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DIRECTING ROLE OF ORGANIC ANION TRANSPORTERS IN THE EXCRETION OF MERCAPTURIC ACIDS OF ALKYLATED POLYCYCLIC AROMATIC HYDROCARBONS

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Running Title: EXCRETION OF BENZYLIC MERCAPTURIC ACIDS OF POLYCYCLICS

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ABBREVIATIONS: DMPMA, benzylic mercapturic acid of 1,8-dimethylpyrene; DMSO, dimethylsulfoxide; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hOAT, human organic anion transporter(s); MPMA, benzylic mercapturic acid of 1-methylpyrene; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; OAT, organic anion transporter(s); PAH, polycyclic aromatic hydrocarbon(s); UPLC, ultra performance liquid chromatography.

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ABSTRACT

Excretion of mercapturic acids of a xenobiotic is a good indicator for the formation of electrophilic intermediates. However, the route of excretion, urine or feces, is important for usage of a given mercapturic acid as a biomarker in humans. In the present study we investigated the excretion routes of 1-methylpyrenyl mercapturic acid (MPMA) and 1,8-dimethylpyrenyl mercapturic acid (DMPMA) formed from the corresponding benzylic alcohols in rats. While MPMA was primarily excreted in urine (72% of the total urinary and fecal level), DMPMA clearly preferred the fecal route (88%). We then examined interactions of these mercapturic acids with renal basolateral organic anion transporters (OAT) using HEK293 cells stably expressing human OAT1 and OAT3. The uptake rates of MPMA by OAT1- and OAT3-expressing cells were 2.8- and 1.7-fold, respectively, higher than that by control cells. MPMA was a competitive inhibitor of *p*-aminohippurate uptake by OAT1 and estrone sulfate uptake by OAT3 with K_i values of 14.5 μ M and 1.5 μ M, respectively. In contrast, DMPMA was not transported by OAT1 and only modestly transported by OAT3 (1.25-fold over control). Thus, we suspect that the substrate specificities, alone or together with other factors, played a directing role in the excretion of MPMA and DMPMA. While the mechanistic link requires verification, our results clearly show that a minute structural difference (the presence or absence of an additional methyl group in an alkylated four-ring polycyclic hydrocarbon) can strongly affect the interaction with transporter proteins and direct the excretion route of mercapturic acids.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are common environmental pollutants and include potent carcinogens, which are activated to reactive metabolites that form DNA adducts and induce mutations (Luch, 2005). While epoxidation is a major mechanism for the activation of many PAH, an additional pathway – formation of reactive benzylic sulfuric acid esters – has been observed for alkylated PAH (Surh and Miller, 1994; Glatt, 2005).

Mono- and dimethylated pyrenes are abundant in the environment. Thus, 1-methylpyrene was detected in cigarette smoke condensate, cellulose pyrolysates, car exhausts, used motor oils, sea sediments, smoked cheese, olive oils, oysters, crabs and finfish (Glatt et al., 2007). It showed carcinogenic activity in animal models (Rice et al., 1987). The activation mechanism of 1-methylpyrene involves side chain hydroxylation and subsequent sulfation to a highly reactive ester (Engst et al., 1999; Glatt, 2000). Likewise, 1,8-dimethylpyrene, another environmental contaminant, forms benzylic DNA adducts in rats, and is converted to 1-hydroxymethyl-8-methylpyrene and 1-sulfooxymethyl-8-methylpyrene by rat and human enzymes *in vitro*; 1-sulfooxymethyl-8-methylpyrene showed higher mutagenic activity in bacteria and mammalian cells than 1-sulfooxymethylpyrene (Donath C, Batke M, Seidel A and Glatt HR, manuscript in preparation).

Benzylic sulfuric acid esters as well as other electrophilic compounds can be conjugated with glutathione and then converted into mercapturic acids (*N*-acetyl-L-cysteine *S*-conjugates). In a previous study, 1-methylpyrenyl mercapturic acid (MPMA, structural formula in Fig. 1) was identified in urine, feces and plasma of rats treated with 1-hydroxymethylpyrene or 1-sulfooxymethylpyrene (Ma et al., 2000). Furthermore, excretion of MPMA correlated with the levels of DNA adducts found in tissues of the treated animals (Ma et al., 2002). Thus, mercapturic acids might be useful as non-invasive biomarkers for demonstrating the uptake and metabolic activation of alkylated PAH in humans. Urinary mercapturic acids have already been used in the monitoring of the occupational exposure to

various other compounds (Commandeur et al., 1995; Haufroid and Lison, 2005). However, metabolites of xenobiotics, especially of large molecules, may not only be excreted in urine, but also in feces, whose collection and analysis is not feasible in large-scale biomonitoring. Therefore, information on the routes and molecular mechanisms of excretion is important in the development of new urinary biomarkers.

Previous studies in rats and mice *in vivo* (Inoue et al., 1981; Inoue et al., 1982) as well as with isolated renal cortical tubules and slices (Inoue et al., 1984; Lock et al., 1986) showed that various mercapturic acids are actively absorbed by renal proximal tubule cells from the blood and then excreted into the lumen. Renal uptake of mercapturic acids was inhibited by probenecid in a similar mode as seen for *p*-aminohippurate, a prototypical substrate of the renal organic anion transport system (Stevens and Jones, 1989). The basolateral organic anion transporters OAT1 and OAT3 mediate the uptake from blood into renal proximal tubule cells, which is the rate-limiting step in this excretion system (Burckhardt and Burckhardt, 2003; Wright and Dantzler, 2004). Genes of both transporters were cloned for several species, including rat and man, and are highly conserved across species (Sekine et al., 2000; van Aabel et al., 2000). Both transporters exchange their substrates for intracellular α -ketoglutarate. They show overlapping substrate tolerance for a wide range of structurally diverse organic anions, including dicarboxylates, prostaglandins, cyclic nucleotides, drugs and drug metabolites (Burckhardt and Burckhardt, 2003). Moreover, they are involved in the renal accumulation of potentially toxic compounds, such as uremic toxins, mercuric species and ochratoxin A (Sweet, 2005) as well as reactive sulfuric acid esters (Bakhiya et al., 2006). Recently, it has been shown that several mercapturic acids, such as benzyl, allyl and dichlorovinyl mercapturic acids, are substrates for human (h) OAT1 (Pombrio et al., 2001). OAT3 has not yet been studied for transport of mercapturic acids, and large mercapturic acids, such as those of alkylated PAH, have not been investigated with either transporter.

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We have studied the routes of the excretion of MPMA and DMPMA in rats, and examined the involvement of human OAT1 and OAT3 in the excretion of these mercapturic acids using human embryonic kidney cells (HEK293) stably expressing human OAT1 and OAT3 as model systems.

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Methods

Radiochemicals. [6,7-³H(N)]estrone sulfate (57.3 Ci/mmol) and [glycyl-2-³H]*p*-aminohippurate (4.18 Ci/mmol) were purchased from Perkin-Elmer (Boston, USA). The purity of the radiochemicals was > 97%. The specific radioactivity of *p*-aminohippurate was reduced to 0.5 Ci/mmol by adding unlabeled compound.

Analytical Procedures Used in the Syntheses. Silica gel 60 (0.063-0.200 μ m, Merck) was used for column chromatography. UV spectra were recorded on a Shimadzu UV-1601 spectrophotometer. NMR spectra were obtained on a Bruker AMX400 or a Bruker DRX500 spectrometer in either CDCl₃ or DMSO-d₆. Melting points were determined on a Büchi 510 Melting Point.

Syntheses of Benzylic Alcohols. 1-Hydroxymethylpyrene was synthesized as described previously (Glatt et al., 1993). 1-Hydroxymethyl-8-methylpyrene was prepared from 1,8-diacetylpyrene (Harvey et al., 1984) via 1,8-pyrenedicarboxylic acid, 1,8-dimethylpyrene and 1-formyl-8-methylpyrene as follows:

Bromine (18 mmol) was added dropwise to a stirred solution of NaOH (65 mmol) in 12 ml of water at 0°C. The solution was heated to 35°C, and a solution of 1,8-diacetylpyrene (1.7 mmol) in 45 ml of dioxane was added dropwise. Excess bromine was destroyed by addition of NaHSO₃ (40% solution in H₂O), the mixture then diluted with 100 ml of water, and the dioxane distilled off. The yellow precipitate was dissolved in 1000 ml of 5% NaOH in water. The solution was filtrated and acidified with concentrated hydrochloric acid. The precipitated product was filtrated, washed with water, and dried. Crystallization from chlorobenzene gave 0.44 g (87% yield) of pale yellow crystals, 1,8-pyrenedicarboxylic acid, with the following properties: melting point > 280°C; UV (CH₃OH) λ_{\max} (nm) (ϵ [cm² mmol⁻¹]) 203 (32,800) 245 (37,300) 286 (25,000) 357 (22,600); ¹H-NMR (400 MHz, DMSO-d₆) δ [ppm] 9.52 (s, 2, H_{9,10}), 8.86 (d, 2, H_{2,7}, J_{2,3} = 8.0 Hz, J_{6,7} = 8.0 Hz) 8.66 (d, 2, H_{3,6}), 8.59 (s, 2, H_{4,5}).

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A solution of 1,8-pyrenedicarboxylic acid (14 mmol) and trichlorosilane (0.30 mol) in 40 ml of acetonitrile was refluxed for 1 h. After cooling in an ice bath, tri-*n*-propylamine (126 mmol) was added so that the temperature did not exceed 15°C. After the mixture had been refluxed for 16 h, it was diluted with 400 ml diethyl ether and filtrated. The filtrate was concentrated under vacuum, whereupon 70 ml of methanol was added and the solution was refluxed for 1 h. Subsequently, potassium hydroxide (1.0 mol), dissolved in a mixture of methanol (95 ml) and water (25 ml), was slowly added. The resulting suspension was refluxed for 20 h and then diluted with 600 ml of water and extracted with dichloromethane. The organic phase was washed with 50 ml of hydrochloric acid (2 N). Removal of the solvent gave a crude product, which was chromatographed on silica gel using dichloromethane to provide 1.75 g (55% yield) of yellow crystals, 1,8-dimethylpyrene acid, with the following properties: melting point 128-129°C; UV (CH₃OH) λ_{\max} (nm) (ϵ [cm² mmol⁻¹]) 243 (56,300) 268 (24,500) 279 (44,800) 294 (3,100) 319 (11,300) 334 (25,800) 351 (34,500) 380 (600); ¹H-NMR (400 MHz, CDCl₃) δ [ppm] 8.16 (s, 2, H_{9,10}), 7.95 (d, 2, H_{3,6}, J_{2,3} = 7.7 Hz, J_{6,7} = 7.7 Hz), 7.84 (s, 2, H_{4,5}) 7.74 (d, 2, H_{2,7}, J_{2,3} = 7.7 Hz, J_{6,7} = 7.7 Hz), 2.87 (s, 6, (-CH₃)₂).

A suspension of 1,8-dimethylpyrene (4.4 mmol) and iodoxybenzoic acid (4.3 mmol) in 15 ml of dimethylsulfoxide (DMSO) was heated at 90°C for 18 h. The crude product was precipitated by adding 50 ml of water. The precipitate was collected, washed with water and dried. Chromatography on silica gel using dichloromethane as solvent gave 0.3 g (28% yield) of orange crystals, 1-formyl-8-methylpyrene, with the following properties: melting point 129-130°C; UV (CH₃OH) λ_{\max} (nm) (ϵ [cm² mmol⁻¹]) 204 (28,500) 235 (33,600) 280 (18,300) 291 (24,700) 367 (17,000) 403 (17,400); ¹H-NMR (400 MHz, CDCl₃) δ [ppm] 10.61 (s, 1, -CHO), 9.28 (d, 1, H₁₀, J_{9,10} = 9.6 Hz), 8.33 (d, 1, H₉) 8.26 (d, 1, H₂, J_{2,3} = 7.9 Hz), 8.06 (d, 1, H₃), 8.03 (d, 1, H₆, J_{6,7} = 7.7 Hz), 7.94 (AB-sys., 2, H_{4,5}, J_{4,5} = 8.9 Hz), 7.78 (d, 1, H₇), 2.88 (s, 3, -CH₃).

A suspension of 1-formyl-8-methylpyrene (1.23 mmol) and sodium borohydride (13 mmol) in 40 ml of ethanol was stirred at room temperature for 18 h. The mixture was diluted with 100 ml of water, the ethanol distilled off, and the separated product filtrated, washed with water and dried. Chromatography on silica gel using a mixture of dichloromethane and methanol (19:1, v/v) provided 0.171 g (57% yield) of white crystals, 1-hydroxymethyl-8-methylpyrene, with the following properties: melting point 160-161°C; UV (CH₃OH) λ_{\max} (nm) (ϵ [cm² mmol⁻¹] 243 (60,500) 268 (23,900) 279 (44,400) 318 (11,100) 333 (25,700) 349 (35,800) 378 (1,200); ¹H-NMR (400 MHz, DMSO-d₆) δ [ppm] 8.31 (d, 1, H₁₀, J_{9,10} = 9.5 Hz), 8.23 (d, 1, H₉), 8.13 (d, 1, H₂, J_{2,3} = 7.8 Hz), 8.07 (d, 1, H₆, J_{6,7} = 7.6 Hz) 8.01 (d, 1, H₃), 7.97 (s, 2, H_{4,5}), 7.83 (d, 1, H₇), 2.84 (s, 3, -CH₃).

The purity of 1-hydroxymethylpyrene and 1-hydroxymethyl-8-methylpyrene was >98%, as determined by HPLC with fluorescence detection. They were dissolved in DMSO within 3 h before they were administered to animals.

Syntheses of Mercapturic Acids. MPMA was prepared from 1-chloromethylpyrene (Glatt et al., 1993). *N*-Acetyl-L-cysteine (3.0 mmol) in 5 ml of methanol was added to a solution of sodium methoxide (6.0 mmol) in 5 ml of methanol. After stirring for 10 min at room temperature, a solution of 1-chloromethylpyrene (3.0 mmol) in 5 ml of tetrahydrofuran was added. After stirring at room temperature for 3 d, the mixture was concentrated. Chromatography on silica gel using methanol and dichloromethane (1:4, v/v) as eluent gave 0.51 g (45% yield) pale yellow crystals, MPMA, with the following properties: melting point 168-170°C (decomposition); UV (CH₃OH) λ_{\max} (nm) (ϵ [cm² mmol⁻¹] 243 (44,500) 267 (18,900) 277 (30,200) 315 (10,200) 329 (23,900) 346 (34,400); ¹H-NMR (400 MHz, DMSO-d₆) δ [ppm] 8.67 (d, 1, H₁₀, J_{9,10} = 9.2 Hz), 8.51 (d, 2, H_{6,8}, J_{6,7} = J_{7,8} = 7.7 Hz), 8.41-8.51 (m, 2, H_{3,9}), 8.36 (AB-sys., 2, H_{4,5}, J_{4,5} = 5.5 Hz), 8.25-8.31 (m, 2, H_{2,7}), 7.99-8.05 (m, 1, -NH), 4.67-4.77 (m, 2, benzylic CH₂), 4.55-4.62 (m, 1, -CH_{cyst.}), 3.26-3.30 (m, 1, CH_{2cyst.}), 3.02-3.08 (m, 1, CH_{2cyst.}), 2.13 (s, 3, -CH_{3cyst.}).

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DMPMA was prepared from 1-hydroxymethyl-8-methylpyrene. A solution of 1-hydroxymethyl-8-methylpyrene (0.30 mmol) in CH_2Cl_2 (18 ml) was added to a stirred solution of *N*-acetyl-L-cysteine (0.30 mmol) in a mixture of trifluoroacetic acid (0.3 ml) and dichloromethane (1.8 ml) dropwise over a period of 1 h at 0°C. Stirring was continued for 30 min at room temperature. Subsequently, methanol (1.0 ml) and water (4.0 ml) were added, the mixture was extracted with chloroform (5 x 10 ml), and the solvent was removed under vacuum. Purification by chromatography on silica gel 60 RP-18 using a mixture of CH_3OH and water (9:1, v/v) gave 14.3 mg (12% yield) of white crystals, DMPMA, with the following properties: melting point 205-206°C (decomposition); UV (CH_3OH) λ_{max} (nm) (ϵ [$\text{cm}^2 \text{mmol}^{-1}$]) 245 (44,800) 270 (19,300) 281 (31,000) 323 (10,600) 337 (23,800) 354 (33,000) 380 (1,700); ^1H -NMR (400 MHz, $\text{DMSO}-d_6$) δ [ppm] 8.65 (d, 1, H_{10} , $J_{9,10} = 9.5$ Hz), 8.53 (d, 1, H_9), 8.50 (d, 1, -NH), 8.37 (d, 2, $\text{H}_{3,6}$, $J_{2,3} = 7.8$ Hz, $J_{6,7} = 7.8$ Hz) 8.26 (AB-sys., 2, $\text{H}_{4,5}$, $J_{4,5} = 8.9$ Hz), 8.18 (d, 1, H_2), 8.13 (d, 1, H_7), 4.71-4.73 (m, 3, benzylic CH_2 , $-\text{CH}_{\text{cyst.}}$), 3.14 (s, 3, aryl- CH_3), 3.10-3.13 (m, 1, $\text{CH}_{2\text{cyst.}}$), 2.92-2.97 (m, 1, $\text{CH}_{2\text{cyst.}}$), 2.13 (s, 3, $-\text{CH}_{3\text{cyst.}}$).

The purity of MPMA and DMPMA was >97%, as determined by analytical reversed phase HPLC and ^1H -NMR. Stock solutions in DMSO were stored at -20°C.

Treatment of Animals. Male rats [Wistar (Han), 175-200 g] were purchased from Charles River Laboratories (Sulzfeld, Germany) and were acclimatized for one week prior to treatment. 1-Hydroxymethylpyrene or 1-hydroxymethyl-8-methylpyrene (83.3 $\mu\text{mol/kg}$ body weight) was administered intraperitoneally, using DMSO (0.5 ml/kg body weight) for delivery. Control animals only received DMSO. Animals were kept individually in metabolic cages 48 h before and 24 h after administration of the test compound. Urine and feces were stored at -80°C until analysis.

Sample Preparation. Urine was adjusted to pH 1.6 with citrate buffer (20 mM, pH 2) and HCl (1 M). Aliquots (1 ml) were passed through a Chromabond C18 ec column [1 ml, 100 mg, {Macherey-Nagel, Düren, Germany}], conditioned with 1 ml citrate buffer. The

mercapturic acid was eluted with 1 ml ethyl acetate. The eluate was brought to near dryness in a Savant SpeedVac (ThermoQuest, Egelsbach, Germany) after adding 10 μ l of DMSO. The residue was dissolved in 90 μ l methanol and analyzed by UPLC-MS/MS as described below. Urine from animals treated only with DMSO was used as a negative control. It was spiked with MPMA and DMPMA (50 pmol/ml), respectively, to create a standard and study the rate of recovery.

Feces was homogenized with an Ultraturrax after adding 3 ml of water per g of feces. An aliquot (equivalent to 0.25 g feces) was acidified with 1 M HCl (75 μ l) and thoroughly mixed with Matrix Solid Phase Dispersion (MSPD) C18 sorbent (1 g, Separtis, Grenzach-Wyhlen, Germany) in a mortar. The mixture was filled into a filter column (8 ml) connected to a Chromabond C18 ec column (1 ml, 100 mg, Macherey-Nagel), which had been conditioned with 1 ml methanol and 1 ml citrate buffer (20 mM, pH 1.8). After washing with 5 ml citrate buffer (20 mM, pH 1.8) and removing the residual water by centrifugation at 3000 g for 10 min, the mercapturic acid was eluted with 5 ml methanol. The eluate was brought to near dryness in a Savant SpeedVac after adding 20 μ l of DMSO. The residue was dissolved in 180 μ l methanol and then analyzed by UPLC-MS/MS. Feces collected from animals before treatment was used as a negative control. It was spiked with MPMA (0.04 pmol per mg feces), respectively, to create a standard and study the rate of recovery.

Detection of Mercapturic Acids. The analyses were carried out on a tandem quadrupole mass spectrometer (Quattro Premier XE, Waters Micromass, Manchester, UK) interfaced with an Acquity UPLC System (Waters, Milford, Massachusetts, USA). Separation was conducted on an Acquity BEH Phenyl column (1.7 μ m; 2.1 x 100 mm) (Waters), kept at 37°C. Samples were cooled to 4°C and aliquots of 2 μ l were given onto the column. Elution was performed using gradient of A (9:1, 10 mM ammonium acetate:methanol, pH 7.4) and B (19:1 acetonitrile:methanol) at a flow rate was 0.45 ml/min: 1 min 95% A, linear gradient to 30% A in 6 min, followed by reconditioning to 95% A. The mass spectrometer was in

negative electrospray mode and the collision-induced dissociation involved Argon as the target gas at 3.0×10^{-3} mbar. Other parameters were: capillary voltage 0.4 kV, cone voltage 27 V, extraction lens voltage 3 V, and radio-frequency lens voltage 0.1 V, source temperature 100°C, desolvation temperature 450°C, cone gas (N₂) 52 l/h, desolvation gas (N₂) 950 l/h, low- and high-mass resolutions at quadrupoles 1 and 2: 13 and 13.5, respectively, entrance at the traveling wave -2, at exit +2, and setting of the multiplier 630 V.

For detecting the mercapturic acid, the neutral losses of the *N*-acetyl-2-aminopropionic acid moiety (130 Da) and the entire side chain (162 Da) were used for quantification and identification, respectively, in parallel multiple reaction monitoring (MRM) measurements. For MPMA, m/z 376 represented the molecular ion $[M - H]^-$ with product ions m/z 247 (first transition) and m/z 201 (second transition), detected using a collision energy of 18 eV and 37 eV, respectively. The values for 1,8-DMPMA amounted to m/z 390 ($[M - H]^-$), m/z 261 (product ion, first transition) and m/z 215 (product ion, second transition). The limit of quantification was 10 ppb for both mercapturic acids and the recovery of 1-MPMA was 91% and 94% in urine and fecal samples respectively.

Tissue Culture. hOAT1 and hOAT3 were stably expressed in the human embryonic kidney cell line HEK293 as described previously (Bakhiya et al., 2006). Cells were grown in flasks containing Dulbecco's modified minimum essential medium (high glucose) supplemented with fetal bovine serum (10%), penicillin (100 units/ml) and streptomycin (100 µg/ml). The medium for the recombinant cells, but not for parental HEK293 cells, additionally contained hygromycin (175 µg/ml). Cultures were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Cultures were split in a 1:5 ratio every third to fourth day.

Inhibition of Uptake of Standard Substrates. Cells were seeded in 24-well plates (2 x 10⁵ cells in 1 ml medium per well) two days before the experiment. Uptake of [³H]*p*-aminohippurate (for OAT1) and [³H]estrone sulfate (for hOAT3) was assayed at 37°C in

Ringer's solution (130 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 20 mM HEPES, 1 mM NaH₂PO₄, 18 mM glucose, pH 7.4) for 2 min in the absence or presence of mercapturic acid. The uptake was terminated by 3 washes with ice-cold buffer (0.5 ml). Cells were then solubilized in 0.5 ml of 1 N NaOH. After neutralization with 0.5 ml of 1 N HCl, their ³H content was assayed by liquid scintillation counting. The results were standardized to the level of protein determined using the bicinchoninic acid assay (Pierce, Rockford, USA) with bovine serum albumin as the standard.

For quantifying the inhibitory activity, we used varying concentrations of the substrate (10 and 50 μM [³H]*p*-aminohippurate for hOAT1; 50 and 250 nM [³H]estrone sulfate for hOAT3) and mercapturic acid (≥5 concentrations, up to 30 μM for hOAT1 and 5 μM for hOAT3). Data were plotted and analyzed according to Dixon (1953).

Uptake of Mercapturic Acids into Cells. Cells were incubated in Ringer's solution containing 20 μM mercapturic acid at 37°C for 15 min. After three washes with ice-cold Ringer's solution, the cells were lysed with 0.25 ml 1 N NaOH and, after neutralization with 0.25 ml of 1 N HCl and protein precipitation with 1 ml of acetone, an aliquot of 2 μl of the supernatant was analyzed by UPLC-MS/MS. For trans-stimulation experiments cells were preloaded with glutarate for 2 h at 37°C. After three washes with warm Ringer's solution uptake of mercapturic acids was studied.

Results

Excretion of MPMA and DMPMA in Rats. The levels of mercapturic acids were analyzed in urine and feces collected in a 24-h period after dosing rats with 1-hydroxymethylpyrene and 1-hydroxymethyl-8-methylpyrene. The analytical method used, UPLC-MS/MS in the MRM mode, was highly specific and sensitive. No signals that might be confounded with MPMA or DMPMA were detected in samples from control animals (only treated with the delivering agents). The limit of detection was 50 fmol per injection for MPMA and DMPMA in standard samples. With the injection volume used, 2 μ l (which could be raised up by a factor two or possibly more if required), this limit was equivalent to the excretion of approximately 2.5 and 5 pmol mercapturic acid in 24-h urine and feces, respectively, in an animal.

MPMA and DMPMA were detected in all urine and feces samples from animals dosed with 1-hydroxymethylpyrene and 1-hydroxymethyl-8-methylpyrene, respectively (Table 1). In agreement with a previous study (Ma et al., 2000), in which rats were treated with 1-hydroxymethylpyrene and 1-sulfooxymethylpyrene, only a minor part of the total dose was excreted as the mercapturic acid, 177 ± 60 ppm of 1-hydroxymethylpyrene and 24 ± 10 ppm of 1-hydroxymethyl-8-methylpyrene (mean \pm SD of 4 and 5 rats, respectively). As in the previous study (Ma et al., 2000), MPMA was primarily excreted in urine ($72 \pm 17\%$ of the total urinary and fecal level). Surprisingly, DMPMA was preferentially excreted via the fecal route ($88 \pm 11\%$). While the fecal excretion of both mercapturic acids was in the same range (36 ± 16 ppm of the dose for MPMA, 22 ± 22 for DMPMA), the urinary level of MPMA (141 ± 125) was 100-fold greater than that of DMPMA (1.4 ± 0.4).

OAT-mediated Uptake of MPMA and DMPMA into Cells. To test whether benzylic mercapturic acids are transported by renal OAT, we exposed parental HEK293 and genetically engineered cells, expressing hOAT1 or hOAT3, to MPMA and DMPMA (20 μ M) for 15 min. The uptake rate of MPMA by OAT1- and OAT3-expressing cells was 2.8- and

1.7-fold higher than that of control cells; the differences were statistically significant (Fig. 2A). In contrast, uptake of DMPMA was equal in control and hOAT1-expressing cells and was marginally (1.25-fold, not significantly) increased in cells expressing hOAT3 (Fig. 2B). The findings indicate that hOAT1 is able to transport MPMA, but not DMPMA. While hOAT3 transported MPMA less efficiently than hOAT1, it might also transport DMPMA (although at an even lower rate than MPMA) – as confirmed in experiments using trans-stimulation (next section).

Cis-Inhibition and Trans-Stimulation of the Uptake of Mercapturic Acids by hOAT1 and hOAT3. Probenecid and glutarate, characteristic OAT inhibitors, were used to corroborate the participation of OAT in the uptake of MPMA. Their presence decreased the uptake rates in hOAT1- and hOAT3-expressing cells to the level observed in control cells (Fig. 3), indicating that MPMA uptake was specifically mediated by OAT. Next, we preloaded the cells with glutarate, an intracellular substrate for the organic anion/dicarboxylate exchange mediated by OAT. As shown in Fig. 4, MPMA uptake by hOAT1- and hOAT3-expressing cells was significantly trans-stimulated by glutarate preloading, providing further evidence that MPMA is substrate for both OAT.

Trans-stimulation by glutarate was used to re-examine the ambiguous increase in DMPMA uptake in hOAT3-expressing cells compared to control cells. As seen in Fig. 5, DMPMA uptake in hOAT3-expressing cells was trans-stimulated by glutarate preloading and became significantly different from the uptake in control cells. In addition, probenecid decreased the uptake rate in hOAT3-expressing cells to the level observed in control cells.

Inhibition of hOAT1- and hOAT3-mediated Uptake of Model Substrates by MPMA and DMPMA. Transporter proteins have to bind and release their substrates. Therefore, insufficient as well as excessive affinity may be unfavorable for a transport. To learn more about the affinity of mercapturic acids for OAT we conducted inhibition studies using model substrates. In the initial experiment, the mercapturic acids were used at a concentration of 20

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μM . Both mercapturic acids were strong inhibitors of hOAT3, using estrone sulfate as the substrate (Fig. 6B). At the concentration tested MPMA showed somewhat stronger inhibition (80%) than DMPMA (65%). MPMA also decreased hOAT1-mediated uptake of *p*-aminohippurate (by 60%), whereas DMPMA showed lower interaction with this transporter (15% decrease in transport rate, not statistically significant) (Fig. 6A).

We then studied the inhibition using varying concentrations of the reference substrate and MPMA (the more potent inhibitor in the initial experiment). K_i values were determined from Dixon plots. Representative plots are shown in Fig. 7. Similar results were obtained in repeat experiments. MPMA competitively inhibited the organic anion transport mediated by hOAT1 and hOAT3. The mean K_i value of MPMA for hOAT1 was determined at 14.5 μM (15.6, 12.1 and 16.0 μM in three experiments). The corresponding value for hOAT3 was 1.5 μM (1.6, 1.4 and 1.6 μM in three experiments).

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Discussion

We recently compared the DNA adduct formation and biotransformation of 1-hydroxymethylpyrene and 1-hydroxymethyl-8-methylpyrene in the rat in the presence of various metabolic modulators (Batke M, Donath C, and Glatt HR, manuscript in preparation). The study included the determination of MPMA and DMPMA as potential biomarkers indicating the formation of electrophilic benzylic sulfates. Despite their structural similarity (differing only in the presence or absence of a methyl group), these mercapturic acids strongly differed in the preferred excretion routes. The main excretion route for MPMA was the urine (72%), which is in agreement with a previous results from our laboratory (Ma et al., 2000). Surprisingly, DMPMA was preferentially excreted in feces (88%). It is known for a long time that organic anions tend to prefer the biliary excretion route with increasing molecular weight (Hirom et al., 1972). However, the difference in molecular weight is small in the present case and the rule is only empirical. It is likely that the substrate specificity of various transmembrane transporters is a key factor in the underlying mechanisms. MPMA and DMPMA differed much more strongly in the level of urinary excretion (100-fold) than in the level of biliary excretion (1.6-fold). Therefore, we studied the interaction of the mercapturic acids with renal transporters. In general, the basolateral uptake, usually mediated by OAT1 and OAT3, appears to be the rate-limiting step for tubular excretion of organic anions (Burckhardt and Burckhardt, 2003). Moreover, we had shown in a previous studies that isomeric 1-sulfooxymethylpyrene – which only differs from MPMA by the nature of the anionic group in the side chain – is a substrate of both transporters (Bakhiya et al., 2006).

Uptake of MPMA was increased in cells engineered for expression of hOAT1 or hOAT3 compared to parental HEK293. This increase in uptake was abolished in the presence of known OAT inhibitors, probenecid or glutarate. OAT1 and OAT3 are known to function as anion exchangers (Race et al., 1999; Bakhiya et al., 2003; Sweet et al., 2003), which use an α -ketoglutarate gradient across the basolateral membrane of the proximal tubule cells as the

driving force for organic anion uptake. Preloading of hOAT1- and hOAT3-expressing cells with glutarate, a non-metabolized homologue of α -ketoglutarate, significantly enhanced MPMA uptake. These modulations clearly demonstrate that the increased MPMA uptake in hOAT1- and hOAT3-expressing cells was indeed mediated by these transporters rather than by an accidental variation in the genetically modified cell lines.

Kinetic studies using MPMA as an inhibitor of the uptake of reference substrate demonstrated that both transporters have a high affinity to MPMA. The K_i value for MPMA was nearly 15 μ M for hOAT1 and 1.5 μ M for hOAT3. These values are similar to the affinity of both transporters to their reference substrates. The apparent K_m of hOAT1 for *p*-aminohippurate uptake determined by different groups ranged from 3.9 μ M (Race et al., 1999) to 22 μ M (Motojima et al., 2002) and the apparent K_m of hOAT3 for estrone sulfate was between 2.2 μ M (Takeda et al., 2001) and 7.5 μ M (Takeda et al., 2000).

Despite the higher affinity of hOAT3, the magnitude of MPMA uptake by hOAT1 was notably higher than that observed for hOAT3. This correlates with our recent data on sulfoxymethylpyrene transport by both OAT (Bakhiya et al., 2006). A possible explanation of this phenomenon is that high-affinity binding of MPMA to hOAT3 could be less favorable for the transport rate, impeding the release of the substrate after the translocation into the cell and, thus, slowing the transporter turnover rate.

Unlike MPMA, its congener DMPMA was not transported by hOAT1 and did not significantly inhibit hOAT1-mediated cellular uptake of *p*-aminohippurate. Thus, the negative transport result might be due to low affinity of DMPMA for hOAT1. Perhaps, the methyl group – in spite of its small size – sterically perturbs the interaction with a critical structure of the substrate-binding site. Similarly, position and number of methyl groups has been shown to effect affinity of xanthine-related compounds to hOAT1 (Sugawara et al., 2005). The presence of the methyl group in DMPMA had a less profound effect on the interaction with hOAT3 compared to hOAT1. DMPMA inhibited hOAT3-mediated cellular

uptake of estrone sulfate to a similar (marginally lower) extent than observed with MPMA. Both mercapturic acids were translocated at a relatively low rate by hOAT3, although this activity was only unambiguous for DMPMA after enhancing the driving force for anion exchange by preloading the cells with glutarate. The findings with DMPMA agree with a common property of hOAT3 to prefer, in comparison to hOAT1, larger and more lipophilic substrates (Burckhardt and Burckhardt, 2003). In this concept, MPMA rather than DMPMA would deviate from empirical rules. Despite its large hydrophobic group it was efficiently transported by hOAT1.

From early *in vivo* studies in rats and mice it is known that various mercapturic acids are secreted by the renal proximal tubule (Inoue et al., 1981; Inoue et al., 1982). The secretion process may be critical for the clearance of mercapturic acids, because they bind to albumin and thus cannot be efficiently filtrated (Okajima et al., 1985). Pombrio et al. (2001) recently investigated transmembrane transport of mercapturic acids in *Xenopus* oocytes expressing rat OAT1. 2,4-Dinitrophenol mercapturic acid was found to be a high-affinity substrate for OAT1 (K_m of 2 μ M) and several other mercapturic acids (including leukotriene E_4 mercapturic acid and benzyl mercapturic acid) were competitive inhibitors of OAT1. Later, the OAT1-mediated uptake of *N*-acetylcysteine conjugates of Hg^{2+} and CH_3-Hg^+ has been observed in Madin-Darby canine kidney cells and *Xenopus* oocytes expressing hOAT1 (Aslamkhan et al., 2003; Zalups and Ahmad, 2005). These studies showed that OAT1 is involved in the renal excretion of mercapturic acids and related *N*-acetylcysteine conjugates. However, little data is available on the transport of mercapturic acids by OAT3. Rat OAT3 expressed in *Xenopus* oocytes enhanced the uptake of the *N*-acetylcysteine conjugate of Hg^{2+} (Aslamkhan et al., 2003), but not of a corresponding conjugate of CH_3-Hg^+ (Koh et al., 2002). In the present study, we have showed for the first time that some mercapturic acids in the narrow sense (as opposed to complexes of *N*-acetylcysteine with metal ions) are substrate

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of hOAT3. Thus, OAT3 could be another candidate transporter contributing to the basolateral uptake of mercapturic acids in the renal proximal tubule.

The relative contribution of the transporters to the uptake of benzylic mercapturic acids *in vivo* remains an open question. Our present observation that MPMA, but not DMPMA, was chiefly secreted in the urine, together with ability of hOAT1 to transport MPMA, but not DMPMA, suggests that OAT1 may be the critical transporter responsible for the elimination of MPMA *in vivo*. Data obtained with OAT1 knockout mice (Eraly et al., 2005) indicate that the critical role in the functioning of the classical renal pathway for organic anion secretion is played by OAT1. Nevertheless, it would be of great interest to study the impact of knockout of either OAT in the elimination of the mercapturic acids of various alkylated PAH.

In summary, we found that a minute structural differences (the presence or absence of an additional methyl group in a alkylated PAH) can strongly direct the excretion route of mercapturic acids as well as their interaction with hOAT1 and – to a lesser extent – hOAT3. Whether the structural difference also affects the activity of hepatic transporters, remains to be investigated.

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Footnotes

Unnumbered footnote to the title: Research described in this article was supported by Philip Morris USA Inc. and by Philip Morris International. A part of this study has been presented at a meeting (Bakhiya et al., 2007).

Legends for Figures

FIG. 1. Structural formulas of 1-methylpyrenyl mercapturic acid (MPMA) and 1,8-dimethylpyrenyl mercapturic acid (DMPMA).

FIG. 2. Uptake of MPMA (A) and DMPMA (B) by hOAT1- and hOAT3-expressing cells. HEK293-derived cells that express hOAT1 or hOAT3 were exposed to 20 μ M MPMA or DMPMA for 15 min. After washing and lysing the cells, the intracellular levels of the mercapturic acid were determined using UPLC-MS/MS. Data are expressed as a percent of the uptake into control (untransformed) cells studied concurrently. Values are means \pm SE of four experiments conducted on separate occasions. The absolute uptake of MPMA and DMPMA into control cells amounted to 2.8 ± 0.2 and 2.6 ± 0.2 nmol/mg protein in 15 min, respectively. * $p < 0.05$ compared to control cells (paired Student's t-test using the absolute uptake as the statistical unit).

FIG. 3. Influence of extracellular glutarate and probenecid on MPMA uptake by parental HEK293 cells (control) and cells expressing hOAT1 and hOAT3. Confluent cultures were incubated in medium containing 20 μ M MPMA in the absence or presence of 1 mM of glutarate or probenecid for 15 min. The MPMA levels in the cells were determined by UPLC-MS/MS. Data are means \pm SE of three cultures. * $p < 0.05$; ** $p < 0.01$ compared to corresponding transporter cells in the absence of the inhibitor (bars labeled "none") (unpaired Student's t-test).

FIG. 4. Influence of intracellular glutarate on MPMA uptake by parental HEK293 cells (control) and cells expressing hOAT1 and hOAT3. Cells were exposed to 2 mM glutarate ("glutarate") or maintained in normal medium ("none") for 2 h prior to uptake studies. They were then exposed to 20 μ M MPMA in Ringer's solution for 15 min. The MPMA levels in

the cells were determined by UPLC-MS/MS. Data are means \pm SE of three cultures. * $p < 0.05$; ** $p < 0.01$ compared to the uptake in the corresponding cells without preloading (unpaired Student's t-test).

FIG. 5. Influence of intracellular glutarate or extracellular probenecid on DMPMA uptake by hOAT3. Confluent cultures of parental HEK293 cells and hOAT3-expressing cells were incubated in medium containing 20 μ M DMPMA for 15 min. Cells were incubated in the absence of modulator (none), after pre-loading with glutarate (glutarate, as described in legend of Fig. 4) or in the presence of 1 mM probenecid. Data are means \pm SE of three cultures. * $p < 0.05$; compared to control (parental HEK293) cells under the corresponding experimental conditions (unpaired Student's t-test).

FIG. 6. Inhibition of hOAT1- and hOAT3-mediated substrate uptake by MPMA and DMPMA. HEK293-derived cells expressing hOAT1 or hOAT3 were assayed in the presence of MPMA or DMPMA (20 μ M) and in their absence ("DMSO") for uptake of [3 H]*p*-aminohippurate (10 μ M; A) and [3 H]estrone sulfate (50 nM; B). Parental HEK293 cells ("control") were used as a negative control. The treatment was performed for 2 min. Data are expressed as a percentage of the basal uptake (in the presence of mercapturic acid) of the OAT-expressing cells in the corresponding experiment. Values are means and SE of three experiments conducted on separate occasions. The absolute values of the basal uptake (\pm SE) amounted to 79.0 ± 5.9 and 0.90 ± 0.03 pmol/mg protein in 2 min for hOAT1 and hOAT3, respectively. * $p < 0.05$; ** $p < 0.01$, compared to corresponding the same cells in the absence of mercapturic acid (paired Student's t-test using the absolute uptake as the statistical unit).

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FIG. 7. Kinetic analyses of the influence of MPMA on organic anion uptake by hOAT1 and hOAT3. Uptake of [^3H]p-aminohippurate (10 μM or 50 μM) and [^3H]estrone sulfate (50 nM or 250 nM) was assayed in HEK293-derived cell lines stably expressing hOAT1 (A) and hOAT3 (B), respectively, in the presence of varying concentrations of MPMA for 2 min. Uptake was also determined in control cells. This value was subtracted from that observed in OAT-expressing cells. Data are presented as Dixon plots. Values are means \pm SE of three wells from a single representative experiment. The following K_i values were calculated from the plots: 15.6 μM for hOAT1 and 1.6 μM for hOAT3. Similar values were obtained in repeat experiments (see main text).

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Tables

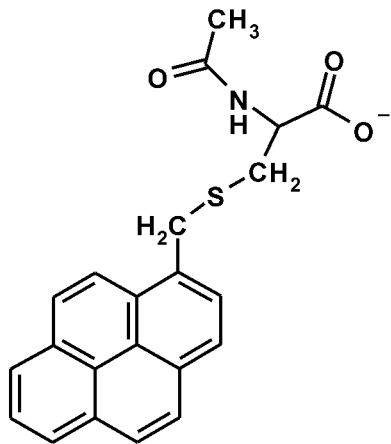
TABLE 1

Urinary and fecal excretion of benzylic mercapturic acids (MPMA and DMPMA) by rats treated with 1-hydroxymethylpyrene and 1-hydroxymethyl-8-methylpyrene, respectively.

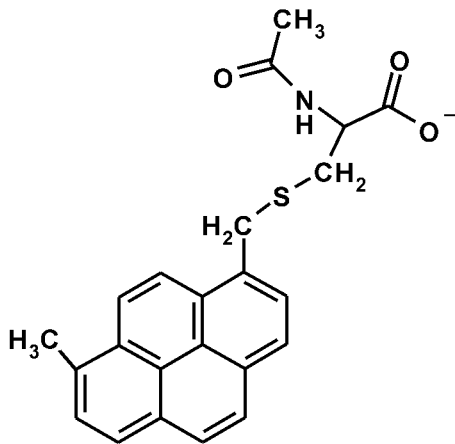
Each test compound (83.3 $\mu\text{mol/kg}$ body weight) was intraperitoneally administrated to 5 male Wistar (Han) rats. One animal treated with 1-hydroxymethylpyrene had to be discarded due to puncturing of the cecum. Urine and feces were collected in metabolic cages for 24 h after the treatment. They were analyzed for levels of mercapturic acids using UPLC-MS/MS. Data are expressed in ppm of the dose and percent of total mercapturic acid excreted (in urine plus feces) for each animal. The percent of urinary and fecal excretion was significantly different between the compounds ($p < 0.01$, Mann-Whitney U-test).

Mercapturic acid	Animal	ppm of dose		Route (% of total)	
		Urine	Feces	Urine	Feces
MPMA	1	43	40	52	48
	2	314	20	94	6
	3	151	57	73	27
	4	57	27	68	33
	Mean \pm SD	141 \pm 125	36 \pm 16	72 \pm 17	28 \pm 17
DMPMA	1	1.3	3.0	30	70
	2	0.7	19.4	4	96
	3	1.8	13.2	12	89
	4	1.7	14.9	10	90
	5	1.4	59.9	2	98
	Mean \pm SD	1.4 \pm 0.4	22 \pm 22	12 \pm 11	88 \pm 11

Fig. 1



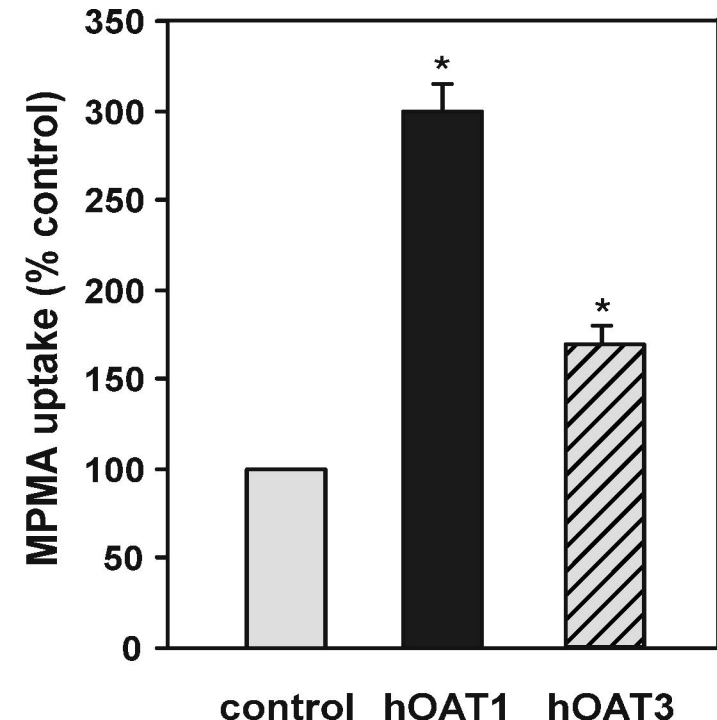
MPMA



DMPMA

Fig. 2

A



B

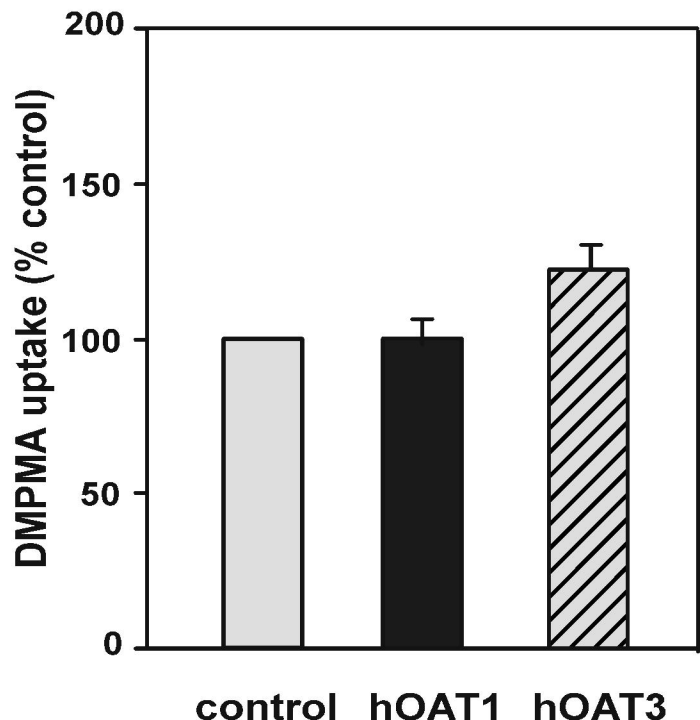


Fig. 3

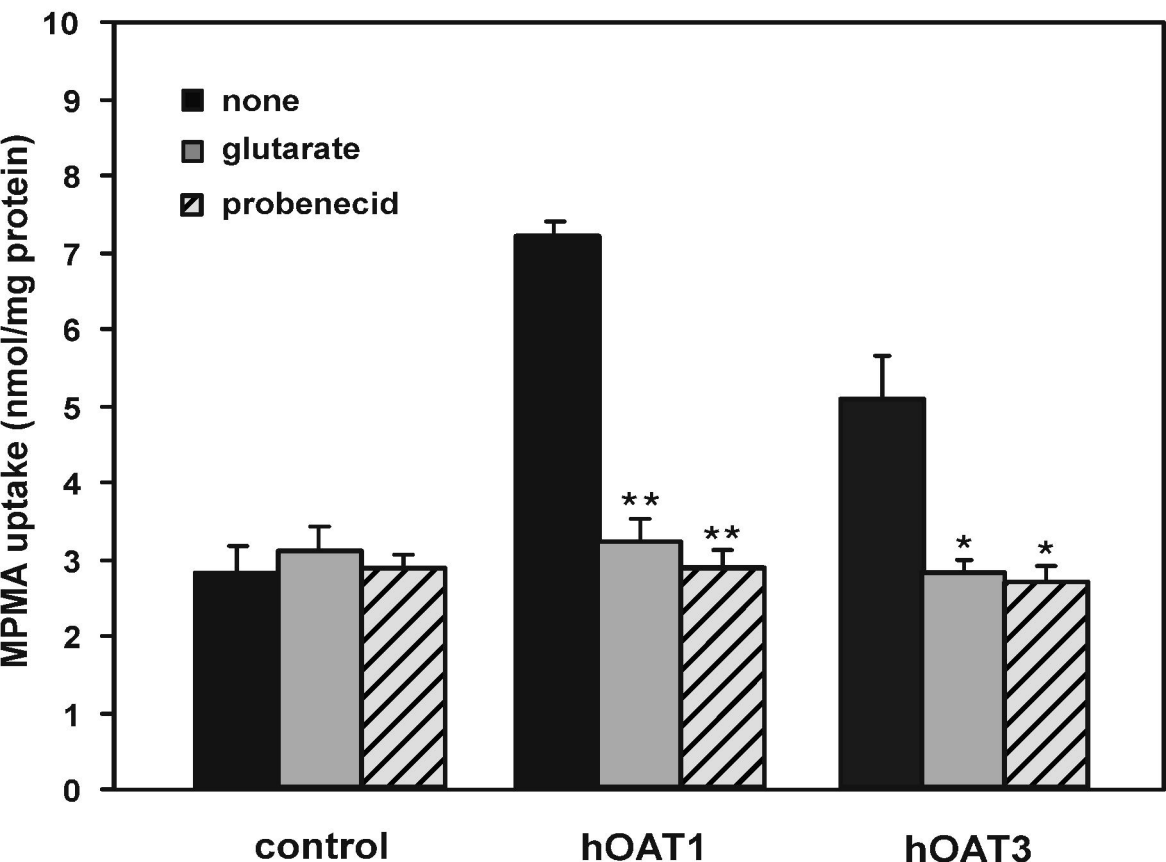


Fig. 4

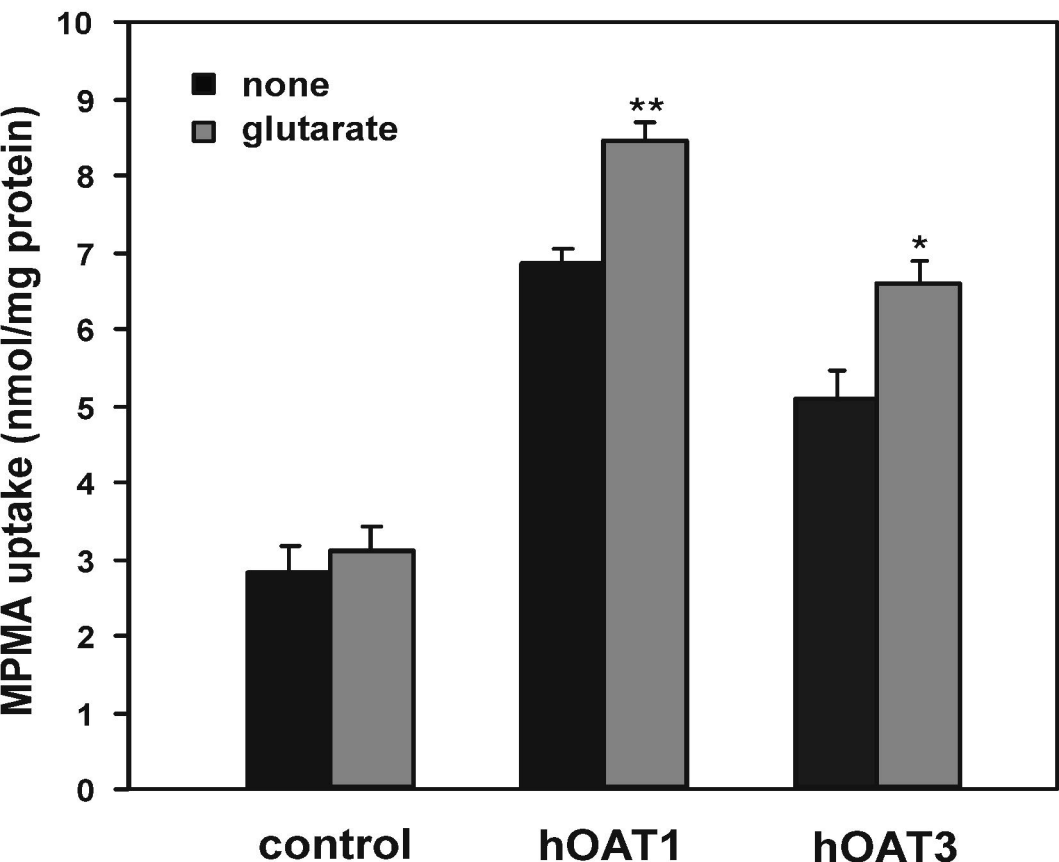


Fig. 5

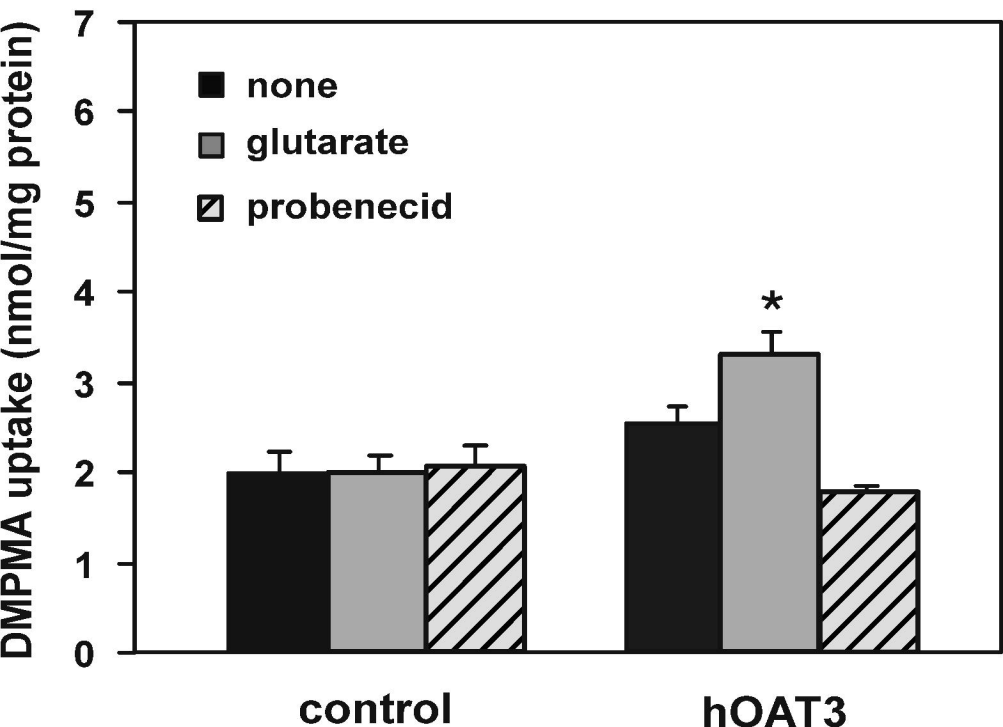


Fig. 6

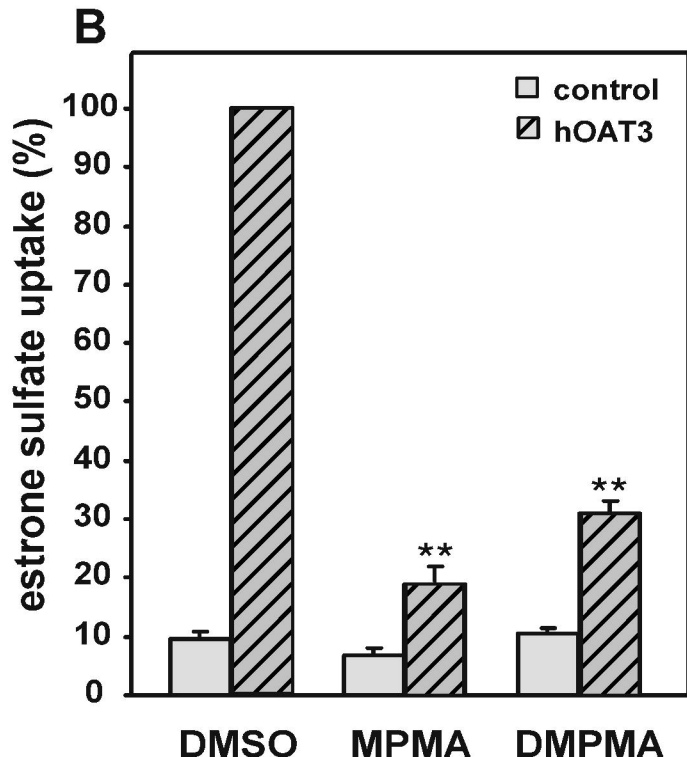
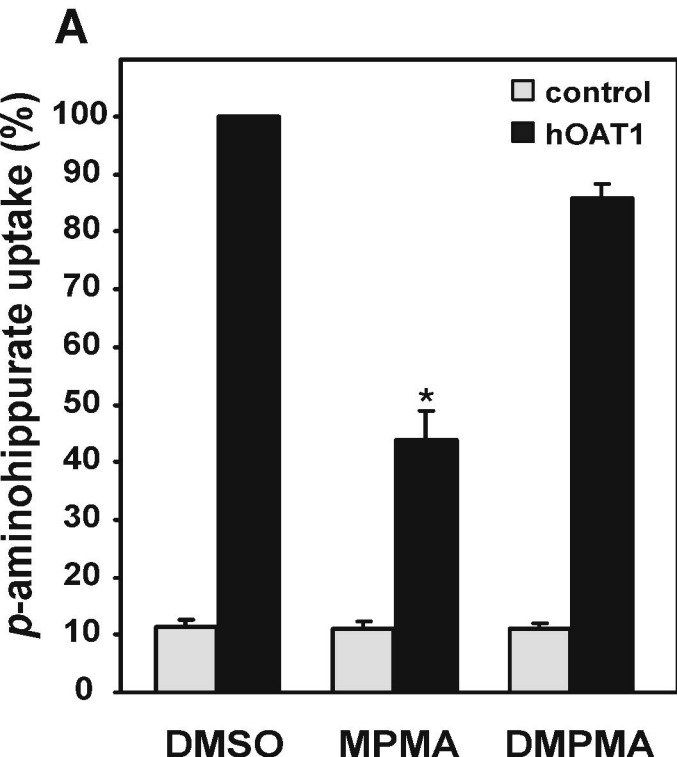


Fig. 7

