FUNCTIONAL CHARACTERIZATION OF HUMAN MONOCARBOXYLATE TRANSPORTER 6 (SLC16A5)

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ABSTRACT:

Human monocarboxylate transporter 6 (MCT6; SLC16A5) is a member of the MCT family, which comprises 14 members (Halestrap and Meredith, 2004) and was first cloned from a placenta cDNA library (Price et al., 1998). MCT6 shares 38% identity with MCT4 at the amino acid level. Northern blot analysis revealed that MCT6 mRNA is highly expressed in the kidney and placenta (Price et al., 1998), but expression at the protein level and the function of MCT6 are still unknown.

The first member of the MCT family was cloned from a mutated Chinese hamster ovary cell line with enhanced mevalonate uptake (Kim et al., 1992). Subsequently, the wild-type protein was demonstrated to catalyze proton-linked transport of L-lactic acid and pyruvic acid and was named monocarboxylate transporter 1 (MCT1) (Garcia et al., 1994a). Based on the nucleotide sequence of hamster MCT1, MCT1 orthologs have been cloned from human, rat, and mouse (Garcia et al., 1994b; Jackson et al., 1995; Takanaga et al., 1995; Carpenter et al., 1996). It has been reported that MCT1 cotransports H+ with L-lactic acid (Kim et al., 1992; Bröer et al., 1997, 1998). MCT1 (SLC16A1), MCT2 (SLC16A7), MCT3 (SLC16A8), and MCT4 (SLC16A3) all transport both L-lactic acid and pyruvic acid (Lin et al., 1998; Grollman et al., 2000; Manning Fox et al., 2000). T-type amino acid transporter 1 (TAT1, SLC16A10), another member of the MCT family, transports aromatic amino acids such as L-tryptophan and succinic acid did not inhibit the MCT6-mediated uptake of [3H]bumetanide. In contrast, short-chain carboxylic acids, such as L-lactic acid and succinic acid did not inhibit the MCT6-mediated uptake of bumetanide. These results suggest that the substrate specificity of MCT6 is distinct from those of other MCTs. Bumetanide would be a good tool for investigating the functional properties of MCT6. It is probable that MCT6 is involved in the disposition of various drugs, including bumetanide.

Materials and Methods

Materials. [3H]Bumetanide (1.85 GBq/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [3H]Probenecid was kindly provided by Fuji Biomedix Co., Ltd. (Saitama, Japan). [14C]Nateglinide and nateglinide were kindly supplied by Ajinomoto Co., Inc. (Tokyo, Japan). Nonradioactive bumetanide was purchased from MP Biomedicals (Irvine, CA). Azosemide, torasemide, and piretanide were kindly supplied by Sanwa Kagaku Kenkyusho Co. (Nagoya, Japan), Taisho Toyama Pharmaceutical Co. (Tokyo, Japan), and Nippon HEXAL Co. (Tokyo, Japan), respectively. Furosemide was purchased from Wako Pure Chemicals (Osaka, Japan). pSP64T-mKV1.3-EC plasmid was obtained from American Type Culture Collection (Manassas, VA). All other chemicals were of the highest grade commercially available.

Methods. Construction of pSP64T-hMCT6. pSP64T vector containing a BglII restriction site was constructed from the pSP64T-mKV1.3-EC plasmid by digesting with BstE II, blunting with Klenow fragment, and ligating with a BglII linker. Next, pSP64T was digested with BglII and ligated with a NotI linker.

ABBREVIATIONS: MCT, monocarboxylate transporter; TAT1, T-type amino acid transporter 1; PCR, polymerase chain reaction; pH, intracellular pH; pHi, external pH; ES, estrone-3-sulfate; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; PAH, p-aminohippuric acid.
Uptake of each compound in MCT6-expressing oocytes or uninjected control oocytes was measured at room temperature and pH 7.4 or 6.0 for 30 min. Each value represents the mean ± S.E.M. of three to five separate experiments. Significance of differences between the uptake in uninjected oocytes and MCT6-expressing oocytes was determined by the unpaired Student’s t test (*p < 0.05).

The plasmid containing MCT6 cDNA (clone KAT01693; Genbank accession no. AK000416) was obtained from New Energy and Industrial Technology Development Organization (NEDO) human cDNA project. MCT6 cDNA was amplified by polymerase chain reaction (PCR). PCR primers were designed to contain the 5′- and 3′-untranslated region sequences of MCT6. The forward primer was 5′-AAG ATT AAC CGG CCG CAA GAA AAG AGC ACT TCC CTG C-3′, and the reverse primer was 5′-TGA GGC CGG GGT CAA TTT GCC CAA CTC-3′. Each primer contains a Nol restriction site (underlined). The PCR products were digested with Nol and ligated into the pSp64T-Nol vector. Finally, we obtained pSp64T-mMCT6. The identity of products was confirmed by means of sequencing.

cRNA Synthesis and Preparation of Xenopus oocytes. The capped cRNA of MCT6 was transcribed in vitro from BamHI-linearized pSP64T-hMCT6 with SP6 RNA polymerase. Oocytes were prepared from ovaries removed from female Xenopus laevis as previously described (Kobayashi et al., 2004). Oocytes (stages V–VI) were injected with cRNA and incubated in Barth’s solution.

Table 1 by uninjected oocytes was essentially the same as that of oocytes injected with water both at pH 7.4 and 6.0, uninjected oocytes were used as the control instead of water-injected oocytes throughout this study. Values of uptake of [3H]bumentanide, [3H]probenecid, [3H]prostaglandin F2α, and [3H]nateglinide by uninjected oocytes at pH 7.4 were 0.06 ± 0.01, 0.51 ± 0.03, 0.19 ± 0.02, and 0.25 ± 0.02 (mean ± S.E., three separate experiments), respectively, and were not significantly different from those by water-injected oocytes: 0.10 ± 0.01, 0.58 ± 0.01, 0.26 ± 0.02, and 0.26 ± 0.01, respectively. The uptake by oocytes (uptake, μl/oocyte) is presented as the ratio of radioactivity in the sample (dpm/oocyte) to the initial concentration in the uptake buffer (dpm/μl).

MCT6-specific uptake was calculated by subtraction of the uptake by uninjected oocytes from that by MCT6 cRNA-injected oocytes. To estimate the kinetic parameters of the saturable uptake via MCT6, the Michaelis-Menten eq. 1 was fitted to the experimental data by means of nonlinear least-squares regression analysis (Yamaoka et al., 1981).

\[ JIC = J_{max}(K + C) \]

where J and C represent the uptake rate (pmol/oocyte/30 min) and the concentration of substrate (μM), respectively, J_{max} and K represent the maximum uptake rate for the saturable component and the half-maximal concentration.

For inhibition curves, the following sigmoidal inhibition eq. 2 was fitted to the data by means of nonlinear least-squares regression analysis.

\[ E = 100 - 100 \cdot C/(IC_{50} + C) \]

where E, IC_{50}, and C represent the relative uptake rate of substrate (percentage of control), the concentration of inhibitor which gives 50% inhibition, and the concentration of inhibitor, respectively.

**Statistical Analysis.** Statistical analysis was performed using the unpaired Student’s t test or analysis of variance followed by Dunnett’s test. A difference between means was considered statistically significant when the P value was less than 0.05.

**Results**

Transport of Organic Compounds via MCT6. [14C]Bumetanide was found to be taken up specifically by oocytes injected with MCT6 cRNA (MCT6-expressing oocytes) in comparison with uninjected control oocytes, showing that bumetanide is a substrate of MCT6. We therefore examined the uptake of various compounds via MCT6 using bumetanide as positive control. Uptake of [3H]estrone-3-sulfate [ES]; a substrate of organic anion transporter (OAT) 3, OAT4, and organic anion transporting polypeptide (OATP) 1A2, OATP1B1, OATP1B3, OATP2B1, and OATP3A1] (Kullak-Ublick et al., 2001; Hagenbuch and Meier, 2004), [14C]l-phenylalanine, [14C]salicylic acid, [3H]hexanoic acid, and [3H]-nateglinide by MCT6-expressing oocytes was similar to that by control oocytes at both pH 7.4 and 6.0 (Table 1). The uptake of [3H]valproic acid...
acid was lower than that by control oocytes. However, uptake of [14C]nateglinide or [3H]prostaglandin F2α by MCT6-expressing oocytes was significantly higher than that by control oocytes (Table 1).

The uptake rate of [14C]nateglinide at 8 μM was higher than that at 57 μM but still lower than that of [3H]bumetanide (Table 1). The values of uptake of [3H]probenecid by MCT6-expressing oocytes at pH 7.4 and 6.0 were significantly higher and lower, respectively, than those by control oocytes (Table 1). MCT6 did not mediate the uptake of [14C]L-lactic acid, a typical substrate of MCT1-MCT4, or [3H]tryptophan, a typical substrate of TAT1 (data not shown). Uptake of [3H]bumetanide by MCT1- and MCT4-expressing oocytes were similar to that by control oocytes (data not shown).

**Functional Characteristics of MCT6.** The uptake of [3H]bumetanide via MCT6 linearly increased up to 60 min and reached a steady state at 90 to 120 min (Fig. 1A). In the following experiments, the uptake was examined at 30 min. When the concentration dependence of [3H]bumetanide uptake by MCT6-expressing oocytes was examined, the uptake was saturable with regard to substrate concentration (Fig. 1B). Kinetic analysis with the Michaelis-Menten eq. 1 yielded a $K_t$ value of 84.2 (56.3, 125.9; mean ± S.D., mean ± S.D.) μM and a $J_{max}$ value of 45.2 ± 21.88 pmol/oocyte/30 min (mean ± S.D. of ten separate experiments). Figure 1C shows the effect of extracellular pH on the uptake of [3H]bumetanide via MCT6. At each pH between 8.0 to 5.5, the uptake by the MCT6-expressing oocytes was higher than that by control oocytes. The uptake rate of [3H]bumetanide via MCT6 increased with decreasing extracellular pH.

The $K_t$ and $J_{max}$ values at pH 6.0 were 83.4 (64.9, 107.2; mean ± S.D., mean ± S.D.) μM and 83.1 ± 27.0 pmol/oocyte/30 min (mean ± S.D. of four separate experiments), respectively. Although the $K_t$ values were comparable at pH 7.4 and 6.0, the $J_{max}$ value at pH 6.0 was about twice that at pH 7.4. Therefore, the increase of uptake activity of MCT6 for [3H]bumetanide at acidic extracellular pH can be ascribed to an increased maximum transport rate, rather than to increased affinity for MCT6.

When NaCl in uptake buffer was replaced with N-methyl-D-glucamine, mannitol, or sodium gluconate to investigate the involvement of sodium ion and chloride ion, there was no significant change of uptake of [3H]bumetanide by MCT6 at pH 7.4 (Table 2). We also examined the effect of membrane potential on the uptake of [3H]bumetanide by MCT6. Under the condition of intracellular K concentration ([K]in) attained by preincubation with the high-potassium buffer containing 10 mM NaCl (control) 100

<table>
<thead>
<tr>
<th>Uptake</th>
<th>% of control</th>
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<tbody>
<tr>
<td>NaCl (control)</td>
<td>100</td>
</tr>
<tr>
<td>NaCl--free</td>
<td>97.6 ± 7.5</td>
</tr>
<tr>
<td>N-Methyl-D-glucamine</td>
<td></td>
</tr>
<tr>
<td>NaCl-- and Cl--free</td>
<td>128.9 ± 19.2</td>
</tr>
<tr>
<td>Mannitol</td>
<td></td>
</tr>
<tr>
<td>Cl--free</td>
<td>130.2 ± 16.7</td>
</tr>
</tbody>
</table>

TABLE 2

Effects of extracellular Na+, and K+, and Cl- on the uptake of [3H]bumetanide by MCT6

The uptake of [3H]bumetanide into MCT6-expressing oocytes or uninjected control oocytes was performed at room temperature and pH 7.4 for 30 min in the standard uptake buffer (NaCl) or Na+-- and/or Cl--free buffer. Each value represents the mean ± S.E.M. of three separate experiments.

For the figures, the solid line in B represented the fitting line obtained from the data fitted with eq. 1 using nonlinear least-squares regression analysis.

**Fig. 1.** Time course (A), concentration dependence (B), and pH dependence (C) of the uptake of [3H]bumetanide by MCT6. Uptake of 0.1 μM [3H]bumetanide into MCT6-expressing (closed circles; A and C) or uninjected control oocytes (open circles) was measured at room temperature and pH 7.4 (A and B) for 30 min (C). MCT6-mediated uptake (open squares) was calculated by subtracting the uptake amount of control oocytes from that of MCT6-expressing oocytes. Each point represents the mean ± S.E.M. ($n = 3$–$5$; * $p < 0.05$ versus uninjected). The solid line in B represented the fitting line obtained from the data fitted with eq. 1 using nonlinear least-squares regression analysis.
valinomycin, the uptake of \[^{3}H\]bumetanide by MCT6 was significantly higher than that in standard uptake buffer (2.5 mM K\(^+\)) or the high-potassium uptake buffer (120 mM K\(^+\)) containing 10 \(\mu\)M valinomycin at pH 7.4 for 30 min, uptake of \[^{3}H\]bumetanide into MCT6-expressing or un.injected control oocytes for 30 min was measured in the same buffer. MCT6-mediated uptake is shown (mean ± S.E.M., three separate experiments; *, p < 0.05 versus control). B, after preincubation in the high-potassium uptake buffer (120 mM K\(^+\)) containing 10 \(\mu\)M valinomycin at pH 7.4 for 30 min, uptake of \[^{3}H\]bumetanide into MCT6-expressing or uninjected control oocytes for 10 min was measured at pH 7.4, 6.0, or 5.5. MCT6-mediated uptake is shown (mean ± S.E.M., n = 5). C and D, after preincubation, uptake of \[^{14}C\]lactic acid by MCT1-expressing oocytes (C) and uptake of \[^{3}H\]bumetanide by MCT6-expressing oocytes was measured under each condition as follows: open triangles, preincubation and uptake buffers were the high-potassium buffer containing 10 \(\mu\)M valinomycin at pH 7.4 ([K\(^+\)]\(_{in}\) = [K\(^+\)]\(_{out}\), pH\(_i\) = pH\(_o\)); closed triangles, preincubation and uptake buffers were the high-potassium buffer containing 10 \(\mu\)M valinomycin and 20 \(\mu\)M nigericin at pH 7.4 ([K\(^+\)]\(_{in}\) = [K\(^+\)]\(_{out}\), pH\(_i\) = pH\(_o\)); open circles, after preincubation in the high-potassium buffer containing 10 \(\mu\)M valinomycin at pH 7.4, uptake was measured in the high-potassium buffer containing 10 \(\mu\)M valinomycin at pH 6.0 ([K\(^+\)]\(_{in}\) = [K\(^+\)]\(_{out}\), pH\(_i\) > pH\(_o\)); closed circles, preincubation and uptake buffers were the high-potassium buffer containing 10 \(\mu\)M valinomycin and 20 \(\mu\)M nigericin at pH 6.0 ([K\(^+\)]\(_{in}\) = [K\(^+\)]\(_{out}\), pH\(_i\) = pH\(_o\)). MCT1- or MCT6-mediated uptake is shown (mean ± S.E.M., n = 5; *, p < 0.05 versus the value in the absence of nigericin).

We also examined the effect of HCO\(_3^-\) on the MCT6-mediated \[^{3}H\]bumetanide uptake under the condition of high extracellular K\(^+\) concentration. The uptake of \[^{3}H\]bumetanide via MCT6 for 30 min after preincubation in high K\(^+\) and HCO\(_3^-\)-containing buffer for 30 min was 0.91 ± 0.11 \(\mu\)l/oocyte (mean ± S.E., n = 5) and was not significantly different from that after preincubation in high K\(^+\) but not HCO\(_3^-\)-containing buffer, 0.95 ± 0.06 \(\mu\)l/oocyte.

**Inhibitory Effect of Various Compounds on the Uptake of Bumetanide Mediated by MCT6.** Figure 3 shows the inhibitory effects of several loop diuretics (furosemide, piretanide, azosemide, and torasemide) on the uptake of \[^{3}H\]bumetanide. All of these drugs concentration-dependently inhibited the MCT6-mediated uptake of \[^{3}H\]bumetanide. The IC\(_{50}\) values of furosemide, piretanide, azosemide, and torasemide were 46.1 ± 16.4, 163.2 ± 74.7, 20.8 ± 5.7,
and 13.0 ± 9.4 μM, respectively (estimate ± S.E.). Table 3 shows the inhibitory effects of various compounds other than loop diuretics on the MCT6-mediated uptake of [3H]bumetanide. L-Lactic acid (a typical substrate of TAT1), pyruvic acid (a substrate of MCT1, MCT2, and MCT4), succinic acid, and citric acid did not affect the MCT6-mediated uptake of [3H]bumetanide. The effects of cationic compounds were subsequently examined. The MCT6-mediated uptake of [3H]bumetanide was inhibited by cimetidine but not by choline. In addition, PAH and ES inhibited the MCT6-mediated uptake of [3H]bumetanide. Thiazides, probenecid, glibenclamide, and nateglinide also inhibited the uptake of [3H]bumetanide.

**Discussion**

Price et al. (1998) identified human MCT6 cDNA and examined the tissue distribution of MCT6 mRNA by Northern blot analysis, but the present study is the first to describe the functional characteristics of MCT6. We found that MCT6 transports bumetanide, but not L-lactic acid, a typical substrate of MCT1-MCT4, or L-tryptophan, a typical substrate of TAT1. In contrast, MCT1 and MCT4 transported L-lactic acid but not bumetanide. These results suggest that the substrate specificity of MCT6 is different from those of other MCTs, such as MCT1-MCT4 and TAT1. The substrates of other MCTs are endogenous compounds such as L-lactic acid, thyroid hormone, and aromatic amino acids (Halestrap and Meredith, 2004), and there is no evidence that the other MCTs mediate the transport of therapeutic drugs. In contrast, MCT6 mediated the transport of bumetanide, probenecid, and nateglinide and was inhibited by various drugs (Tables 1 and 3). Therefore, it seems likely that MCT6 participates in the membrane transport of various drugs. There are similar peptide sequences of rat (ex. XM_346905) and mouse (ex. XM_126601) in the GenBank database (http://www.ncbi.nlm.nih.gov). Further studies will be required to elucidate the difference in MCT6-mediated drug transport between humans and rodents. In addition, the physiological and pharmacokinetic roles of human MCT6 remain to be elucidated.

The uptake of [3H]probenecid by MCT6-expressing oocytes at pH 6.0 was lower than that by control oocytes. Further investigation is necessary to compare the properties of probenecid uptake by MCT6 with those of bumetanide uptake and to understand the action of pH on the transport activity of MCT6.

MCT6 did not transport PAH and ES, typical substrates of organic anion transporters such as OATs and OATPs, which are involved in

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**TABLE 3**

Inhibitory effects of anions and cations on the uptake of [3H]bumetanide by MCT6

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (μM)</th>
<th>pH 7.4</th>
<th>pH 6.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Aminophenyl mercaptan</td>
<td>5</td>
<td>123.8</td>
<td>76.8</td>
</tr>
<tr>
<td>Chlorothiazide</td>
<td>0.5</td>
<td>52.0</td>
<td>77.5</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>0.5</td>
<td>71.0</td>
<td>68.6</td>
</tr>
<tr>
<td>Trichlormethiazide</td>
<td>0.5</td>
<td>20.7</td>
<td>64.2</td>
</tr>
<tr>
<td>Probenecid</td>
<td>0.5</td>
<td>46.3</td>
<td>82.6</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>0.1</td>
<td>31.3</td>
<td>56.3</td>
</tr>
<tr>
<td>Nateglinide</td>
<td>0.001</td>
<td>40.8</td>
<td>47.1</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. control.
mediated uptake of [3H]bumetanide (Fig. 3), as did several thiazides, semide, piretanide, azosemide, and torasemide, inhibited the MCT6-inhibited by PAH and ES (Table 3). Kinetic analysis of the inhibitory

The is expressed predominantly in the kidney (Price et al., 1998), MCT6 side and hOAT4 localized on the apical side of the proximal tubules suggested to involve hOAT1 to hOAT3 localized on the basolateral this hypothesis, further electrophysiological studies are needed. The transepithelial transport of bumetanide in the kidney has been suggested to involve hOAT1 to hOAT3 localized on the apical side of the proximal tubules (Hasannejad et al., 2004; Kobayashi et al., 2005). Since MCT6 mRNA is expressed predominantly in the kidney (Price et al., 1998), MCT6 may be involved in the renal secretion or reabsorption of bumetanide. The $K_v$ values of bumetanide for hOAT2, hOAT3, and hOAT4 were 7.52, 1.59, and 0.31 $\mu$M, respectively (Hasannejad et al., 2004; Kobayashi et al., 2005), but the affinity of bumetanide for MCT6 ($K_v$ at pH 7.4: $84 \mu$M) was lower. The clinical plasma concentration and plasma protein binding of bumetanide were reported to be about 0.27 $\mu$M and 95%, respectively (Davies et al., 1974; Pentikainen et al., 1977). Consequently, the estimated plasma unbound concentration of bumetanide is 0.014 $\mu$M, which is lower than $K_v$ values of MCT6, hOAT2, hOAT3, and hOAT4. Therefore, none of these transporters should be saturated, and they are all possibly involved in the renal secretion and/or reabsorption of bumetanide. The renal localization of MCT6 and its quantitative contribution to the renal secretion and/or reabsorption of bumetanide needs further study. Even under the condition of membrane depolarization, the uptake of bumetanide by MCT6 was elevated by extracellular acidic pH. However, our results indicate that the pH gradient across the cell membrane is not the driving force of MCT6. The mRNA of MCT6 is highly expressed in placenta and kidney. In kidney, the intracellular pH of tubular epithelial cells is lower than the pH of the extracellular fluid, and urine is acidic under normal conditions. In placenta, fetal blood is slightly more acidic (pH 7.3) than maternal blood (pH 7.4). In addition, Na$^+$/H$^+$ exchanges on the luminal and basolateral membrane of tubular epithelium and brush-border membrane of trophoblast cells produce a microacidic environment. Although the physiologic meaning of the pH sensitivity of MCT6 activity is still unknown, MCT6 could be regulated by the acidic environment in these tissues.

In conclusion, we identified bumetanide for the first time as an MCT6 ligand. MCT6 transports bumetanide in a pH- and membrane potential-sensitive but not proton gradient-dependent manner, and the substrate specificity of MCT6 is different from those of the other MCTs. It is probable that MCT6 is involved in the disposition of various drugs, including bumetanide. Bumetanide should be a good tool for investigating the functional properties of MCT6 and the differences of substrate recognition sites among MCT isoforms.

References


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