiol-17β-D-glucuronide (Fig. 4D), respectively. The uptake of [3H]cidofovir by hMRP4, hBCRP- and hMRP2-expressing membrane vesicles was examined (Fig. 5, A–C, respectively). No significant ATP-dependent transport of [3H]cidofovir via hMRP4, hBCRP, and hMRP2 was observed.

**Relative Expression of Oats, Bcrp, and Mrp2 mRNAs in the Kidney in Wild-Type and Mrp4 Knockout Mice.** In addition to Mrp4, Oat1, Oat3, Bcrp, and Mrp2 mRNA were expressed at various levels in the kidney, as shown in Fig. 6. No significant difference was observed in the expression level of Oat1, Oat3, Bcrp, and Mrp2 in the kidney between wild-
type and Mrp4 knockout mice. In Mrp4 knockout mice, Mrp4 expression was below the limit of detection.

**Renal Excretion of Adefovir, Tenofovir, and Cidofovir in Mrp4 Knockout and Wild-Type Mice.** [3H]adefovir, [3H]tenofovir, and [3H]cidofovir were administered by constant intravenous infusion. The plasma concentration of adefovir was higher in Mrp4 knockout mice than in wild-type mice, whereas the urinary excretion rate was similar for the three drugs (Fig. 7, B, E, and H). Compared with the wild-type mice, the kidney concentration of adefovir and tenofovir was almost 2-fold greater in Mrp4 knockout mice (Fig. 7, C and F), which probably reflects a reduced ability to remove adefovir and tenofovir. The pharmacokinetic parameters of adefovir, tenofovir, and cidofovir are summarized in Tables 1 to 3.

**Discussion**

In the present study, the involvement of MRP4 in the tubular secretion of acyclic nucleotide phosphonates was examined to demonstrate its importance as one of the crucial factors governing the incidence of nephrotoxicity. Using membrane vesicles expressing hMRP4, the uptake of adefovir, tenofovir, and cidofovir in the presence of ATP or AMP was determined. The renal clearance of adefovir, tenofovir, and cidofovir with regard to the kidney concentration, representing the efflux transport activity across the BBM, was compared between wild-type and Mrp4 knockout mice.

ATP-dependent uptake of adefovir and tenofovir was detected in MRP4-expressing membrane vesicles (Fig. 2, A and D). Consistent with the previous inhibition study in which adefovir had a minimal effect on the ATP-dependent uptake of [3H]estradiol-17β-β-glucuronide and [3H]cGMP by MRP4 at 1 mM (van Aubel et al., 2002; Reid et al., 2003), the ATP-dependent uptake of adefovir by MRP4-expressing membrane vesicles is carrier-mediated, but there is no binding to the membrane vesicles. A similar observation...
was obtained for tenofovir (Fig. 2E). The $K_m$ values of adefovir and tenofovir for MRP4 were quite high ($> 1$ mM) (Fig. 2B). This is also supported by an inhibition study in which 1 mM adefovir had no effect on the ATP-dependent transport of DHEAS by MRP4 (data not shown). Therefore, adefovir and tenofovir are poor MRP4 substrates characterized by low $K_m$ values.

![Fig. 7](image.png)

**Fig. 7.** Time profiles of the plasma concentration, urinary excretion, and kidney accumulation of $[^{3}H]$adefovir, $[^{3}H]$tenofovir, and $[^{3}H]$cidofovir in Mrp4 knockout and wild-type mice. The plasma concentration, urinary excretion rate, and kidney concentration of $[^{3}H]$adefovir (A–C), $[^{3}H]$tenofovir (D–F), and $[^{3}H]$cidofovir (G–I) were determined during constant intravenous infusion into wild-type (○) and Mrp4 knockout mice (●). Each point represents the mean ± S.E. ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ statistically significant difference.

**TABLE 1**
Pharmacokinetic parameters of adefovir during constant infusion into wild-type and Mrp4 knockout mice

Data are taken from Fig. 7, A–C. Data represent the mean ± S.E. ($n = 3$).

<table>
<thead>
<tr>
<th></th>
<th>$\text{CL}_{\text{total}}$ (ml/min/kg)</th>
<th>$\text{CL}_{\text{renal,plasma}}$ (ml/min/kg)</th>
<th>$\text{CL}_{\text{renal,kidney}}$ (ml/min/kg)</th>
<th>GFR (ml/min/kg)</th>
<th>$K_p$,kidney</th>
<th>$K_p$,liver</th>
<th>$K_p$,brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>24.7 ± 1.2</td>
<td>23.7 ± 1.6</td>
<td>0.86 ± 0.04</td>
<td>12.9 ± 0.9</td>
<td>13.1 ± 0.7</td>
<td>0.82 ± 0.08</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>Mrp4(−/−)</td>
<td>18.8 ± 0.3**</td>
<td>18.7 ± 0.2**</td>
<td>0.32 ± 0.03**</td>
<td>10.8 ± 0.3</td>
<td>19.5 ± 1.3*</td>
<td>0.98 ± 0.20</td>
<td>0.09 ± 0.04</td>
</tr>
</tbody>
</table>

$K_p$,kidney: $K_p$, value obtained by dividing the kidney concentration by $C_{\text{plasma}}$ (at 90 min).

* $P < 0.05$ and ** $P < 0.01$, statistically significant difference.

**TABLE 2**
Pharmacokinetic parameters of tenofovir during constant infusion into wild-type and Mrp4 knockout mice

Data are taken from Fig. 7, D–F. Data represent the mean ± S.E. ($n = 3$).

<table>
<thead>
<tr>
<th></th>
<th>$\text{CL}_{\text{total}}$ (ml/min/kg)</th>
<th>$\text{CL}_{\text{renal,plasma}}$ (ml/min/kg)</th>
<th>$\text{CL}_{\text{renal,kidney}}$ (ml/min/kg)</th>
<th>GFR (ml/min/kg)</th>
<th>$K_p$,kidney</th>
<th>$K_p$,liver</th>
<th>$K_p$,brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>28.0 ± 2.2</td>
<td>24.9 ± 0.7</td>
<td>0.860 ± 0.073</td>
<td>14.5 ± 1.2</td>
<td>12.7 ± 0.3</td>
<td>1.17 ± 0.16</td>
<td>0.0146 ± 0.0029</td>
</tr>
<tr>
<td>Mrp4(−/−)</td>
<td>28.1 ± 2.0</td>
<td>28.0 ± 1.3</td>
<td>0.394 ± 0.073</td>
<td>14.8 ± 2.1</td>
<td>29.5 ± 5.5*</td>
<td>1.31 ± 0.15</td>
<td>0.0191 ± 0.0110</td>
</tr>
</tbody>
</table>

$K_p$,kidney: $K_p$, value obtained by dividing the kidney concentration by $C_{\text{plasma}}$ (at 90 min).

* $P < 0.05$ statistically significant difference.
transport activity and a high $K_m$ value. Another nucleotide analog, cidofovir, exhibited no ATP-dependent uptake in the membrane vesicles expressing MRP4 (Fig. 5). This is consistent with a lack of attenuation of cidofovir-induced cytotoxicity by overexpression of MRP4 (Reid et al., 2003). Therefore, MRP4 specifically transports adefovir and tenofovir, whereas OAT1, a basolateral organic anion transporter, accepts all acyclic nucleotide phosphonates as substrates with similar transport activity (Ho et al., 2000). Adefovir and tenofovir have a purine base in their structure, whereas cidofovir has a pyrimidine base. MRP4 may prefer purine analogs since urate (a purine end metabolite), cAMP, cGMP, adefovir, and tenofovir all contain a purine base.

The involvement of MRP4 in the renal clearance of adefovir and tenofovir was evaluated in Mrp4 knockout mice, which have kidneys that exhibit no change in the mRNA expression of Oat1, Oat3, Bcrp, and Mrp2 in comparison with wild-type mice (Fig. 6). The total clearance of adefovir and tenofovir was comparable with the renal clearance with respect to the circulating plasma, indicating that renal elimination is the major elimination pathway (Tables 1 and 2). In view of the fact that renal clearance is accounted for by hybrid parameters of protein binding, glomerular filtration rate, and tubular secretion, the tubular secretion and glomerular filtration rate account equally for the renal clearance since the plasma protein binding of adefovir and tenofovir is quite small, and, at most, 4 and 0.7%, respectively. Both adefovir and tenofovir accumulated to a high degree in the kidney (Tables 1 and 2). Considering the predominant expression of OAT1 in the proximal tubules, the local tissue-to-plasma concentration ratios of adefovir and tenofovir will be much greater. In Mrp4 knockout mice, the renal clearance of adefovir and tenofovir was reduced and the kidney concentration was 2-fold greater in Mrp4 knockout mice. Moreover, the ratio of clearance of tenofovir because there is no published information about the fraction of reabsorption of these compounds. If substantial amounts of adefovir and tenofovir undergo reabsorption in the kidney, the contribution of Mrp4 to net efflux clearance across the BBM will become smaller. However, this seems unlikely because the increased $K_p$ value in Mrp4 knockout mice indicates that Mrp4 plays a significant role in the net efflux from the proximal tubular cells. Since the increase in $K_{\text{efflux}}$ of tenofovir due to impairment of Mrp4 was greater than that of adefovir, it seems that MRP4 makes a greater contribution to the metabolites of tenofovir than adefovir in the kidney. However, because we observed a similar contribution of MRP4 to the luminal efflux of adefovir in Mrp4 knockout mice, it is possible that the basolateral efflux clearance of adefovir is greater than that of tenofovir. This may explain why the plasma concentration of adefovir was increased in Mrp4 knockout mice, whereas that of tenofovir was unaffected. The relationship between the basolateral and luminal efflux clearances governs the rate-limiting process for plasma elimination. For example, if the luminal efflux is greater than the basolateral efflux, the transporters responsible for influx will be the rate-limiting factors governing net secretion from the blood.

Adefovir undergoes intracellular phosphorylation by intracellular enzymes, such as adenylate kinase and/or 5-phosphoribosyl 1-pyrophosphate synthetase, although contradictory reports are available (Balzarini and De Clercq, 1991; Robbins et al., 1995). HPLC analysis demonstrated the existence of mono- and diphosphorylated metabolites in the kidney, whereas none of these metabolites were detected in the blood and urine (data not shown). The phosphorylated form of adefovir is associated with nephrotoxicity and the kidney concentrations of these metabolites were significantly greater in Mrp4 knockout mice. Moreover, the ratio of metabolites to intact adefovir was almost identical in Mrp4 knockout and wild-type mice. Therefore, Mrp4 indirectly regulates the kidney concentration of phosphorylated metabolites. Whether Mrp4 is involved in the direct efflux of the phosphorylated metabolites in the kidney remains unknown.

### TABLE 3
Pharmacokinetic parameters of cidofovir during constant infusion into wild-type and Mrp4 knockout mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>Mrp4(-/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{total}}$ (ml/min/kg)</td>
<td>34.4 ± 6.4</td>
<td>42.7 ± 0.9</td>
</tr>
<tr>
<td>$C_{\text{renal plasma}}$ (ml/min/kg)</td>
<td>26.1 ± 5.7</td>
<td>29.6 ± 0.5</td>
</tr>
<tr>
<td>$C_{\text{renal kidney}}$ (ml/min/kg)</td>
<td>0.60 ± 0.08</td>
<td>0.60 ± 0.08</td>
</tr>
<tr>
<td>GFR (ml/min/kg)</td>
<td>13.8 ± 2.5</td>
<td>12.6 ± 0.4</td>
</tr>
<tr>
<td>$K_{p,kidney}$</td>
<td>22.2 ± 1.7</td>
<td>27.3 ± 5.8</td>
</tr>
<tr>
<td>$K_{p,liver}$</td>
<td>0.31 ± 0.03</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>$K_{p,brain}$</td>
<td>0.045 ± 0.006</td>
<td>0.056 ± 0.001</td>
</tr>
</tbody>
</table>

$K_{p,kidney}$: $K_p$ value obtained by dividing the kidney concentration by $C_{\text{renal plasma}}$ (at 90 min)

**Fig. 8.** Characterization of the metabolites of adefovir in the kidney. At 90 min after constant intravenous infusion of adefovir into wild-type (A) and Mrp4 knockout mice (B), kidney was isolated and tissue extracts were analyzed by anion exchange HPLC followed by determination of the radioactivity in the elute. The inset represents a magnification of the radioactivity in the elute from 45 to 65 min.
Considering the importance of the phosphorylated forms in the nephrotoxic reaction, this should be examined in further studies.

In addition to the kidney, MRP4 is expressed in other normal tissues, such as liver, and tissue barriers, such as the brain capillaries and choroid plexus, where it is expressed on the plasma membrane facing the circulating blood (Leggas et al., 2004). In particular, the liver is the most important organ as far as the therapeutic efficacy of adefovir is concerned. There was no significant change in the $K_v$ values of adefovir in the liver and brain (Table 1); thus, Mrp4 makes only a minor contribution to the total cellular sequestration pathways in these tissues.

The renal clearance of adefovir in Mrp4 knockout mice was still greater than the glomerular filtration rate, suggesting the involvement of other efflux transporters expressed on the BBM of the kidney proximal tubules. In addition to MRP4, two ATP-binding cassette transporters, MRP2 and BCRP, have been identified on the BBM (Schaub et al., 1997; Jonker et al., 2002). In the membrane vesicle study, neither hMRP2 nor hBCRP accepted adefovir as a substrate (Fig. 4). Consistent with the in vitro study, there was no significant difference in the urinary excretion of adefovir between hereditarily Mrp2-deficient mutant rats (Esai hyperbilirubinemic rat) and normal rats (data not shown). Therefore, these ATP-binding cassette transporters do not account for the luminal efflux of adefovir remaining in Mrp4 knockout mice. In addition, in vivo studies in mice showed that cidofovir undergoes tubular secretion. None of the membrane vesicles expressing MRP2, MRP4 or BCRP exhibited ATP-dependent transport of cidofovir (Fig. 5), and there was no change in the kinetic parameters in Mrp4 knockout mice (Table 3). The luminal efflux mechanism of cidofovir remains unclear. Further studies are required to identify the transporters responsible for the efflux of adefovir and cidofovir across the BBM.

Accumulation of phosphorylated metabolites of adefovir is closely linked to nephrotoxicity, and it seems its dose limiting at maximal doses when used to treat hepatitis B. In vivo studies using mice, high concentrations of adefovir accumulated in the kidney, consistent with the tissue distribution of OAT1. Mono- and diphosphorylated metabolites accumulated in Mrp4 knockout mice in proportion to the increased accumulation of adefovir. In view of the expression of Mrp4 in the proximal tubules of the kidney and that adefovir causes nephrotoxicity by affecting the renal tubules, it is likely that Mrp4 plays an important role in protecting the kidneys from the renal toxicity of adefovir. Mrp4 function might be associated with the incidence of nephrotoxicity. Assem et al. (2004) have demonstrated that activation of the constitutive androgen receptor promotes up-regulation of Mrp4 in the kidney (Assem et al., 2004) and, recently, it was found that feeding mice ursodeoxycholate resulted in higher Mrp4 expression in the kidney due to an unknown mechanism (9-fold) (Zollner et al., 2006). In addition to inhibitors of uptake transporters, it is possible that ursodeoxycholate as well as constitutive androgen receptor agonists may reduce the risk of nephrotoxicity and contribute to an increase in the nephrotoxic dose for therapy.

In conclusion, using Mrp4 knockout mice and membrane vesicles expressing Mrp4, we have demonstrated the functional involvement of MRP4 in the urinary excretion of adefovir and tenofovir but not cidofovir. Changes in the adefovir-uptake-efflux equilibrium in proximal tubules may lead to nephrotoxicity seen in some adefovir-treated patients. Our findings provide new insights into the efflux mechanisms of drugs in the kidney.

Acknowledgments

We thank Dr. Michael J. M. Hitchcock (Gilead Sciences, Foster City, CA) for providing unlabeled adefovir and cidofovir. We also thank Drs. Kathleen M. Giacomini and Debbie W. Lin (University of California San Francisco, San Francisco, CA) for helpful discussions and advice and Dr. Yoshitane Nozaki (University of Tokyo, Tokyo, Japan) for helpful comments.

References


Address correspondence to: Dr. Yuichi Sugiyama, Department of Molecular Pharmaceutics, Graduate School of Pharmaceutical Sciences, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan. E-mail: sugiyama@mol.f.u-tokyo.ac.jp