IN Volvement of Uric Acid Transporter in Increased Renal Clearance of the Xanthine Oxidase Inhibitor Oxypurinol Induced by a Uricosuric Agent, Benzbromarone

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ABSTRACT:
Benzbromarone has been reported to increase the renal clearance of oxypurinol, an active metabolite of allopurinol. We examined the renal transport of oxypurinol to determine whether such a change in renal clearance could be explained by altered transporter-mediated reabsorption. Since the first step of reabsorption takes place at the renal epithelial apical membrane, we focused on membrane transporters. Benzbromarone is an inhibitor of reabsorption of uric acid mediated by the uric acid transporter (URAT1) URAT1 (SLC22A12), which is expressed at the apical membrane of proximal tubular cells in humans. Uptake of oxypurinol by Xenopus oocytes injected with complementary RNA of URAT1 was significantly higher than that by water-injected oocytes, and the uptake was saturable, with a $K_m$ of about 800 μM. Moreover, benzbromarone inhibited the oxypurinol uptake by URAT1 at concentrations as low as 0.01 μM. The uptake of oxypurinol by another organic anion transporter (OAT), OAT4 (SLC22A11), which is also expressed at the apical membrane of proximal tubular epithelial cells, was negligible, whereas the uptake of [3H]estrone-3-sulfate by OAT4 was significantly inhibited by oxypurinol. Furthermore, neither the transport activity of organic cation/carnitine transporter (OCTN) 1 nor OCTN2 was affected by oxypurinol or benzbromarone. These results indicate that URAT1 is involved in renal reabsorption of oxypurinol, and the increment of renal clearance of oxypurinol upon concomitant administration of benzbromarone could be due to drug interaction at URAT1.

Allopurinol is widely used for the treatment of hyperuricemia and gout by inhibiting xanthine oxidase, which is the rate-limiting enzyme for uric acid formation. Allopurinol itself is a substrate of xanthine oxidase and its major metabolite, oxypurinol, is also a potent inhibitor of xanthine oxidase (Elion et al., 1966; Rundles, 1985). The inhibitory effect of allopurinol on xanthine oxidase can be largely attributed to oxypurinol, which has a longer plasma half-life than allopurinol (Elion et al., 1968; Hande et al., 1972; Appelbaum et al., 1982; Yamamoto et al., 1991). Fractional excretion of oxypurinol is about 20% of glomerular filtration in humans, and oxypurinol shows negligible binding to plasma protein (Elion et al., 1966). This observation suggests that about 80% of oxypurinol is reabsorbed after glomerular filtration, although the reabsorption mechanism remains to be clarified (Colin et al., 1986; Yamamoto et al., 1991; Turhheim et al., 1999).

Furthermore, concomitant use of benzbromarone and probenecid caused an increase in the renal clearance and a decrease in plasma concentration of oxypurinol (Colin et al., 1986; Yamamoto et al., 1991; Muller et al., 1993). Colin et al. (1986) reported that renal clearance of oxypurinol was increased to 0.69 ml/s from 0.43 ml/s, and plasma area under the curve (0-∞) of oxypurinol was decreased to half by concomitant use of 20 mg of benzbromarone in healthy subjects. Benzbromarone and probenecid are used as uricosuric agents, based on their inhibition of the renal reabsorption of uric acid at proximal tubular epithelial cells (Steere and Boner, 1973; Sinclair and Fox, 1975; de Vries and Sperling, 1979). On the other hand, a decrease in urinary excretion of oxypurinol was reported in the case of coadministration of pyrazinamide, an antituberculous agent (Yamamoto et al., 1991). It has been reported that pyrazinamide reduces the renal secretion of uric acid via activation of the exchange transport of uric acid with pyrazinamide, which is a metabolite of pyrazinamide, through the uric acid transporter URAT1 (SLC22A12) (de Vries and Sperling, 1979; Enomoto et al., 2002). These observations, i.e., that uricosuric agents increase the renal excretion of oxypurinol, whereas an antiuricosuric agent decreases its renal clearance, imply that renal handling of oxypurinol is similar to that of uric acid. As described above, renal reabsorption of uric acid is due to URAT1, which was identified as a member of the OAT family of organic anion transporters and is expressed at the apical membrane of renal tubular epithelial cells (Enomoto et al., 2002). Enomoto et al. (2002) showed that the uptake of uric acid by URAT1-expressing oocytes was decreased in the presence of uricosuric agents, such as benzbromarone and probenecid, whereas it was increased by preloading of pyrazinecarboxylic acid in URAT1-expressed oocytes. These findings are similar to those in the case of oxypurinol and further support the idea that renal transport of oxypurinol is similar to that of uric acid. Urinary excretion of uric acid accounts for approximately 70% of the daily production of uric acid. The so-called "four-component hypothesis", involving glomerular filtration, pressecretory reabsorption, secretion, and postsecretory reabsorption, has

ABBREVIATIONS: URAT1, uric acid transporter; OAT, organic anion transporter; OCTN, organic cation/carnitine transporter; HEK, human embryonic kidney; cRNA, complementary RNA; LC-MS/MS, liquid chromatography-tandem mass spectrometry.
been proposed to explain the renal handling of uric acid, and approximately 10% of uric acid that is filtered through the glomerulus is finally excreted in urine (Sica and Schoolwerth, 2000). It was suggested that URAT1 plays a dominant role in uric acid reabsorption at proximal tubular cells, and it is considered to be one of the sites of action of uricosuric and antiuricosuric agents to regulate blood uric acid level.

Therefore, we hypothesized that URAT1 is involved in renal reabsorption of oxypurinol, and that reabsorption of oxypurinol by URAT1 is disturbed by benz bromarone, resulting in increased urinary excretion. Accordingly, we examined the uptake of oxypurinol by URAT1 and the inhibitory effect of benz bromarone on oxypurinol transport by URAT1, using URAT1-expressing oocytes. We also examined the possible involvement in this interaction of organic anion transporter 4 (OAT4, SLC22A11), which is classified into the same SLC22A family as URAT1, and organic cation/carnitine transporters OCTN1 and OCTN2; all of these transporters are expressed at the apical membrane of proximal tubular epithelial cells and transport xenobiotics (Tamai et al., 2001, 2004; Ekaratanawong et al., 2004).

Materials and Methods

Chemicals. [14C]Uric acid (1.92 TBq/mmol), [3H]estrone-3-sulfate (2.12 TBq/mmol), [3H]tetraethylammonium (2.04 TBq/mmol), and [3H]carnitine (2.96 TBq/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA), PerkinElmer Life and Analytical Sciences (Boston, MA), American Radiolabeled Chemicals, Inc. (St. Louis, MO), and GE Healthcare (Little Chalfont, Buckinghamshire, UK), respectively. Oxypurinol, benz bromarone, and uric acid were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were purchased from Sigma-Aldrich, Wako Pure Chemicals (Osaka, Japan), and Kishida Chemicals (Osaka, Japan).

cDNA Cloning of URAT1 and OAT4. The URAT1 gene was PCR-amplified using QUICK-Clone human kidney cDNA (BD Biosciences Clontech, Palo Alto, CA) as a template, with upstream primer 5’-AGGTGTCAT-GGCATTTTCTGAC-3’ and downstream primer 5’-CCTGCGCAATCAACATGCTTCT-3’, based on the reported URAT1 gene sequence (Enomoto et al., 2002) (GenBank accession no. AB071863). A major 2.0-kilobase polymerase chain reaction product was ligated into pCR 2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA), and then URAT1-cDNA was digested with BamHI and XbaI and ligated into the expression vector pGEMHE, which was kindly provided by Dr. E. R. Liman (Liman et al., 1992). The OAT4 gene was PCR-amplified using Cap Site cDNA of human kidney (Nippon Gene, Tokyo, Japan) as a template, with upstream primer 5’-GAATTCATGTCATGCGGCTCAGA-3’ and downstream primer 5’-AGGCTCTGATATGGTGCGG-GGGTGAGA-3’ (both synthesized by Hokkaido System Science, Sapporo, Japan) and Ex Taq DNA polymerase (Takara Shuzo Co., Shiga, Japan), based on the reported OAT4 gene sequence (Chu et al., 2000). GenBank accession no. AB026116. A major 2.2-kilobase polymerase chain reaction product was ligated into the TA cloning vector pGEM-T Easy (Promega, Madison, WI), and then OAT4 cDNA was digested with EcoRI and HindIII and ligated to pGEMHE. The obtained cDNA sequences were analyzed and confirmed to be the same as the reported ones.

Uptake Experiments Using Xenopus laevis Oocytes and HEK293 Cells Expressed with Transporter Genes. Complementary RNAs (cRNAs) of URAT1 and OAT4 were prepared by in vitro transcription with T7 RNA polymerase in the presence of ribonuclease inhibitor and an RNA cap analog using a mMESSAGE mMACHINE kit (Ambion, Austin, TX). For experiments with URAT1, defolliculated oocytes were injected with 25 ng of URAT1 cRNA or the same volume of water and incubated in modified Barth’s solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, and 5 mM HEPES, pH 7.4) containing 50 μg/ml gentamicin at 18°C for 3 days as we reported previously (Nozawa et al., 2005). The oocytes were transferred to a 24-well plate and preincubated in ND96 buffer containing 5 mM glutarate at 25°C for 60 min. After this preincubation, the buffer was replaced with 0.5 ml of ND96 buffer containing [14C]tetraethylammonium by HEK293 cells expressed with human OCTN1 and OCTN2, which is classified into the same SLC22A family as URAT1 and OAT4 and which inhibits the uptake reaction. The oocytes were incubated at 25°C for 60 min and then washed three times with 1 ml of ice-cold ND96 buffer to terminate the uptake reaction. The oocytes were solubilized with 5% sodium dodecyl sulfate solution for 120 min. The radioactivity was measured using a liquid scintillation counter (PerkinElmer Life and Analytical Sciences) after the addition of scintillation cocktail Clearsol I (Nacalai Tesque, Kyoto, Japan).

Uptake was expressed as the cell-to-medium ratio (microliter/oocyte), obtained by dividing the uptake amount by the concentration of substrate in the incubation buffer. To estimate the kinetic parameters for the uptake by URAT1 or OAT4, the initial uptake rates by URAT1 or OAT4 (obtained after subtraction of the uptake by water-injected oocytes from that by URAT1 or OAT4 cRNA-injected oocytes) were fitted to the following equation by means of nonlinear least-squares regression analysis using KaleidaGraph software version 3.5 (Synergy Software, Reading, PA):

\[ V = V_{\text{max}} \times s/(K_s + s) \] (1)

where \( V \) is the initial uptake rate of substrate (pmol/min/oocyte), \( s \) is the substrate concentration in the medium (μM), \( K_s \) is the Michaelis-Menten constant (μM), and \( V_{\text{max}} \) is the maximum uptake rate (pmol/min/oocyte).

The inhibition studies for OCTN1- or OCTN2-mediated transport of their typical substrates were examined in the absence or presence of inhibitors (1 mM oxypurinol or 1 μM benz bromarone). The transport experiments of [14C]tetraethylammonium by HEK293 cells expressed with human OCTN1 was carried out as described previously (Tamai et al., 2004). The transport experiment of [3H]carnitine by HEK293 cells expressed with human OCTN2 was carried out as described previously (Tamai et al., 2001).

LC-MS/MS Analysis. LC-MS/MS analysis was carried out using a high-performance liquid chromatography system consisting of an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA), a vacuum degasser, and an autosampler with a 100-μl loop interface to an API 3000 SCIEX triple-quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Oxypurinol, xanthine (oocyte-derived), and the internal standard (3-methylxanthine) were separated on a Mightysil Aqua RP-18 GP 2.0 × 150-mm column (Kanto Chemical, Tokyo, Japan). The mobile phase consisted of 0.1% acetic acid containing 5% methanol. The sample was delivered at a flow rate of 0.2 ml/min, and each analysis lasted for 10 min. The mass spectrometer was operated in the turbo ion spray mode with negative ion detection. The turbo ion spray temperature was maintained at 550°C, and a voltage of −3.5 kV was applied to the sprayer needle. Nitrogen was used as the turbo ion spray and nebulizing gas. The detection and quantification of analytes were performed using the multiple reactions monitoring mode with the transition m/z 151.0 to 107.9 for oxypurinol and m/z 165.0 to 122.0 for 3-methylxanthine as the internal standard. All raw data were processed with MDS SCIEX Analyst software, version 1.3. Calibration curves were linear from 10 to 1000 nM for oxypurinol, and the coefficient of variation was less than 15%.

Statistical Analysis. For analysis of differences between the water-injected group and cRNA-injected group, the two-tailed unpaired Student’s t test was...
used. For the inhibition studies of URAT1 and OAT4, the statistical significance of differences was determined by using one-way analysis of variance with Dunnett’s post hoc test, with \( P < 0.05 \) as the criterion.

**Results**

**Uptake of Oxyipurinol by Xenopus Oocytes Expressing URAT1.** Our method to evaluate URAT1 activity using Xenopus oocytes was validated with the uptake of \([^{14}C]\)uric acid (Fig. 1A). Uptake of \([^{14}C]\)uric acid at 60 min by oocytes injected with URAT1 cRNA was significantly higher than that by oocytes injected with water (control). Then, the time course of oxyipurinol uptake by *Xenopus* oocytes injected with URAT1 cRNA or water was examined. The uptake of oxyipurinol by *Xenopus* oocytes injected with URAT1 cRNA was linearly increased with time over 60 min (Fig. 1B) and was significantly greater than that by water-injected oocytes \(( P < 0.05)\). Thus, uptake at 60 min was routinely used for initial uptake rate measurement in the subsequent studies. The concentration dependence of oxyipurinol uptake by URAT1 was studied in the concentration range from 300 to 3000 \(\mu\)M; all values were corrected by subtraction of the uptake by water-injected oocytes (Fig. 2). The uptake was saturable, and the \( K_m \) and \( V_{max} \) values of oxyipurinol transport by URAT1 were 794 \(\pm\) 77.9 \(\mu\)M (mean and standard error) and 1.37 \(\pm\) 0.05 pmol/min/oocyte, respectively.

**Inhibitory Effects of Benzbromarone and Uric Acid on Oxyipurinol Uptake by URAT1.** The plasma concentration of oxyipurinol is affected by concomitant use of benzbromarone (Colin et al., 1986; Yamamoto et al., 1991; Muller et al., 1993). Therefore, to examine the involvement of URAT1 in the drug interaction between oxyipurinol and benzbromarone, we examined the inhibitory effects of benzbromarone on oxyipurinol uptake by URAT1. Benzbromarone, which is a potent inhibitor of URAT1 (Enomoto et al., 2002), significantly reduced the oxyipurinol uptake at a concentration as low as 10 \(\mu\)M and completely blocked it at 1 \(\mu\)M. Furthermore, uric acid, which is a substrate of URAT1, significantly reduced the uptake of oxyipurinol in a concentration-dependent manner (Fig. 3). The \( IC_{50} \) value of uric acid for inhibition of oxyipurinol uptake was estimated to be about 170 \(\mu\)M.

**Studies for the Involvement of OAT4, OCTN1, and OCTN2 in the Interaction between Oxyipurinol and Benzbromarone.** The anion exchanger OAT4 is expressed at the apical membrane of proximal tubular epithelial cells (Ekaratanaawong et al., 2004) and is a member of the SLC22A family, like URAT1. The amino acid sequence predicted by the URAT1 cDNA exhibits 42% amino acid identity with that of OAT4 (Enomono et al., 2002). We therefore examined whether OAT4 transports oxyipurinol and characterized the inhibitory effects of benzbromarone, uric acid, and oxyipurinol on the uptake of \([^{3}H]\)estrone-3-sulfate, a substrate of OAT4 (Fig. 4). Uptake of \([^{3}H]\)estrone-3-sulfate by OAT4 was reduced in the presence of oxyipurinol and uric acid at 1000 \(\mu\)M or higher concentration, whereas no decrease in the uptake of estrone-3-sulfate was observed at 1 \(\mu\)M benzbromarone, which completely inhibited URAT1-mediated transport of oxyipurinol. Furthermore, uptake of oxyipurinol at the concentration of 300 \(\mu\)M was comparable in oocytes injected with OAT4 cRNA and with water (Fig. 5). Accordingly, although oxyipurinol has affinity for OAT4, it does not appear to be transported by OAT4.

We also examined the involvement of organic cation/carnitine transporters OCTN1 and OCTN2, since they are present at the apical membrane of the renal tubular epithelial cells (Tamai et al., 2001, 2004) and are known to transport xenobiotics. As a result, uptake of
the typical substrate of OCTN1 (tetaethylammonium) by the HEK293 cells expressed with human OCTN1 in the presence of 1 mM oxypurinol and 1 μM benzbromarone were 103 and 96.7% of control, respectively. Similarly, the uptakes of typical substrate of OCTN2 (carnitine) by the HEK293 cells expressed with human OCTN2 in the presence of those two compounds were 95.0 and 111% of control, respectively. Accordingly, neither of OCTN1 nor OCTN2 is involved in the interaction between oxypurinol and benzbromarone.

Discussion

We examined the renal transport mechanism of oxypurinol, an active metabolite of the xanthine oxidase inhibitor allopurinol, to clarify the mechanism of the pharmacokinetic interaction between oxypurinol and the uricosuric agent benzbromarone, since concomitant use of benzbromarone causes increased renal clearance of oxypurinol, which may lead to reduced efficacy of allopurinol/oxypurinol (Colin et al., 1986; Yamamoto et al., 1991; Muller et al., 1993). Previous observations, that approximately 80% of glomerularly filtered oxypurinol is reabsorbed (Turnheim et al., 1999) and that benzbromarone is a strong inhibitor of URAT1-mediated transport of uric acid (Enomoto et al., 2002), suggest the involvement of a transporter such as URAT1 in the reabsorption of oxypurinol, and inhibition of the transporter by benzbromarone could explain the drug interaction between oxypurinol and benzbromarone. Accordingly, the present study was mainly focused on URAT1, although attention was also paid to another apical membrane transporter, OAT4, which is classified into the SLC22A family (as is URAT1) and is expressed at the apical membrane of renal tubular epithelial cells (Ekaratanawong et al., 2004) as well as organic cation/carnitine transporters OCTN1 and OCTN2, which are also expressed at the apical membrane and transport xenobiotics (Tama et al., 2001, 2004).

The uptake of oxypurinol by oocytes injected with URAT1 cRNA was significantly higher than that by water-injected oocytes (Fig. 1B). The initial uptake of oxypurinol mediated by URAT1 was saturable with a Michaelis constant of 794 μM (Fig. 2). In addition, this URAT1-mediated uptake of oxypurinol was inhibited by benzbromarone in a concentration-dependent manner (Fig. 3). Accordingly, it was demonstrated that oxypurinol is a substrate of URAT1 and that the transport was inhibited by benzbromarone.

On the other hand, although oxypurinol inhibited the transport of estrone-3-sulfate by OAT4 (Fig. 4), it appeared not to be a substrate of OAT4, at least in Xenopus oocytes expressing OAT4 (Fig. 5). Furthermore, we examined the involvement of organic cation/carnitine transporters OCTN1 and OCTN2, since they are present at the apical membrane of the renal tubular epithelial cells (Tama et al., 2001, 2004) and are known to transport xenobiotics. However, we could not see any effect of oxypurinol or benzbromarone on the transport activity of OCTN1 or OCTN2. Therefore, we conclude that the contribution of OAT4, OCTN1, and OCTN2 to the renal reabsorption of oxypurinol is negligible and that URAT1 plays a key role in the renal reabsorption of oxypurinol. Since the clinical concentration of oxypurinol is as high as 30 μM in plasma, and the plasma protein binding of oxypurinol is negligible (Elion et al., 1966), the estimated clinical concentration of oxypurinol in plasma or glomerular filtrate is 30 μM or less. Here, the \( K_m \) value of oxypurinol to URAT1 (794 μM) is higher than the clinical concentration (30 μM). Accordingly, it was suggested that URAT1 is not saturated at the clinical concentration of oxypurinol, and oxypurinol can be transported efficiently by URAT1.

According to the basic product information of benzbromarone from the pharmaceutical company (Unirion; Torii Pharmaceutical Co. Ltd., Tokyo, Japan), the maximum plasma concentration of benzbromarone reached 5.4 μM after oral administration of the clinical dose of 100 mg in humans, and at 24 h after the administration, 0.5 μM benzbromarone remained in plasma (Okawa et al., 2004). Since plasma protein binding of benzbromarone is approximately 97%...
The inhibition of urate transporter 1 (URAT1) by benzbromarone may lead to a decrease in uric acid reabsorption in the proximal tubule. This effect is mediated by the organic anion transporter 4 (OAT4). The interaction between URAT1 and OAT4 is thought to be a major contributor to the altered renal handling of uric acid in patients taking benzbromarone. Since uric acid exhibits negligible plasma protein binding, its concentration in the proximal tubular lumen is expected to be comparable with that in plasma. Accordingly, renal reabsorption of uric acid might also be decreased by uric acid under physiological conditions.

In conclusion, oxypurinol was clarified to be a substrate of URAT1, not just of OAT4. Inhibitory effects on URAT1 were coadministered with benzbromarone or other agents. The results suggest that oxypurinol could be transported efficiently by URAT1 in vivo, as described above. Accordingly, benzbromarone should be able to reduce the reabsorptive transport of oxypurinol mediated by URAT1 at clinical doses of the two drugs.

On the other hand, URAT1-mediated uptake of oxypurinol was inhibited by uric acid, which is a substrate of URAT1. In Guideline for the Management of Hyperuricemia and Gout (Japanese Society of Gout and Nucleic Acid Metabolism, 2002), hyperuricemia is defined as a plasma concentration of uric acid of 7 mg/dl (417 μM) or more. Since uric acid exhibits negligible plasma protein binding, its concentration in the proximal tubular lumen is expected to be comparable with that in plasma. Accordingly, renal reabsorption of oxypurinol might also be decreased by uric acid under physiological conditions.

References


