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Functional involvement of multidrug resistance associated protein 4 (MRP4/ABCC4) in the renal elimination of the anti-viral drugs, adefovir and tenofovir

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Abbreviations:

MRP, multidrug resistance associated protein; OAT, organic anion transporter; BCRP, breast cancer resistance protein; HEK, human embryonic kidney; GFR, glomerular filtration rate; CL, clearance; GFP; green fluorescent fusion protein, BBM; brush border membrane

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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 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 BCRP. 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/gram tissue), respectively), and 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 di-phosphorylated 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.

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Introduction

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 like 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 di-phosphorylated metabolite is responsible for their antiviral activity (Balzarini et al., 1991; Eisenberg et al., 2001; Ho et al., 1992). 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 CMV therapy and high-dose adefovir HIV therapy (Kahn et al., 1999; Lalezari et al., 1997). Although tenofovir is an analog of adefovir (Fig.1), patients treated with tenofovir exhibit a lower incidence of renal dysfuction 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 multispecific organic anion and cation transport systems (Inui et al., 2000; Koepsell and Endou, 2004; Lee and Kim, 2004; Sekine et al., 2006; Sweet, 2005; Wright, 2005). 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, and, therefore, OAT1 inhibitors, such as nonsteroidal anti-inflammatory drugs (NSAIDS), 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 4 (MRP4/*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 homologue 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*-aminohippurate, urate, dehydroepiandrosterone sulfate, methotrexate and estradiol-17β-D-glucuronide as well as adefovir (Schuetz et al., 1999; van Aubel et al., 2002; Van Aubel et al., 2005; Zelcer et al., 2003). In addition, glutathione modulates the transport activity of MRP4, and the ATP-dependent uptake of taurocholate by MRP4 is stimulated by co-transport 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) have 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.

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Materials and Methods

Materials

[^{3}H]Adefovir (10 Ci/mmol), [^{3}H]cidofovir (9 Ci/mmol) and [^{3}H]tenofovir (10 Ci/mmol) were purchased from Moravek Biochemicals (Brea,CA). [^{14}C]Inulin (8 mCi/mmol) and [^{3}H]dehydroepiandrosterone sulfate (DHEAS) (74 Ci/mmol) were purchased from Perkin Elmer Life Science Products (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 (12-15weeks 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 reported in this manuscript 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) (Hirouchi et al., 2004; 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 48h after infection, and then the membrane vesicles were isolated from 1 to 2 × 10^{8} 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 1h on ice in the

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presence of 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin. The cell lysate was centrifuged at 100,000 *g* for 30 min at 4 °C, and the resulting pellet was suspended in 10 ml 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 280,000 g for 60 min at 4 °C. The turbid layer at the interface was collected, diluted to 23 ml with TS buffer, and centrifuged at 100,000 g for 30 min at 4 °C. The resulting pellet was suspended in 400 μl 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 transport medium (10 mM Tris-HCl, 250 mM sucrose, and 10 mM $MgCl_2$, 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 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 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 stop solution. The radioactivity retained on the filter was determined in a liquid scintillation counter (LS6000SE; Beckman Coulter, Inc., Fullerton, CA) after the addition of scintillation cocktail (Clear-sol I; Nacalai Tesque, Tokyo, Japan).

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In vivo infusion study in mice

Female BL6-129 and Mrp4 knockout mice weighing approximately 20 to 30 g were used for these experiments. Under pentobarbital anesthesia (30 mg/kg), the jugular vein was cannulated with a polyethylene catheter (PE-10) for the injection of [^{3}H]adefovir, [^{3}H]tenofovir and [^{3}H]cidofovir. The mice then received a constant infusion of adefovir, tenofovir and cidofovir at a dose of 124 pmol (1.24 μCi) /min/kg. Blood samples were collected from the jugular vein and urine was collected in preweighed test tubes at 30-min intervals throughout the experiment. To examine the GFR, [^{14}C]inulin (0.4 mg; 4.2 μCi/ min/kg) was infused via the jugular vein. Plasma was prepared by centrifugation of the blood samples (10,000 *g*, Microfuge; Beckman Coulter). The mice were 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 55 °C for 4 to 6 h after the addition of 2 ml Soluene-350 (PerkinElmer Life and Analytical Sciences) to dissolve the tissues. Radioactivity was determined in a liquid scintillation counter after the addition of scintillation cocktail. For further HPLC analysis, the kidney homogenate was treated with ice-cold methanol followed by centrifigation at 10,000 g. The supernatant was analyzed by HPLC.

HPLC analysis

Urine, serum and kidney extracts were analyzed by HPLC on an anion exchange Whatman Partisil 10 SAX column (10 μm, 4.6 mm×250 mm). To separate the metabolites of adefovir, a phosphate gradient was used from 100 % buffer A (7 mM $(NH_4)H_2PO_4$ adjusted to pH 3.8) to 100 % buffer B (0.25 M $(NH_4)H_2PO_4$ and 0.5 M KCl adjusted to pH 4.5) (Naesens et al., 1992). After equilibration of the Partisil 10 SAX column with 100 % buffer A, elution was performed at a flow rate of 1.5 ml/min with an isocratic phase of buffer A (6 min), followed by a linear gradient from buffer A to buffer B (20 min), and an isocratic elution with 100 % buffer B (25 min).

Pharamacokinetic analysis

Total plasma clearance (CL_{total}), renal clearance normalized by the circulating plasma

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concentration ($CL_{renal,plasma}$), and renal clearance normalized by the kidney concentration ($CL_{renal,kidney}$) were calculated from the equations

$$CL_{total} = I/C_{ss,plasma} \quad (1)$$

$$CL_{renal,plasma} = V_{urine}/C_{ss,plasma} \quad (2)$$

$$CL_{renal,\ kidney} = (V_{urine} - f_B\ GFR\ C_{ss,plasma})/C_{ss,kidney} \quad (3)$$

where I, $C_{ss,plasma}$, V_{urine}, $C_{ss,kidney}$ and GFR represent the infusion rate (μg/min/kg), plasma concentration at steady-state (ng/ml), urinary excretion rate at steady-state (μg/min/kg), kidney concentration at steady-state (ng/ml) and glomerular filtration rate (ml/min/kg), respectively. $C_{ss,plasma}$ was determined using the value of the plasma concentration at 60min. V_{urine} was determined as the mean value of the renal excretion rate of adefovir from 30 to 60 min and from 60 to 90 min. $C_{ss,kidney}$ was determined as the kidney concentration at the end of the *in vivo* experiment. To calculate $C_{ss,kidney}$, the specific gravity of the kidney was assumed to be unity. Thus, the amount in the kidney (ng/g kidney) can be regarded as the kidney concentration (ng/g), and the units of $CL_{renal,kidney}$ are ml/min/kg. GFR was determined simultaneously, and calculated from the urinary excretion rate of [^{14}C]inulin from 60 min to 90 min divided by the plasma concentration of [^{14}C]inulin at 90 min.

Quantitative PCR analysis

To quantify the expression of mRNA of Oats, Bcrp and Mrp2 mRNA in the kidney of wild-type and Mrp4 knockout mice (female BL6-129 and Mrp4 knockout mice weighing approximately 20 to 30 g), real-time quantitative PCR was performed using a LightCycler™ and the appropriate software (Version 3.53, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. cDNA used for the quantification was prepared as already described. PCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA). The protocol for PCR was as follows: 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s, 40 cycles. An external standard was generated by dilution of the target PCR product, which had been purified and had its concentration measured previously. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and gel electrophoresis. All gene expressions in each reaction

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were normalized by the expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Statistical differences were analyzed by using Student's t-test to identify significant differences between two sets of data and by one-way ANOVA with Bonferroni adjustment for multiple pairwise comparisons.

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Results

ATP-dependent transport of [^{3}H]adefovir and [^{3}H]tenofovir via human MRP4

Membrane vesicles were prepared from HEK293 cells either infected with recombinant adenovirus harboring hMRP4 or GFP. Plasma membrane vesicles were prepared and immunoblots revealed high levels of hMRP4. hMRP4 is known to transport DHEAS (Zelcer et al., 2003) and this compound was used as a positive control. The uptake of DHEAS by hMRP4 after a 5 min incubation 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 [^{3}H]adefovir and [^{3}H]tenofovir by hMRP4- expressing membrane vesicles is shown in Fig.2A and 2D. The uptake of [^{3}H]adefovir and [^{3}H]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.2B and 2E. 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.2C and 2F). DHEAS inhibited the ATP-dependent uptake of [^{3}H]adefovir and [^{3}H]tenofovir by MRP4 expressing membrane vesicles in a concentration-dependent manner.

Osmotic sensitivity of the uptake of [^{3}H]adefovir into membrane vesicles expressing hMRP4

Osmotic sensitivity was studied by examining the uptake of [^{3}H]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 binding to the vesicle surface. As shown in Fig.3, the uptake of [^{3}H]adefovir was reduced as the sucrose concentration medium increased. The y-intercept for the relationship between the amount of [^{3}H]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.

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Uptake of [^{3}H]adefovir and[^{3}H] cidofovir via hBCRP- and hMRP2-expressing vesicles

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

Renal excretion of adefovir, tenofovir and cidofovir in Mrp4 knockout and wild-type mice.

[^{3}H]adefovir, [^{3}H]tenofovir and [^{3}H]cidofovir were administered by constant intravenous infusion. The plasma concentration of adefovir was higher in Mrp4 knockout mice than in wild-type mice, while the urinary excretion rate was similar for the three drugs (Fig.7B, 7E and 7H). Compared with the wild-type mice, the kidney concentration of adefovir and tenofovir was almost 2-fold greater in Mrp4 knockout mice (Fig.7C and 7F), which probably reflects a reduced ability to remove adefovir and tenofovir. The pharmacokinetic parameters of adefovir, tenofovir and cidofovir are summarized in Tables1-3. $CL_{renal,plasma}$, the renal clearance with respect to the concentration in the plasma, was reduced in Mrp4 knockout mice only in the case of adefovir. $CL_{renal,\ kidney}$, the tubular secretion clearance with respect to the concentration in the kidney, of adefovir and tenofovir was significantly reduced in Mrp4 knockout mice (Tables1 and 2). There was no significant change in the pharmacokinetic parameters of cidofovir between wild-type and Mrp4 knockout mice (Fig.7G- 7I, Table 3).

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Adefovir metabolites in the kidney

Mono- and di-phosphorylated adefovir (adefovirp and adefovirpp, respectively) levels were determined in the kidney of wild-type and Mrp4 knockout mice after a 90 min infusion of [^{3}H]adefovir (Fig.8). Only intact adefovir was detected in blood and urine within 100 min (retention time;15 min) (data not shown), while, in the kidney, intact adefovir, adefovirp and adefovirpp were detected in both wild-type (retention times: adefovir, 15min, adefovirp, 28min adefovirpp, 54min) and Mrp4 knockout mice (retention times;adefovir, 14min, adefovirp 28 min and adefovirpp 55 min, respectively). The ratio of adefovirp and adefovirpp to intact adefovir was unchanged between wild-type mice and Mrp4 knockout mice (30.0 and 1.6%, and 30.1 and 1.1%). These studies indicate that the metabolism of adefovir was unchanged in the Mrp4- deficient animals and that the overall increase in adefovir is consistent with the previous findings that indicate adefovir is an Mrp4 substrate and that phosphorylated forms are not MRP4 substrates (Reid et al., 2003; Schuetz et al., 1999).

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.

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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.2A and 2D). Consistent with the previous inhibition study in which adefovir had a minimal effect on the ATP-dependent uptake of [^{3}H]estradiol-17β-D-glucuronide and [^{3}H]cGMP by MRP4 at 1 mM (Reid et al., 2003 ; van Aubel et al., 2002), the ATP-dependent uptake of adefovir was not saturated at the maximum concentration examined (Fig2B). Inhibition by DHEAS (Fig. 2C), and osmotic sensitivity (Fig. 3) indicated that 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 will be quite high (> 1mM) (Fig.2B). This is also supported by an inhibition study in whichi adefovir (1 mM) 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 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, while 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

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Mrp4 knockout mice which have kidneys which 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 (Table 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 than the values in wild-type mice (Fig.7). Taking these parameters into consideration, the intrinsic clearance for the luminal efflux of adefovir and tenofovir in Mrp4 knockout mice was 37, and 46 %, respectively, of the corresponding control values. It should be noted that these values were calculated assuming negligible reabsorption of adefovir and 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 $K_{p,\ kidney}$ of tenofovir due to impairment of Mrp4 was greater than that of adefovir, it appears that MRP4 makes a greater contribution to the net efflux 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, while 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

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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 di-phosphorylated metabolites in the kidney, while 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 phosphylated metabolites in the kidney remains unknown. 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_p values of adefovir in the liver and brain (Table1) and, 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 kidney proximal tubules. In addition to MRP4, two ABC transporters, MRP2 and BCRP, have been identified on the BBM (Jonker et al., 2002; Schaub et al., 1997). In the membrane vesicle study, neither hMRP2 nor hBCRP accepted adefovir as a substrate (Fig4). Consistent with the *in vitro* study, there was no significant difference in the urinary excretion of adefovir between hereditarily Mrp2 deficient mutant rats (eisai hyperbilirubinemic rat; EHBR) and normal rats (data not shown). Therefore, the luminal efflux of adefovir remaining in Mrp4 knockout mice is not accounted for by

these ABC transporters. 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 appears that it is dose-limiting at maximal doses when used to treat hepatitis B. In *in vivo* studies using mice, high concentrations of adefovir accumulated in the kidney, consistent with the tissue distribution of OAT1. Mono- and di-phosphorylated 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 the fact 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 have demonstrated that activation of the constitutive androsterone receptor (CAR) produces 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., 2005). In addition to inhibitors of uptake transporters, it is possible that ursodeoxycholate as well as CAR agonists may reduce the risk of nephrotoxicity, and contribute to an increase in the maximum 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.

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Footnotes

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Legends for figures

Figure 1 Structures of adefovir, tenofovir and cidofovir

Figure 2 Time-profiles, concentration-dependence and inhibitory effect of DHEAS on the uptake of [^{3}H]adefovir and [^{3}H]tenofovir by human MRP4-expressing membrane vesicles.

A and D. The uptake of [^{3}H]adefovir (A, 0.1 μM) and [^{3}H]tenofovir (D, 0.1 μM) by membrane vesicles expressing MRP4 was examined at 37 °C in medium containing 5 mM ATP (closed symbols) or AMP (open symbols). Circles and triangles represent the uptake in MRP4 and GFP- expressing membrane vesicles, respectively. B and E. The concentration-dependence of MRP4-mediated transport of [^{3}H]adefovir (B) and [^{3}H]tenofovir (E) was determined for 5 min. C and F. The inihibitory effect of DHEAS on the MRP4-mediated transport of 0.1 μM [^{3}H]adefovir (C) and [^{3}H]tenofovir (F) was investigated for 5 min. Each point represents the mean ± S.E. (n=3). **, P<0.01 statistically significantly different from control.

Figure 3 Osmotic sensitivity of [^{3}H]adefovir uptake into human MRP4-expressing membrane vesicles.

Membrane vesicles expressing MRP4 were preincubated at 4 °C for 60 min in increasing concentrations of sucrose (0.25 M, 0.33 M, 0.50 M, 0.66 M and 1.0 M) in 10 μl incubation medium containing 10mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, followed by incubation at 37 °C for 10 min in increasing concentrations of sucrose in 20 μl incubation medium containing 10mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and [^{3}H]adefovir (0.1 μM) in the presence (closed symbols) or absence (open symbols) of ATP. Each point represents the mean ± S.E. (n=3).

Figure 4 Uptake of [^{3}H]adefovir by hBCRP- and hMRP2- expressing membrane vesicles

The uptake of 0.1 μM [^{3}H]adefovir into membrane vesicles expressing hBCRP(A), hMRP2 (C) and GFP was examined at 37 °C for 5 min in medium containing 5 mM ATP or AMP. The uptake of 0.1 μM [^{3}H]estrone sulfate (B) and 0.1 μM [^{3}H]estradiol-17β-D-glucuronide (D) into membrane vesicles

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expressing hBCRP (B), hMRP2 (D) and GFP was determined at 37 °C for 5 min in medium containing 5 mM ATP or AMP. Each point represents the mean ± S.E. (n=3). **: $P<0.01$ statistically significantly difference.

Figure 5 Uptake of [^{3}H]cidofovir by hMRP4-, hBCRP- and hMRP2- expressing membrane vesicles

The uptake of 0.1 μM [^{3}H]cidofovir into membrane vesicles expressing hMRP4 (A), hBCRP (B) and hMRP2 (C) was examined at 37 °C for 5 min in medium containing 5 mM ATP or AMP. Each point represents the mean ± S.E. (n=3).

Figure 6 Expression level of mRNA of various transporters in the kidney of wild-type and Mrp4 knockout mice

Expression of mRNA of various transporters in the kidney of wild-type (open columns) and Mrp4 knockout mice (closed columns) was determined using real-time quantitative PCR. Each point represents the mean ± S.E. (n=3).

Figure 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.

Figure 8 Characterization of the metabolites of adefovir in the kidney

At 90min 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 45min to 65min.

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Table 1 Pharmacokinetic parameters of adefovir during constant infusion into wild-type and Mrp4 knockout mice. Data are taken from Figs.7A, 7B and 7C. Data represent the mean± S.E. (n=3)

	CL_{total}	$CL_{renal,plasma}$	$CL_{renal,kidney}$	GFR	$K_{p,kidney}$	$K_{p,liver}$	$K_{p,brain}$
	ml/min/kg	*ml/min/kg*	*ml/min/kg*	*ml/min/kg*			
Wild type	**24.7±1.2**	**23.7±1.6**	**0.86±0.04**	**12.9±0.9**	**13.1±0.7**	**0.82±0.08**	**0.07±0.02**
Mrp4(-/-)	**18.8±0.3 ****	**16.7±0.2 ***	**0.32±0.03 ****	**10.8±0.3**	**19.5±1.3 ***	**0.98±0.20**	**0.09±0.04**

**P*<0.05; ***P*<0.01 statistically significant difference.

CL_{total}, total clearance; $CL_{renal,plasma}$, renal clearance with respect to circulating plasma. $CL_{renal,kidney}$:renal clearance with respect to the kidney concentration, GFR: glomerular filtration rate, $K_{p,kidney}$,; K_p value obtained by dividing the kidney concentration by $C_{ss,plasma}$ (at 90min)

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Table 2 Pharmacokinetic parameters of tenofovir during constant infusion into wild-type and Mrp4 knockout mice. Data are taken from Figs. 7D, 7E and 7F. Data represent the mean± S.E. (n=3)

	CL_{total}	$CL_{renal,plasma}$	$CL_{renal,kidney}$	GFR	$K_{p,kidney}$	$K_{p,liver}$	$K_{p,brain}$
	ml/min/kg	*ml/min/kg*	*ml/min/kg*	*ml/min/kg*			
Wild type	**28.0±2.2**	**24.9±0.7**	**0.860±0.073**	**14.5±1.2**	**12.7±0.3**	**1.17±0.16**	**0.0146±0.0029**
Mrp4(-/-)	**28.1±2.0**	**26.0±1.3**	**0.394±0.079 ***	**14.8±2.1**	**29.5±5.5 ***	**1.31±0.15**	**0.0191±0.0110**

*P<0.05 statistically significant difference **.**

CL_{total}, total clearance; $CL_{renal,plasma}$, renal clearance with respect to circulating plasma.$CL_{renal,kidney}$:renal clearance with respect to the kidney concentration, GFR: glomerular filtration rate, $K_{p,kidney}$,; Kp value obtained by dividing the kidney concentration by $C_{ss,plasma}$ (at 90min)

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Table 3 Pharmacokinetic parameters of cidofovir during constant infusion into wild-type and Mrp4 knockout mice. Data are taken from Figs. 7G, 7H and 7I. Data represent the mean± S.E. (n=3)

	CL_{total}	$CL_{renal,plasma}$	$CL_{renal,kidney}$	GFR	$K_{p,kidney}$	$K_{p,liver}$	$K_{p,brain}$
	ml/min/kg	*ml/min/kg*	*ml/min/kg*	*ml/min/kg*			
Wild type	**34.4±6.4**	**26.1±5.7**	**0.53±0.16**	**13.8±2.5**	**22.2±1.7**	**0.31±0.03**	**0.045±0.006**
Mrp4(-/-)	**42.7±0.9**	**29.6±0.5**	**0.60±0.08**	**12.6±0.4**	**27.3±5.8**	**0.34±0.04**	**0.056±0.001**

CL_{total}, total clearance; $CL_{renal,plasma}$, renal clearance with respect to circulating plasma.$CL_{renal,kidney}$:renal clearance with respect to the kidney concentration, GFR: glomerular filtration rate, $K_{p,kidney}$,; K_p value obtained by dividing the kidney concentration by $C_{ss,plasma}$ (at 90min)

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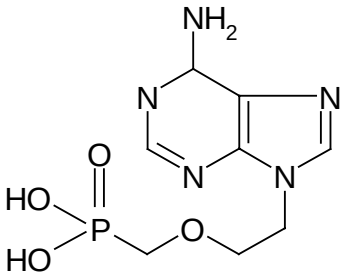
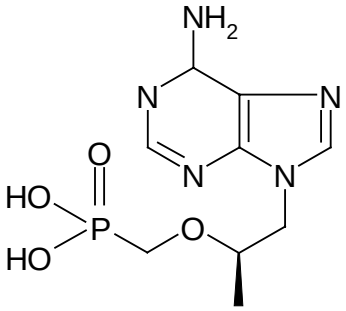
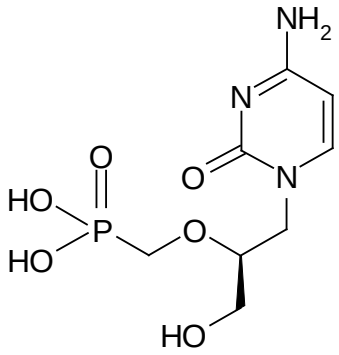
Figure1



Adefovir



Tenofovir



Cidofovir

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Figure2

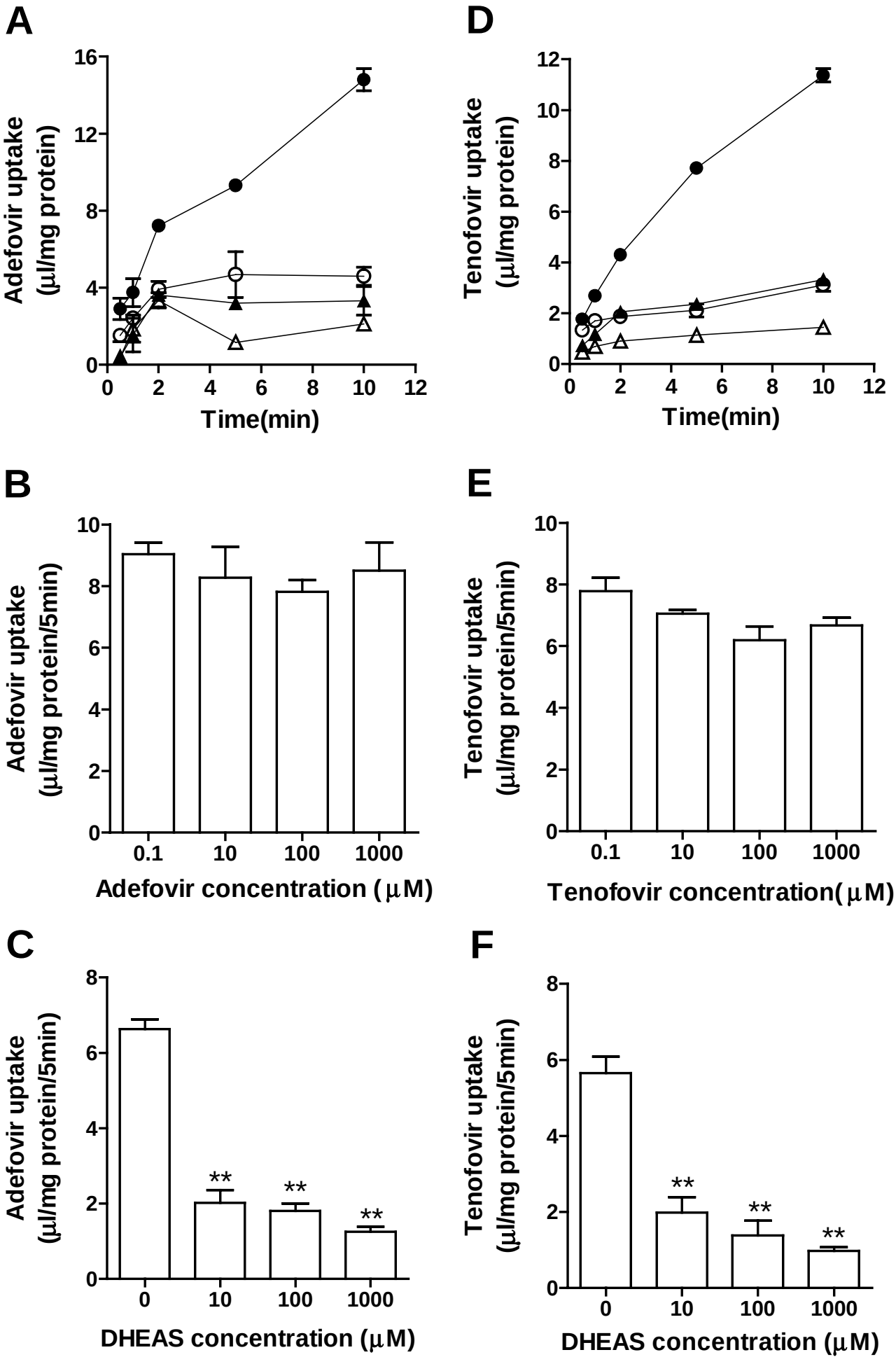

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Figure 3

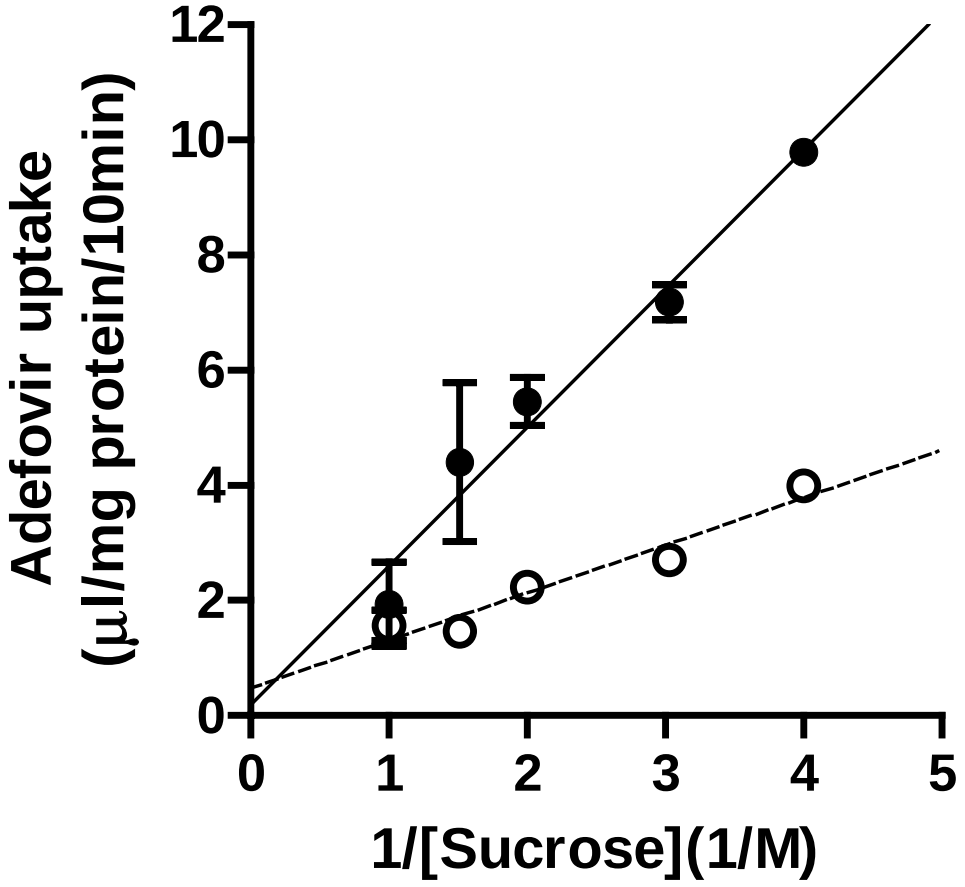

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Figure 4

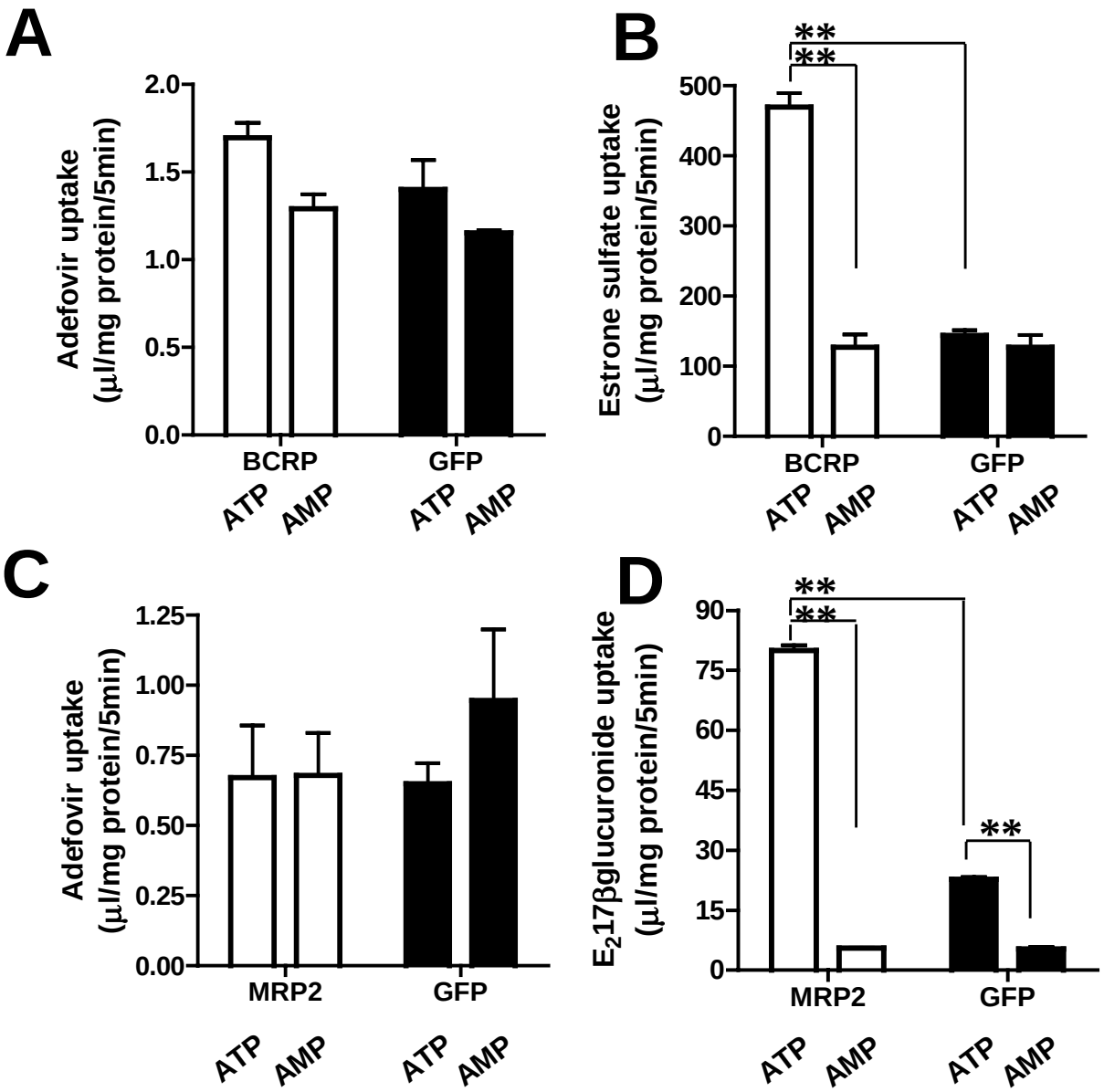

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Figure 5

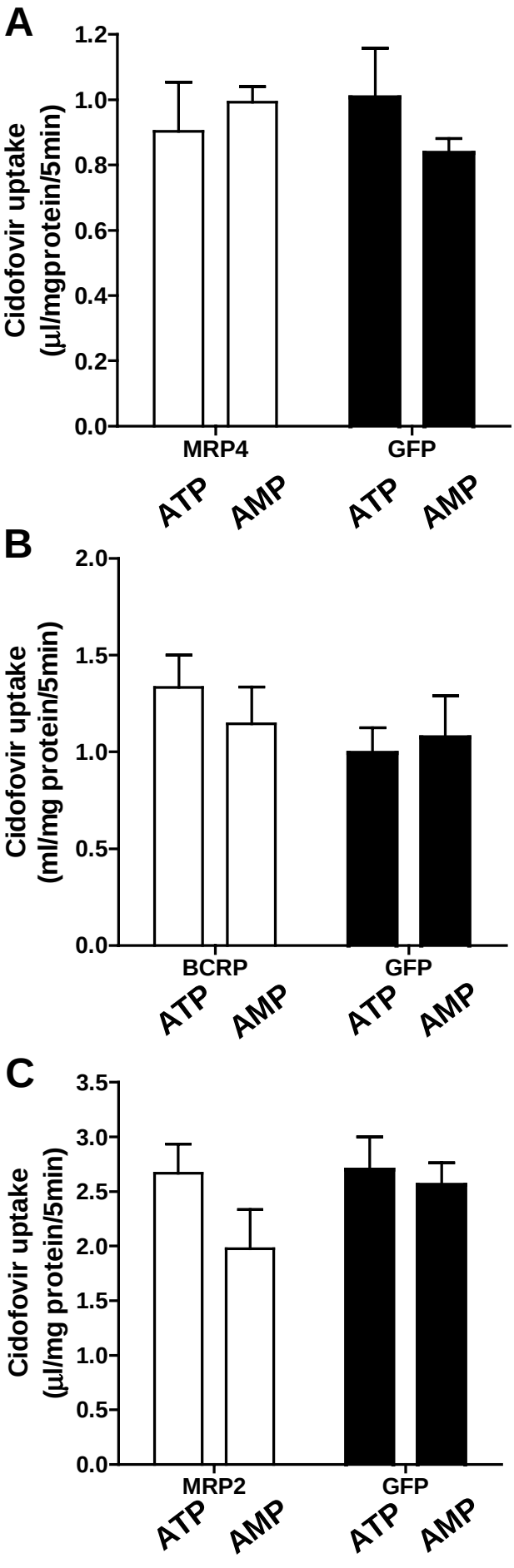

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Figure 6

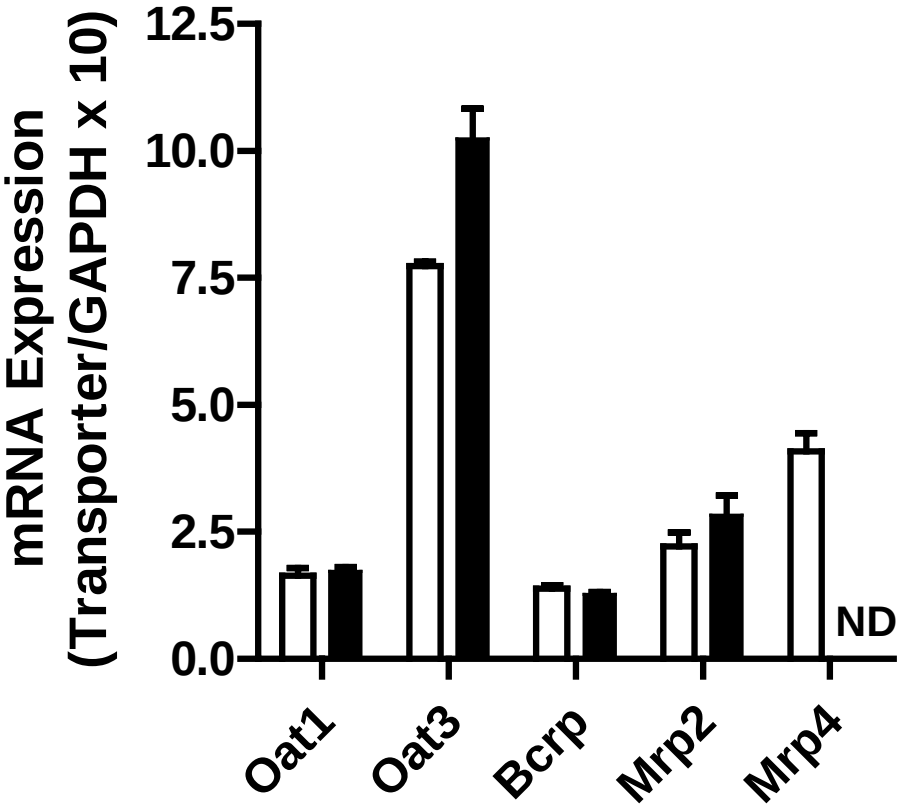

Figure 7

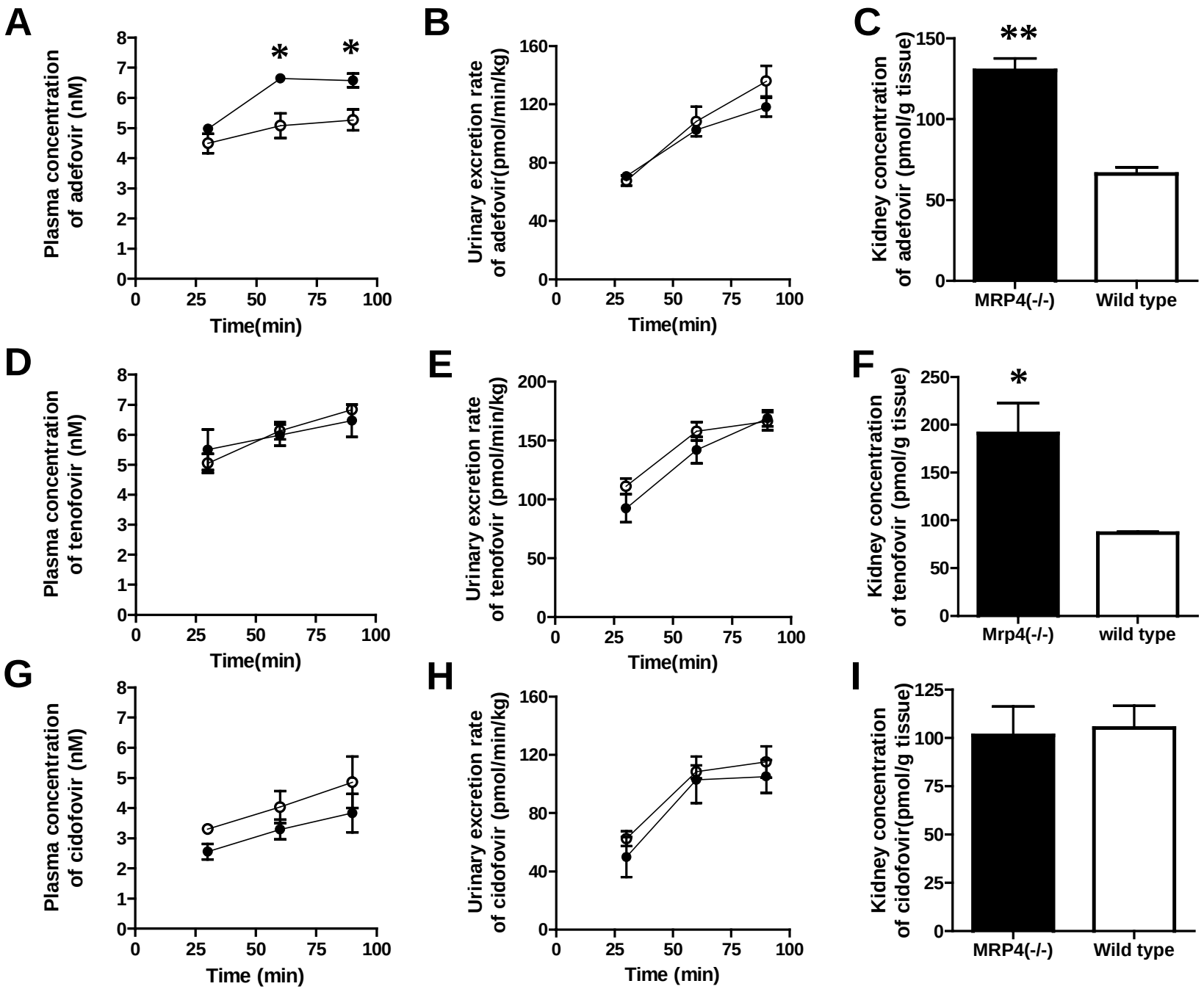

Figure 8