

Handling of the Homocysteine S-Conjugate of Methylmercury by Renal Epithelial Cells: Role of Organic Anion Transporter 1 and Amino Acid Transporters

Rudolfs K. Zalups and Sarfaraz Ahmad

Division of Basic Medical Sciences, Mercer University, School of Medicine, Macon, Georgia

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ABSTRACT

Recently, the activity of the organic anion transporter 1 (OAT1) protein has been implicated in the basolateral uptake of inorganic mercuric species in renal proximal tubular cells. Unfortunately, very little is known about the role of OAT1 in the renal epithelial transport of organic forms of mercury, such as methylmercury (CH_3Hg^+). Homocysteine (Hcy) S-conjugates of methylmercury [(S)-(3-amino-3-carboxypropylthio)(methyl)mercury ($\text{CH}_3\text{Hg-Hcy}$)] have been identified recently as being potentially important biologically relevant forms of mercury. Thus, the present study was designed to characterize the transport of $\text{CH}_3\text{Hg-Hcy}$ in Madin-Darby canine kidney (MDCK) cells (which are derived from the distal nephron) that were transfected stably with the human isoform of OAT1 (hOAT1). Data on saturation kinetics, time dependence, substrate specificity, and temperature dependence demonstrated that $\text{CH}_3\text{Hg-Hcy}$ is a

transportable substrate of hOAT1. However, substrate-specificity data from the control MDCK cells also showed that $\text{CH}_3\text{Hg-Hcy}$ is a substrate of one or more transporter(s) that is/are not hOAT1. Additional findings indicated that at least one amino acid transport system was probably responsible for this transport. It is noteworthy that the activity of amino acid transporters accounted for the greatest level of uptake of $\text{CH}_3\text{Hg-Hcy}$ in the hOAT1-expressing cells. Furthermore, rates of survival of the hOAT1-transfected MDCK cells were significantly lower than those of corresponding control MDCK cells when they were exposed to cytotoxic concentrations of $\text{CH}_3\text{Hg-Hcy}$. Collectively, the present data indicate that $\text{CH}_3\text{Hg-Hcy}$ is a transportable substrate of OAT1 and amino acid transporters and, thus, is probably a transportable mercuric species taken up in vivo by proximal tubular epithelial cells.

Homocysteine (Hcy) is a sulfhydryl-containing amino acid formed from methionine after it undergoes demethylation. After its formation, Hcy is broken down by one of two mechanisms: remethylation or transsulfuration. In the remethylation pathway, a methyl group (from N^5 -methyltetrahydrofolate or betaine) is transferred to Hcy to re-form Met. In the alternative pathway, Hcy is converted to cysteine and α -ketobutyrate by trans-sulfuration, which requires the sequential actions of cystathionine- β -synthase and γ -cystathionase (Selhub, 1999).

In blood, Hcy, Cys, and glutathione (GSH) make up a very important pool of nonprotein thiols to which various electro-

philes, including metals such as mercury (Hg) and cadmium (Cd), can interact. Normal plasma levels of Hcy are generally similar to those of Cys and GSH (Lash and Jones, 1985). However, under certain pathophysiological conditions, the plasma levels of Hcy can increase from approximately 5 to 10 μM to as much as 200 μM (Stabler et al., 1988; Malinow, 1990; Selhub, 1999). Hyperhomocysteinemia is a significant clinical problem (resulting from altered intracellular metabolism of Hcy or Met) that has been implicated as a risk factor in the development of cardiovascular diseases.

Recent evidence indicates that the kidneys are major sites where Hcy is metabolized (Dudman et al., 1996; House et al., 1997, 1998; Bostom et al., 1998; Refsum et al., 1998; Selhub, 1999), with most of the metabolism occurring in the epithelial cells lining the segments of the proximal tubule (House et al., 1997). It is noteworthy that the proximal portion of the nephron is also the primary target where inorganic and organic mercuric ions accumulate and exert their toxic effects.

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ABBREVIATIONS: Hcy, homocysteine; DMPS, 2,3-dimercaptopropane-1-sulfonic acid; GSH, glutathione; Hcy-Hg-Hcy, bis(S)-3-amino-3-carboxypropylthio)mercury; EMEM, Eagle's modified essential medium; OAT1, organic anion transporter 1; NAC-S-Hg-S-NAC, di-N-acetylcysteine S-conjugate of inorganic mercury; NAC-S-HgCH₃, N-acetylcysteine S-conjugate of methylmercury; PAH, *p*-aminohippuric acid; HBSS, Hanks' balanced saline solution; hOAT1, human organic anion transporter 1; $\text{CH}_3\text{Hg-Hcy}$, (S)-(3-amino-3-carboxypropylthio)(methyl)mercury; MDCK, Madin-Darby canine kidney cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide.

Various sets of data have recently implicated the formation of mercuric conjugates of Cys, *N*-acetylcysteine (NAC), and GSH in the proximal tubular transport of inorganic and organic forms of mercury (Zalups, 1995, 1998a, 2004; Aslamkhan et al., 2003; Bridges and Zalups, 2004; Bridges et al., 2004; Zalups and Ahmad, 2004). Unfortunately, little attention has been paid to Hcy as a biologically relevant binding ligand of mercuric ions.

In an aqueous environment, Hcy (like Cys and GSH) bonds to inorganic or organic mercuric ions in a linear coordinate covalent manner. Until recently, attention has been paid primarily to mercuric conjugates of Cys and GSH as the biologically relevant forms of mercury involved in the proximal tubular uptake of inorganic or organic mercuric species. Because there is the potential for the formation of linear coordinate covalent complexes between molecules of Hcy and mercuric ions, we had designed experiments to investigate *in vivo* the potential of the Hcy *S*-conjugate of inorganic mercury (Hg^{2+}), bis(*S*)-3-amino-3-carboxypropylthio)mercury (Hcy-Hg-Hcy), as a biologically relevant transportable substrate in the kidneys. We discovered that when Hcy-Hg-Hcy was administered intravenously to rats, Hg^{2+} was taken up at both the luminal and basolateral plasma membranes of renal (proximal) tubular epithelial cells (Zalups and Barfuss, 1998a). More recent findings from our laboratory indicate that amino acid transporters are probably responsible for the luminal uptake of Hcy-Hg-Hcy. In particular, we demonstrated *in vitro* that Hcy-Hg-Hcy is a high-affinity substrate of the heterodimeric amino acid transporter, system $\text{b}^{0,+}$, in renal epithelial cells transfected stably with this transporter (Bridges and Zalups, 2004).

We have also provided recent evidence indicating that the basolateral uptake of Hcy-Hg-Hcy by proximal tubular epithelial cells is mediated by the activity of the organic anion transporter 1 (OAT1) protein. We demonstrated in renal epithelial cells transfected stably with the human isoform of OAT1 (hOAT1) that Hcy-Hg-Hcy is a transportable substrate of OAT1. Thus, Hcy is not only a potential plasma thiol to which mercuric ions can bind but that mercuric conjugates of Hcy are likely important transportable substrates that are taken up by both luminal and basolateral transporters in proximal tubular epithelial cells.

Although there is considerable evidence implicating thiol *S*-conjugates of Hg^{2+} as substrates of OAT1, little is known about the role of this transport protein in the uptake of Hcy *S*-conjugates of organic forms of mercury by renal epithelial cells. Because methylmercury is the primary form of mercury present in the environment, it is important to understand the mechanisms involved in the handling this species of mercury by target cells affected adversely by this toxicant. Consequently, we designed experiments in the present study to characterize mechanistically the potential role of OAT1 in the extracellular-to-intracellular transport of the Hcy *S*-conjugate of methylmercury, (*S*)-(3-amino-3-carboxypropylthio)-(methyl)mercury ($\text{CH}_3\text{Hg-Hcy}$), in Madin-Darby canine kidney (MDCK) cells transfected stably with hOAT1. Because MDCK cells (which are derived from the distal nephron of the dog) do not express organic anion transporters, direct molecular evidence for the participation of OAT1 in the transport of $\text{CH}_3\text{Hg-Hcy}$ could be obtained.

Materials and Methods

Transfection of MDCK II Cells with hOAT1. Mycoplasma-free type II MDCK cells, which were originally developed in the laboratory of Dr. Kai Simons (EMBL, Heidelberg, Germany), were used in the present investigation. As described previously, subclones of these cells were transfected with the cDNA for hOAT1 ligated to pcDNA3.1 (Invitrogen, Carlsbad, CA) using QIAGEN SuperFect Reagent (Valencia, CA) according to the manufacturer's protocol (5 μl of SuperFect/ μg DNA) (Aslamkhan et al. 2003). Subclones of hOAT1-expressing (and wild type) MDCK II cells used in the present investigation were provided as a gift by Dr. John Pritchard at NIEHS, National Institutes of Health. These subclones were maintained in culture media with 200 $\mu\text{g}/\text{ml}$ geneticin (G418; Invitrogen, Carlsbad, CA) and were screened regularly for their ability to transport *p*-aminouppurate (PAH), [^3H]PAH.

Maintaining Cells in Culture. Both wild-type and hOAT1-expressing MDCK cells were grown in a confluent monolayer. While in culture, type II MDCK cells polarize and develop cell-to-cell attachments, which afford the cultured epithelium a transepithelial resistance. All of the MDCK II cells were grown at 37°C in Eagle's minimum essential medium (EMEM; Invitrogen) supplemented with 1 mM sodium pyruvate and 10% fetal bovine serum (Invitrogen). While in culture, the MDCK cells were grown and maintained in a humidified atmosphere consisting of 95% O_2 and 5% CO_2 . Cells were split every 3 to 7 days, and 5 to 10% of the culture was inoculated into new flasks.

Uptake of PAH. Dr. Pritchard's laboratory had established that insertion of hOAT1 protein occurs at both apical and basolateral plasma membranes in the MDCK cells transfected with the cDNA encoding hOAT1. The apical insertion of hOAT1 permitted us to study hOAT1-dependent transport using cells grown on a solid surface. Thus, the uptake of [^3H]PAH (as well as other substrates) was assessed in cells and plated in 24-well (2.0 cm^2/well) cell culture cluster plates (Corning Life Sciences, Acton, MA) containing supplemented EMEM at a density of 0.5×10^6 cells/well (added as 2 ml). The cells were grown in a humidified atmosphere of 95% air and 5% CO_2 for two days at 37°C, with media being changed after the first 24 h.

Immediately before assessing transport parameters, cells were first rinsed with Hanks' balanced saline solution (HBSS) supplemented with 10 mM HEPES (pH 7.4) for three consecutive 5-min periods. At the beginning of each experiment, 350 μl of the aforementioned Hanks' buffer containing 5.0 μM PAH, with or without of 200 μM probenecid, were added to each well. Some of the PAH was in the form of [^3H]PAH (4.54 $\text{mCi}/\mu\text{mol}$; PerkinElmer Life and Analytical Sciences, Boston, MA). After 60 min of exposure to PAH, the cells in each well were rinsed with ice-cold "stop" buffer (4°C) [HBSS supplemented with 10 mM HEPES (pH 7.4)]. To determine the cellular content of [^3H]PAH, cells were first lysed by adding 1 ml of 1 N NaOH to each well. The plates containing NaOH were shaken overnight (for at least 12 h) in an orbital shaker operating at a rate of 500 rpm. Subsequently, 700 μl of cellular lysate from each well were neutralized with 700 μl of 1 N HCl. The total volume of neutralized solution was added to 15 ml of Opti-FLUOR high flash-point liquid scintillation fluid (PerkinElmer Life and Analytical Sciences). The radioactivity in each sample was determined using a Beckman LS6000IC Liquid Scintillation Analyzer (Beckman Coulter Inc., Fullerton CA). Fifty microliters of the remaining cellular lysate from each well were used to determine the total amount of protein per well using the Bradford protein assay (Bradford, 1976).

When 5 μM PAH was applied to extracellular medium, the hOAT1-transfected MDCK cells transported PAH into their cytosolic compartment at a rate of $9.9 \pm 0.16 \text{ pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$. In addition, this rate of transport was reduced to less than 1.0 $\text{pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$ by the addition of 200 μM probenecid to the extracellular compartment. In the wild-type MDCK cells, uptake of PAH was insignificant, averaging only approximately

$0.07 \pm 0.01 \text{ pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$. In addition, probenecid did not affect significantly the low level of uptake of PAH in the wild-type MDCK cells. Thus, these findings confirm that functional hOAT1 protein was being inserted into the plasma membranes of the hOAT1-transfected MDCK cells.

Experimental Design. Extracellular to intracellular transport of $\text{CH}_3\text{Hg-Hcy}$ was characterized and compared in both hOAT1-transfectants and corresponding wild-type control MDCK cells. To determine whether $\text{CH}_3\text{Hg-Hcy}$ is a transportable substrate of hOAT1, concentration-response data for the uptake of CH_3Hg^+ (in the form of $\text{CH}_3\text{Hg-Hcy}$) were fitted to the Michaelis-Menten equation.

$$V = \frac{V_{\max}[S]}{K_m + [S]} \quad (1)$$

In this equation, V represents velocity, V_{\max} is the maximal velocity for transport of the substrate being studied, $[S]$ is the concentration of the substrate being transported, and K_m is the Michaelis-Menten constant. In addition, linear regression analysis was applied to transport data plotted by the Eadie-Hofstee method, where V is plotted against $V/[S]$. The slope of the regression lines determined by this method is equal to $-K_m$. By using these equations, we were able to characterize the transport of Hcy *S*-conjugates of CH_3Hg^+ .

Transport of CH_3Hg^+ in hOAT1-Transfected and Nontransfected MDCK II Cells. In the experiments where the transport of Hcy *S*-conjugates of CH_3Hg^+ were studied, cells were also plated in 24-well (2.0 cm^2) cell culture cluster plates (Corning Life Sciences) containing supplemented EMEM at a density of 0.5×10^6 cells/well (added as 2 ml). They were then grown in a humidified atmosphere of 95% air and 5% CO_2 for two days at 37°C , with media being changed after the first 24 h. During the assessment of transport activity, medium was aspirated from the wells and cells were rinsed three times with 3 ml of HBSS supplemented with 10 mM HEPES (pH 7.4). Three hundred and fifty microliters of transport buffer (specific to each experiment) containing radioactive inorganic mercury ($^{203}\text{Hg}^{2+}$; 8–12 mCi/mg Hg) or methylmercury ($^{14}\text{C}[\text{H}_3\text{Hg}^+]$; 20 mCi/mmol; American Radiolabeled Chemical Inc., St. Louis, MO) were added to each well.

In separate experiments, competitive inhibitors of OAT1, such as PAH or the dicarboxylate, adipate or glutarate, were added to the transport buffer. The rationale for employing adipate and glutarate is that both of these substrates are high-affinity competitive substrates of OAT1. With their use, we were able to better characterize the activity of hOAT1 in the uptake of $\text{CH}_3\text{Hg-Hcy}$.

In addition, the influence of various L-isomers of amino acids (such as leucine, isoleucine, methionine, alanine, phenylalanine, etc.) on the transport of $\text{CH}_3\text{Hg-Hcy}$ was assessed. The rationale for using the amino acids leucine, isoleucine, phenylalanine, lysine, and histidine is that they are known substrates of system $\text{b}^{0,+}$, which has been implicated in the transport of Hcy and Cys *S*-conjugates of inorganic mercury. Moreover, leucine, isoleucine, methionine, threonine, and alanine were chosen because they are known substrates of system L, which has been implicated in the transport of Cys *S*-conjugates of methylmercury. The acidic amino acids glutamate and aspartate are not substrates of system $\text{b}^{0,+}$ or system L but were used to determine whether additional carrier systems may have been involved.

A racemic mixture of Hcy was added in a 2:1 mol ratio with CH_3Hg^+ to ensure that each methylmercuric ion in solution formed a thermodynamically stable linear-I coordinate-covalent complex with a molecule of Hcy. The association constant between mercuric ions and the sulfur atom of low-molecular weight thiols is more than ten orders of magnitude greater than that between mercuric ions and any other biologically occurring nucleophilic groups (Zalups, 2000).

It is important to point out, however, that the transport findings obtained with a mixture of D- and L-isomers may prove to be somewhat different from those obtained with the use of only the L-isomer (or D-isomer), because there are documented differences in the han-

dling of D- and L-isomers of some of the amino acids by a particular transporter. Unfortunately, the L-isomer of Hcy was not commercially available to us at the time of experimentation.

At the conclusion of each period of exposure, cells in each well were rinsed with ice-cold "stop" buffer (4°C) [HBSS supplemented with 10 mM HEPES (pH 7.4) containing 1 mM DMPS and 200 μM probenecid]. DMPS is a very effective dithiol chelator of mercuric ions. It was used to reduce the pool of mercuric ions bound to outer surfaces of the outer surface of the plasma membrane to negligible levels. Because DMPS oxidizes rapidly in aqueous solutions, it was mixed into solution within the first 15 min of its use. Probenecid was used in the stop buffer as an added measure to inhibit the activity of OAT1 at the termination of each experiment.

Cellular content of ^{14}C -labeled CH_3Hg^+ was determined by liquid scintillation spectrometry after the application of 1 ml of 1 N NaOH to each well. After adding the NaOH, the 24-well plates were shaken in an orbital shaker at 500 rpm for 24 h. Seven-hundred microliters of cellular lysate from each well were neutralized with 700 μl of 1 N HCl. The total volume of neutralized solution was added to 15 ml of Opti-FLUOR scintillation fluid. The radioactivity of each sample was determined using a Beckman LS6000IC Liquid Scintillation Analyzer (^{203}Hg counting-efficiency $\sim 80\text{--}90\%$). Fifty microliters of the remaining cellular lysate from each well were used to determine the total amount of protein per well using the Bradford protein assay. Transport data obtained from each well of cells were normalized to the corresponding concentration of cellular protein.

Assessment of Toxicity and Cellular Viability. The effects of $\text{CH}_3\text{Hg-Hcy}$ on cellular viability were measured by the MTT-based toxicity assay (Sigma-Aldrich, St. Louis MO). This assay measures the activity of mitochondrial dehydrogenase by the conversion of the yellow tetrazolium dye MTT to purple formazan crystals. Cells were plated in supplemented EMEM at a density of 5.0×10^4 cells/well (added as 200 μl /well) in sterile 96-well microtiter plates (Corning Life Sciences) and allowed to grow for 48 h in a humidified atmosphere of 5% CO_2 , 95% air at 37°C . Supplemented EMEM was changed after the first 24 h by inversion. Excess medium adhering to the plate was blotted off with sterile gauze (Johnson & Johnson, New Brunswick, NJ). After 48 h, wells were washed again twice with 200 μl of HBSS/well. After washing, test compounds were added to individual wells (200 μl /well) in unsupplemented EMEM, and cells were grown for 6 h in a humidified atmosphere of 5% CO_2 , 95% O_2 at 37°C . At the conclusion of the exposure period, medium was removed by inversion and blotting, wells were washed with 200 μl of HBSS, and 100 μl of 0.5 mg/ml (1.2 mM) MTT in HBSS was added to each well. Cells were incubated for 2 h, and 100 μl of solubilization buffer (10% Triton X-100, 0.1 N HCl in isopropyl alcohol) were added to each well. This buffer both lysed the cells (releasing the formazan) and dissolved the water-insoluble formazan crystals. After an overnight incubation at room temperature, full solubilization had occurred, and plates were read at 595 nm in a Titertek Multiskan MKII plate reader (Fisher Scientific, Suwanee, GA).

Statistical Analyses. Data are expressed as the mean \pm S.E. For uptake studies, a sample size of $n = 3$ or 4 was used. Each experiment was repeated at least twice to establish repeatability. Assuming that each set of data were independent, distributed in a Gaussian manner, and had a level of variance equal to that for the other samples tested, statistical analysis for each parameter was performed by first using a two-way analysis of variance followed by either Tukey's or Dunnett's post hoc test. Data expressed as a percentage were first normalized using the arcsine transformation before applying parametric statistical analyses. This transformation takes the arcsine of the square root of the decimal fraction of the percent score. Differences among means were considered statistically significant at $p < 0.05$.

Results

Concentration-Dependent Uptake of CH_3Hg^+ . The mean values for the uptake of CH_3Hg^+ tended to be greater in the hOAT1-transfected MDCK cell than in the corresponding wild-type MDCK cells when the cells were exposed to 1 to 300 μM $\text{CH}_3\text{Hg-Hcy}$ for 15 min (Fig. 1A). The reason for presenting the transport data as the amount of CH_3Hg^+ transported relates to the fact that one cannot be certain that all of the $\text{CH}_3\text{Hg-Hcy}$ transported into the cells remained intact (as the Hcy conjugate) at the time of determination.

Significant differences in uptake between the two groups of

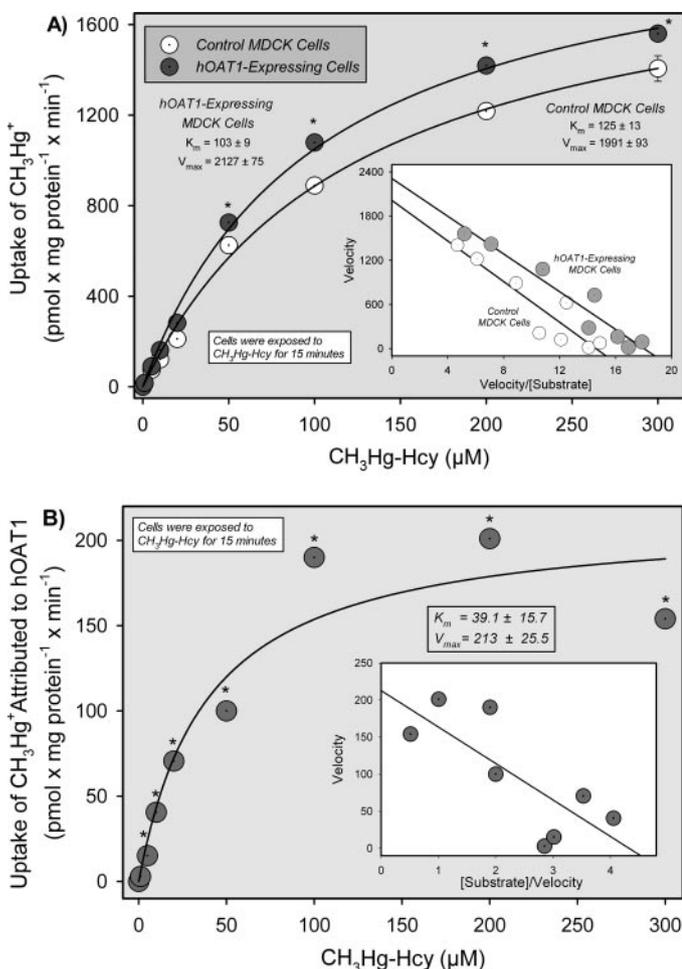


Fig. 1. A shows the concentration-dependent uptake (picomole \times milligram of cellular protein $^{-1} \times$ minute $^{-1}$) of CH_3Hg^+ in control and hOAT1-expressing MDCK II cells exposed to various concentrations of $\text{CH}_3\text{Hg-Hcy}$ for 15 min (at 37°C). The units for K_m and V_{max} are micromole and picomole \times milligrams of protein $^{-1} \times$ minute $^{-1}$, respectively. Values are mean \pm S.E. *, significantly different ($p < 0.05$) from the mean for the corresponding group of control MDCK cells. Data for the control and hOAT1-expressing MDCK cells in A were fitted to the nonlinear regression equation $y = V_{max} \times X / (K_m + X)$, with a resulting $r^2 = 0.9975$ and an $r^2 = 0.9972$, respectively. Linear regression analysis of the Eadie-Hofstee data for the control MDCK cells in A reveal a good fit between the experimental and control data and the predicted lines ($r^2 = 9708$ and $r^2 = 0.9334$, respectively). B shows the kinetic analysis of the concentration-dependent uptake of CH_3Hg^+ attributable to hOAT1. Cellular uptake was studied for 30 min (at 37°C). The units for K_m and V_{max} are micromole and picomole \times milligrams of protein $^{-1} \times$ minute $^{-1}$, respectively. Data for the hOAT1 component in B fit the nonlinear regression equation $y = V_{max} \times X / (K_m + X)$ with an $r^2 = 0.9902$. The data expressed in the Eadie-Hofstee plot for the hOAT1 component in B fit a linear equation with $r^2 = 0.8567$. Values are mean \pm S.E. *, significantly different ($p < 0.05$) from the mean for the corresponding group of control MDCK cells.

cells were particularly evident when the cells were exposed to concentrations of $\text{CH}_3\text{Hg-Hcy}$ between 50 and 300 μM . Although the overall levels of uptake CH_3Hg^+ tended to be greater in the hOAT1-transfected cells, kinetic analysis revealed that carrier-mediated processes were likely involved in the uptake of CH_3Hg^+ in both types of MDCK cells. Based on analysis of the uptake data by both Michaelis-Menten and Eadie-Hofstee methods, the apparent K_m and V_{max} values for the uptake of CH_3Hg^+ in the hOAT1-transfected MDCK cells were approximately 103 μM and 2127 $\text{pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$, respectively. By contrast, the apparent K_m and V_{max} values for the uptake of CH_3Hg^+ in the wild-type MDCK cells were approximately 125 μM and 1991 $\text{pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$, respectively. Analysis of the component for the uptake of CH_3Hg^+ that could be attributed to hOAT1 (hOAT1 data – wild-type data) revealed the apparent K_m and V_{max} for the uptake of CH_3Hg^+ of approximately 39 μM and 213 $\text{pmol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$, respectively (Fig. 1B).

Time-Dependent Uptake of CH_3Hg^+ . During the initial 60 min, uptake of CH_3Hg^+ was significantly greater in the hOAT1-transfected cells than in the corresponding wild-type cells when the cells were exposed to 5 μM $\text{CH}_3\text{Hg-Hcy}$ (Fig. 2, inset). However, at the times studied after approximately 120 min, uptake of CH_3Hg^+ was significantly greater in the wild-type MDCK cells than in the hOAT1-transfected cells.

Effect of PAH and Probenecid on the Uptake of CH_3Hg^+ . During 60 min of exposure to $\text{CH}_3\text{Hg-Hcy}$, uptake of CH_3Hg^+ was significantly greater in the hOAT1-transfected MDCK cells than in the corresponding wild-type MDCK cells in the experiment assessing the effects of PAH and probenecid on the uptake of CH_3Hg^+ (Fig. 3). In the presence of 1 mM PAH or 200 μM probenecid, uptake of CH_3Hg^+ in the hOAT1-transfected cells was significantly less than that in the corresponding group of hOAT1-transfected cells exposed to only 5 μM $\text{CH}_3\text{Hg-Hcy}$. However, the level of uptake of CH_3Hg^+ in the hOAT1-transfected cells exposed to PAH or probenecid was not significantly different from the level of uptake in the corresponding group of wild-type cells treated in the same manner. There were no signif-

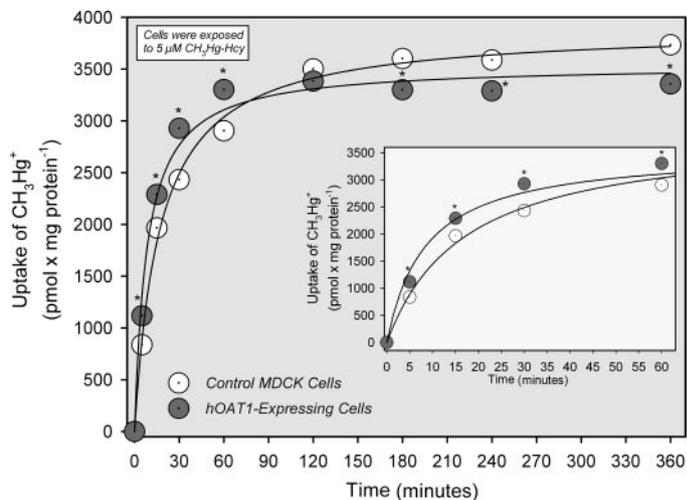


Fig. 2. Time-dependent uptake (picomole \times milligrams of cellular protein $^{-1}$) of CH_3Hg^+ in control and hOAT1-expressing MDCK II cells exposed to 5 μM $\text{CH}_3\text{Hg-Hcy}$. Values are mean \pm S.E. *, significantly different ($p < 0.05$) from the mean for the corresponding group of control MDCK cells.

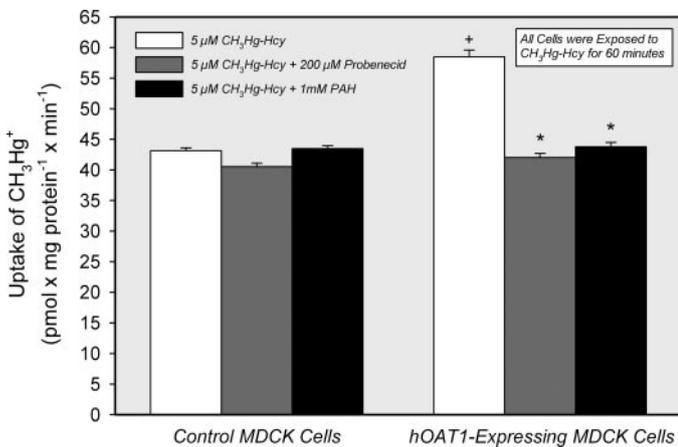


Fig. 3. Effect of 1 mM PAH or 200 μ M probenecid on the uptake (picomole \times milligrams of cellular protein⁻¹) of CH₃Hg⁺ in control and hOAT1-expressing MDCK II cells exposed to 5 μ M CH₃Hg-Hcy for 60 min (at 37°C). Values are mean \pm S.E. *, significantly different ($p < 0.05$) from the mean for the corresponding group of wild-type control or hOAT1-transfected MDCK cells exposed to only 5 μ M CH₃Hg-Hcy. +, significantly different ($p < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells treated in the same manner.

icant differences in the uptake of CH₃Hg⁺ among the three groups of wild-type MDCK cells.

Effect of Dicarboxylates on the Uptake of CH₃Hg⁺. In the experiment designed to assess the influence of dicarboxylates on the uptake of CH₃Hg⁺, uptake of CH₃Hg⁺ was significantly greater in the hOAT1-transfected MDCK cells than in the corresponding wild-type MDCK cells during 60 min of exposure to 5 μ M CH₃Hg-Hcy (Fig. 4). When glutarate or adipate (1 mM) was added to the extracellular medium, uptake of CH₃Hg⁺ in the hOAT1-transfected cells was significantly less than that in the corresponding group of hOAT1-transfected cells exposed to only 5 μ M CH₃Hg-Hcy. In addition, the level of uptake of CH₃Hg⁺ in the hOAT1-transfected cells, exposed to glutarate or adipate, was also significantly less than the level of uptake in the corresponding group of wild-type cells treated in the same manner. No

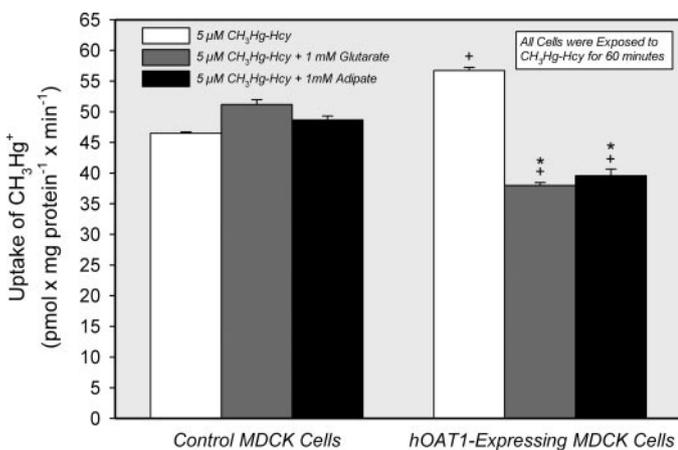


Fig. 4. Influence of 1 mM glutarate or adipate on the uptake (picomole \times milligrams of cellular protein⁻¹) of CH₃Hg⁺ in control and hOAT1-expressing MDCK II cells exposed to 5 μ M CH₃Hg-Hcy for 60 min (at 37°C). Values are mean \pm S.E. *, significantly different ($p < 0.05$) from the mean for the corresponding group of wild-type control or hOAT1-transfected MDCK cells exposed to only 5 μ M CH₃Hg-Hcy. +, significantly different ($p < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells treated in the same manner.

significant differences in the uptake of CH₃Hg⁺ were detected among the three groups of wild-type MDCK cells.

Effect of Temperature on the Uptake of CH₃Hg⁺. Temperature-dependent differences in the extracellular to intracellular transport of CH₃Hg⁺ were detected among the groups of corresponding hOAT1-transfected and wild-type control cells exposed to 5 μ M CH₃Hg-Hcy (Fig. 5). At 37°C, the uptake of CH₃Hg⁺ was significantly greater in the hOAT1-transfected MDCK cells than in the corresponding wild-type control MDCK cells during 1 h of exposure.

When the extracellular temperature was reduced to 21°C, uptake of CH₃Hg⁺ in the hOAT1-transfected and wild-type control MDCK cells was significantly lower than that detected at 37°C. However, their level of uptake CH₃Hg⁺ in the hOAT1-transfected cells remained significantly greater than that in the corresponding wild-type control cells.

Uptake of CH₃Hg⁺ was greatly reduced in both the hOAT1-expressing cells and the wild-type control cells when the temperature of the extracellular medium was reduced to 4°C. Moreover, there was no significant difference in the uptake of CH₃Hg⁺ between the two groups of MDCK cells.

Effect of L-Leucine and/or PAH on the Uptake of CH₃Hg⁺. In the experiment designed to assess the effects of L-leucine and PAH on the uptake of CH₃Hg⁺, uptake of CH₃Hg⁺ in the hOAT1-expressing MDCK cells exposed to only 5 μ M CH₃Hg-Hcy for 15 min was significantly greater than that in the corresponding groups of wild-type cells (Fig. 6). L-Leucine was chosen for this experiment, because it is a transportable substrate of both systems b⁰⁺ and L. In the groups of hOAT1-expressing cells exposed to either 1 mM PAH or 1 mM L-leucine, the uptake of CH₃Hg⁺ was significantly less than that in the group of hOAT1-transfected cells exposed to only 5 μ M CH₃Hg-Hcy. It is noteworthy that the uptake of CH₃Hg⁺ in the group of hOAT1-transfected cells exposed to 1 mM PAH + 1 mM L-leucine was significantly lower than that in any of the other three groups of hOAT1-transfected cells.

Among the control MDCK cells, uptake of CH₃Hg⁺ in the group exposed to 1 mM L-leucine or 1 mM L-leucine + 1 mM

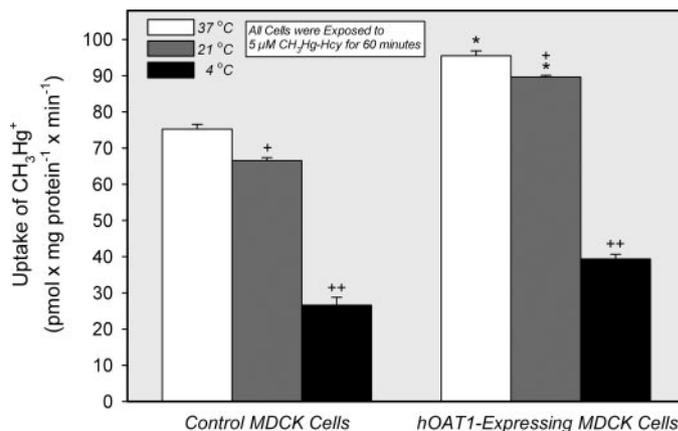


Fig. 5. Effect of temperature on the uptake (picomole \times milligrams of cellular protein⁻¹) of CH₃Hg⁺ in control and hOAT1-expressing MDCK II cells exposed to 5 μ M CH₃Hg-Hcy for 60 min. Values are mean \pm S.E. *, significantly different ($p < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells treated in the same manner. +, significantly different ($p < 0.05$) from the mean for the corresponding group of MDCK cells treated at 37°C. **, significantly different ($p < 0.05$) from the mean for the corresponding group of MDCK cells treated at 37 or 21°C.

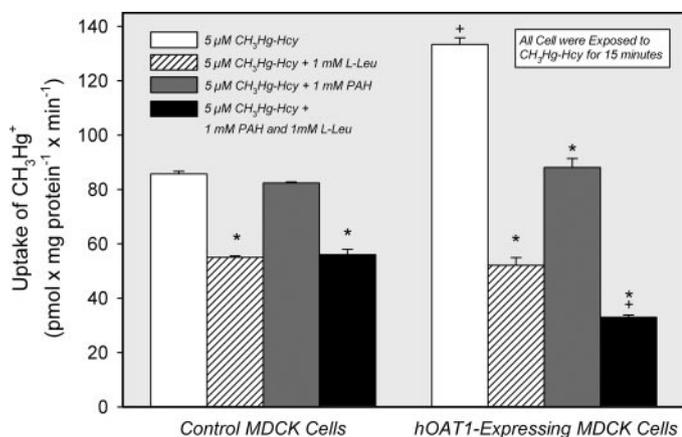


Fig. 6. Effect of adding 1 mM PAH and/or 1 mM L-leucine on the uptake (picomole \times milligrams of cellular protein⁻¹) of CH₃Hg⁺ in control and hOAT1-expressing MDCK II cells exposed to 5 μ M CH₃Hg-Hcy for 30 min (at 37°C). Values are mean \pm S.E. *, significantly different ($p < 0.05$) from the corresponding mean for the group of wild-type control or hOAT1-transfected MDCK cells exposed to only 5 μ M CH₃Hg-Hcy. **, significantly different ($p < 0.05$) from the mean for the corresponding groups of wild-type control or hOAT1-transfected MDCK cells exposed to only 5 μ M CH₃Hg-Hcy or 5 μ M CH₃Hg-Hcy + 1 mM L-leucine (L-Leu). ***, significantly different ($p < 0.05$) from all corresponding means for the groups of wild-type control or hOAT1-transfected MDCK cells. +, significantly different ($p < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells treated in the same manner.

PAH was significantly lower than that in the other two groups of MDCK cells. No significant difference in the uptake of CH₃Hg⁺ was detected between the group of wild-type cells exposed to 1 mM PAH and the group of control MDCK cells exposed to only 5 μ M CH₃Hg-Hcy.

Effect of L-Amino Acids on the Uptake of CH₃Hg⁺. In the experiment designed to assess the effect of various amino acids on the uptake of CH₃Hg⁺, the extracellular-to-intracellular transport of CH₃Hg⁺ was again significantly greater in the hOAT1-transfected MDCK cells exposed to only 5 μ M CH₃Hg-Hcy for 15 min than in the corresponding wild-type control MDCK cells (Fig. 7).

Among the control MDCK cells exposed to CH₃Hg-Hcy, significant effects on the uptake of CH₃Hg⁺ were detected in all groups, with the exception of those exposed to 1 mM L-lysine, L-histidine, or L-methionine. Significant decreases in the uptake of CH₃Hg⁺ were detected in the groups exposed to 1 mM L-Cys, L-leucine, L-isoleucine, L-phenylalanine, or L-tyrosine. By far the greatest decrease in the uptake of CH₃Hg⁺ was detected in the control group of cells exposed to 1 mM Cys. It is noteworthy that enhanced uptake of CH₃Hg⁺ was detected in the control cells exposed to 1 mM alanine, threonine, glutamate, or aspartate.

The addition of 1 mM Cys also had the greatest inhibitory effect on the uptake of CH₃Hg⁺ among the groups of hOAT1-transfected cells. Significant inhibitory effects on the uptake of CH₃Hg⁺ in the hOAT1-transfected cells were also induced by all of the other amino acids studied, although exposure to 1 mM L-alanine, L-threonine, L-glutamate, or L-aspartate had the least inhibitory effects.

Assessment of Toxicity and Cellular Viability. Significant decreases in the cellular viability were detected in both the hOAT1-expressing and control MDCK cells during 6 h of exposure to CH₃Hg-Hcy (Fig. 8). More importantly, significantly greater decreases in survival were detected in the

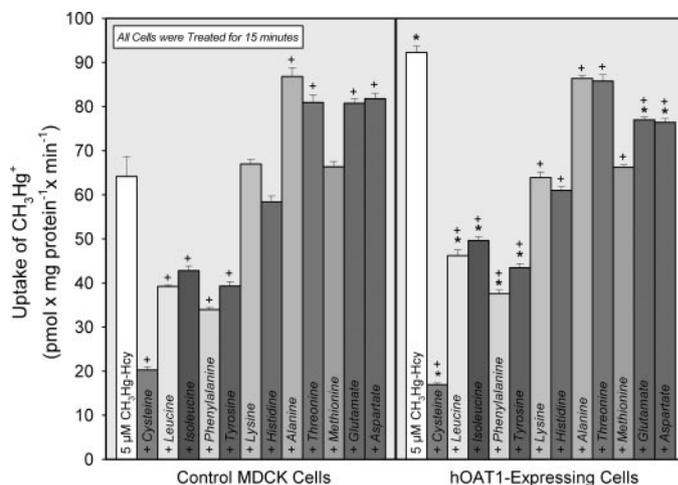


Fig. 7. Influence of extracellular amino acids (1 mM) on the uptake (picomole \times milligrams of cellular protein⁻¹) of CH₃Hg⁺ in control and hOAT1-expressing MDCK II cells exposed to 5 μ M CH₃Hg-Hcy for 60 min (at 37°C). Values are mean \pm S.E. *, significantly different ($p < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells treated in the same manner. +, significantly different ($p < 0.05$) from the mean for the corresponding group of control or hOAT1-expressing MDCK cells exposed to 5 μ M CH₃Hg-Hcy without any additional amino acids added.

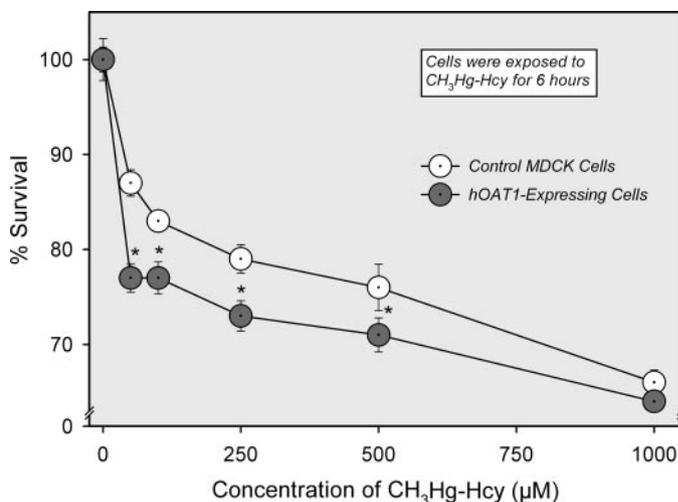


Fig. 8. The percentage of control and hOAT1-expressing MDCK II cells surviving 6 h of exposure to various concentrations of CH₃Hg-Hcy. Values are mean \pm S.E. *, significantly different ($p < 0.05$) from the mean for the corresponding group of control MDCK cells.

hOAT1-expressing cells than in the corresponding control cells at almost all of the CH₃Hg-Hcy concentrations studied.

Discussion

It is well documented that the kidneys are the primary sites in the body where Hg²⁺ is taken up and accumulated, with the preponderance of this accumulation occurring in proximal tubular epithelial cells (Zalups and Barfuss, 1990; Zalups, 1991a,b, 2000). Moreover, both luminal and basolateral mechanisms have been shown to be involved in the proximal tubular transport and handling of inorganic mercuric ions (Zalups, 1995, 1997, 1998a,b; Zalups and Barfuss, 1995, 1998a,b, 2002; Zalups and Minor, 1995; Zalups and Lash, 1997; Zalups et al., 1998).

At the basolateral membrane, one or more PAH-sensitive

transporter(s) have been implicated in the uptake of Hg^{2+} in vivo (Zalups, 1995, 1998a,b; Zalups and Barfuss, 1995, 2002). Because PAH is a substrate of OAT1 (Pritchard and Miller, 1996), we have been designing experiments to test the hypothesis that OAT1 is involved in the basolateral uptake of Hg^{2+} in vivo (Zalups, 1995, 1997, 1998a,b; Zalups and Barfuss, 1995, 1998a,b, 2002; Zalups and Minor, 1995; Zalups and Lash, 1997; Zalups et al., 1998).

In blood, mercuric ions have a very high predilection to bind to the reduced sulfur atom of sulfhydryl groups on selective proteins, such as albumin, and nonprotein thiols, such as Cys, GSH, NAC, and Hcy. This particular binding characteristic of mercuric ions has led us to hypothesize that mercuric conjugates of nonprotein thiols in plasma serve as transportable substrates for OAT1. Recent data obtained from isolated perfused proximal tubular segments and from wild-type and hOAT1-transfected type II MDCK cells demonstrate that NAC, Cys, and Hcy *S*-conjugates of Hg^{2+} are indeed transportable substrates of OAT1 (Zalups and Barfuss, 2002; Aslamkhan et al., 2003; Zalups and Ahmad, 2004; Zalups et al., 2004), thus supporting our hypothesis.

Because Hcy can also serve as a potential binding ligand for organic mercuric ions, we designed the present study to test the hypothesis that the Hcy *S*-conjugate of methylmercury, $\text{CH}_3\text{Hg-Hcy}$, is an additional mercuric species that can be transported by OAT1. Before testing this hypothesis, we first confirmed that expression and membrane-insertion of a fully functional hOAT1 protein was occurring in the hOAT1-transfected MDCK II cells. This was accomplished by demonstrating concentration- and time-dependent extracellular-to-intracellular transport of PAH in only the hOAT1-transfected cells and by confirming that probenecid or small dicarboxylates inhibited the uptake of PAH.

The findings from the present study clearly show that, in addition to gaining the ability to transport PAH, MDCK II cells transfected stably with hOAT1 also gain the ability to transport CH_3Hg^+ in the form of $\text{CH}_3\text{Hg-Hcy}$. Analysis of saturation kinetics, time and temperature dependence, and substrate specificity in the transfected and wild-type cells provide additional supporting evidence that $\text{CH}_3\text{Hg-Hcy}$ is a transportable substrate of hOAT1. In particular, the uptake of $\text{CH}_3\text{Hg-Hcy}$ in the MDCK cells expressing hOAT1 was inhibited partially by PAH, probenecid, adipate, or glutarate; all of which have been shown to be substrates of hOAT1 (Zalups and Barfuss, 2002; Aslamkhan et al., 2003; Zalups and Ahmad, 2004; Zalups et al., 2004). Thus, these data indicate that a PAH-, probenecid-, and dicarboxylate-sensitive component for the uptake of $\text{CH}_3\text{Hg-Hcy}$ was afforded exclusively to hOAT1-expressing cells.

It is noteworthy that a significant level of uptake of $\text{CH}_3\text{Hg-Hcy}$ also occurred in the wild-type control MDCK cells, which was not linked to a mechanism sensitive to PAH, probenecid, adipate, or glutarate. Thus, the wild-type MDCK cells express at least one transport system promoting the extracellular-to-intracellular transport of $\text{CH}_3\text{Hg-Hcy}$ that is not OAT1. Substrate-specificity analyses revealed that this separate mechanism is linked to the activity of one or more amino acid transporters native to MDCK cells. The first set of data implicating an amino acid transporter in the uptake of $\text{CH}_3\text{Hg-Hcy}$ (in both types of MDCK cells) is the set that shows the effects of PAH and/or L-leucine on the uptake of $\text{CH}_3\text{Hg-Hcy}$ (Fig. 8). Inhibition in the uptake of $\text{CH}_3\text{Hg-Hcy}$

in the wild-type cells occurred only during the exposure to 1 mM L-leucine. However, in the hOAT1-expressing cells, exposure to either 1 mM PAH or 1 mM L-leucine inhibited the uptake of $\text{CH}_3\text{Hg-Hcy}$. More importantly, exposure of the hOAT1-expressing cells to both 1 mM PAH and 1 mM L-leucine had an additive effect on inhibiting the uptake of $\text{CH}_3\text{Hg-Hcy}$. Therefore, these data indicate that a combined effect of hOAT1 and one or more amino acid transporters was responsible for the overall level of uptake of $\text{CH}_3\text{Hg-Hcy}$ in the hOAT1-expressing cells. Additional substrate-specificity data (presented in Fig. 7) also implicate the role of one or more amino acid transporters in the uptake $\text{CH}_3\text{Hg-Hcy}$ in both types of MDCK cells. More specifically, exposure to 1 mM L-Cys, L-leucine, L-isoleucine, L-phenylalanine, or L-tyrosine significantly inhibited the uptake of $\text{CH}_3\text{Hg-Hcy}$ in the wild-type and hOAT1-expressing MDCK cells. It is noteworthy that the addition of L-Cys to the extracellular compartment had the greatest inhibitory effect on the transport of $\text{CH}_3\text{Hg-Hcy}$ in both cell-types. By contrast, exposure to 1 mM L-alanine, L-threonine, L-glutamate, or L-aspartate enhanced the uptake of $\text{CH}_3\text{Hg-Hcy}$ in the wild-type MDCK cells but slightly inhibited the uptake of $\text{CH}_3\text{Hg-Hcy}$ in the hOAT1-expressing cells. The reason for the disparity in these findings between the wild-type and hOAT1-expressing cells is not known at present but is likely related to the activity of hOAT1.

An additional variable in the handling of the Hcy *S*-conjugates of CH_3Hg^+ by the MDCK cells relates to the use of the racemic mixture of Hcy. Accordingly, one might expect that the handling the *D*-isomer of the Hcy *S*-conjugate of CH_3Hg^+ (if it proves to be a transportable substrate) is mediated by one or more amino acid transporters different from those involved in the handling of the *L*-isomeric form.

Involvement of amino acid transporters in the uptake of CH_3Hg^+ in wild-type and hOAT-1 transfected MDCK cells has also been documented recently in cells exposed to $\text{CH}_3\text{Hg-NAC}$ (Zalups and Ahmad, 2005a) or $\text{CH}_3\text{Hg-Cys}$ (Zalups and Ahmad, 2005b). A summary of the concentration-dependent uptake of $\text{CH}_3\text{Hg-NAC}$, $\text{CH}_3\text{Hg-Cys}$, and $\text{CH}_3\text{Hg-Hcy}$ in both wild-type and hOAT-1-transfected MDCK cells is provided in Fig. 9. Quite surprisingly, this figure shows that

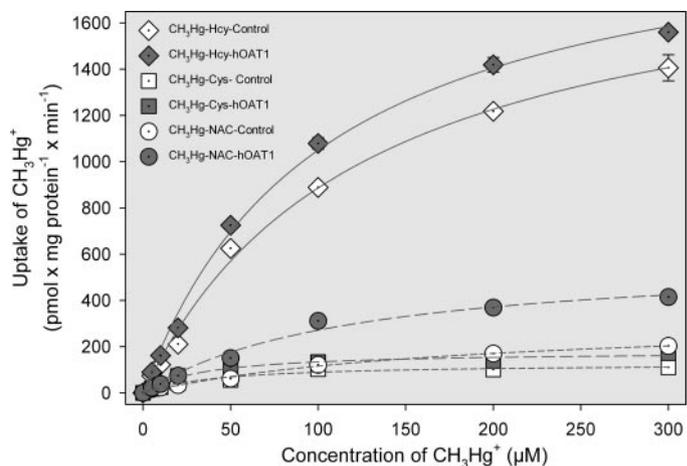


Fig. 9. Concentration-dependent uptake (picomole \times milligrams of cellular protein $^{-1}$) of CH_3Hg^+ in control and hOAT1-expressing MDCK II cells exposed to various concentrations of $\text{CH}_3\text{Hg-NAC}$, $\text{CH}_3\text{Hg-Cys}$, or $\text{CH}_3\text{Hg-Hcy}$. Note the great differences in the level of uptake of $\text{CH}_3\text{Hg-Hcy}$ relative to the uptake of the other methylmercuric conjugates.

the maximal rates of uptake of CH_3Hg^+ in the control and hOAT1-transfected cells exposed to $\text{CH}_3\text{Hg-Hcy}$ are between 3- and 15-fold greater than those in wild-type and hOAT1-transfected MDCK cells exposed to $\text{CH}_3\text{Hg-NAC}$ or $\text{CH}_3\text{Hg-Cys}$. Although hOAT1 seems to participate significantly in the uptake of each thiol *S*-conjugate of CH_3Hg^+ in the hOAT1-transfected cells, the fraction of the total level of uptake that can be attributed to the activity of hOAT1 varies greatly depending on thiol *S*-conjugate. For example, the activity of hOAT1 seems to contribute to no more than ~15% of the total level of uptake of CH_3Hg^+ when the hOAT1-transfected cells are exposed to $\text{CH}_3\text{Hg-Hcy}$. By contrast, the activity of hOAT1 in the transfected cells contributed to approximately 50% of the total level of uptake of CH_3Hg^+ when the cells were exposed to $\text{CH}_3\text{Hg-NAC}$.

It should be stressed that the transport findings implicating amino acid transporters and hOAT1 in the transfected cells were obtained mainly from apically exposed cells. In vivo, OAT1 is expressed almost exclusively at the basolateral plasma membrane. Thus, some of the interactions detected between amino acid transporters and OAT1 in the present study may prove to be different in proximal tubular epithelial in vivo. Moreover, the complement of amino acid transporters present in apical and basolateral plasma membranes of the MDCK cells is also different from that present in proximal tubular epithelial cells. Despite these differences, our collective findings do implicate roles for both amino acid transporters and hOAT1 in the transport of various methylmercuric species.

It is noteworthy to mention that molecular mimicry has been suggested to be a potential mechanism involved in the uptake of the Cys *S*-conjugate of CH_3Hg^+ in glial and endothelial cells (Aschner et al., 1990, 1991; Kerper et al., 1992; Clarkson, 1993; Mokrzan et al., 1995). It has been suggested that $\text{CH}_3\text{Hg-S-Cys}$ serves as a molecular mimic of the amino acid methionine at one or more neutral amino acid transporters (Clarkson, 1993). Recent data obtained from *Xenopus laevis* oocytes expressing (the neutral amino acid transporters) LAT1 or LAT2 provide evidence supporting this hypothesis (Simmons-Willis et al., 2002). Based on the present findings, molecular mimicry may also serve as a mechanism in the uptake of $\text{CH}_3\text{Hg-Hcy}$ in renal epithelial cells, such as MDCK cells. However, because methionine had mixed effects on the uptake of CH_3Hg^+ in the two populations of MDCK cells, $\text{CH}_3\text{Hg-Hcy}$ may not be a molecular mimic of methionine at the transporter(s) responsible for the uptake of $\text{CH}_3\text{Hg-Hcy}$. This does not necessarily preclude the possibility that LAT1 and/or LAT2 are potential transporters involved in the uptake of $\text{CH}_3\text{Hg-Hcy}$ in the MDCK cells, because other substrates of system L, such as L-leucine, L-isoleucine, and L-phenylalanine, were able to significantly inhibit the uptake of this mercuric conjugate. Because system L is colocalized with OAT1 on the basolateral plasma membrane of proximal tubular epithelial cells, this amino acid transport system may serve as an additional mechanism involved in the basolateral uptake of $\text{CH}_3\text{Hg-Hcy}$ in vivo. It is also possible that molecular mimicry is involved in the uptake of $\text{CH}_3\text{Hg-Hcy}$ by OAT1, because this transport protein has a broad range of substrate specificity. Clearly, additional studies are warranted to better define the specific mechanisms involved in the uptake of $\text{CH}_3\text{Hg-Hcy}$ by OAT1 and amino acid transporters.

Significant decreases in the survival of both wild-type control and hOAT1-expressing cells were documented after exposure to various toxic concentrations of $\text{CH}_3\text{Hg-Hcy}$. However, the percentage of cells surviving 6 h of exposure was significantly greater in the wild-type control cells than in the hOAT1-expressing cells. It is interesting that the accumulation of CH_3Hg^+ tended to be greater in the wild-type cells than in the transfected cells after 3 h of exposure. This pattern of accumulation can be explained by the increased level of cellular death in the transfected cells. Initially, as the transfected cells accumulated greater amounts of CH_3Hg^+ , cellular death was induced more rapidly in this population of cells, which in turn resulted in an increased cellular release of CH_3Hg^+ back into the bathing medium. Overall, the toxicological findings provide additional support for the hypothesis that at least two mechanisms were involved in the uptake of $\text{CH}_3\text{Hg-Hcy}$ in the hOAT1-expressing cells, because the amount of cell death induced in both populations of cells correlates with the level of extracellular to intracellular transport of $\text{CH}_3\text{Hg-Hcy}$.

The risk of humans and other organisms exposed and intoxicated by CH_3Hg^+ continues to increase because of pervasive contamination of the environment with various forms of mercury (Zalups, 2000). Burning of fossil fuels is a leading cause of contamination of the environment with mercury. Much of the Hg^{2+} released into the atmosphere during the burning of fossil fuels is eventually converted to CH_3Hg^+ by microorganisms in the soil and water. As the risk of humans exposed to mercury increases, it becomes paramount that scientists and health care providers understand how the various chemical forms of this metal affect target organs and tissues (after they gain entry into systemic circulation) so that better strategies to treat exposed or intoxicated individuals can be developed.

In summary, the findings from the present study represent the first line of direct molecular evidence implicating the basolateral organic anion/dicarboxylate transporter 1 and amino acid transport proteins in the renal cellular uptake of and intoxication by the Hcy *S*-conjugate of CH_3Hg^+ . More importantly, the present findings indicate that $\text{CH}_3\text{Hg-Hcy}$ is likely an important organic mercuric species that is taken up in vivo at the basolateral membrane of renal proximal tubular epithelial cells by both OAT1 and/or selective amino acid transporters.

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Address correspondence to: Dr. Rudolfs K. Zalups, Division of Basic Medical Sciences, Mercer University School of Medicine, 1550 College Street, Macon, GA 31207. E-mail: zalups_rk@mercer.edu
