原著 4

Interaction of zotepine and chlorprothixene with URAT1-mediated urate transport

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Abstract

Zotepine and chlorprothixene are antipsychotic drugs, and they have a broad therapeutic effect in patients with schizophrenia. Zotepine and chlorprothixene have been reported to decrease serum urate levels. In addition, an increase in renal clearance of urate was shown in several studies. However, the molecular mechanism underlying these effects has not yet been clarified. In the present study, we measured [¹⁴C]urate uptake using stable cells expressing human urate transporter 1 (HEK-hURAT1) and mock cells (HEK-mock) to evaluate the uricosuric actions of zotepine and chlorprothixene. Zotepine and chlorprothixene inhibited URAT1-mediated [¹⁴C]urate uptake in a concentration-dependent manner (IC₅₀ values were 14.52 μM for zotepine and 6.08 μM for chlorprothixene). Next, we measured the activity of xanthine oxidase (XO) to determine whether zotepine and chlorprothixene have inhibitory effects on urate production. Urate production mediated by XO was not inhibited. Taken together, the results suggest that URAT1, a major contributor of renal urate reabsorption and a major target of uricosuric drugs such as losartan and benzbromarone, interacted with zotepine and chlorprothixene; however, XO, a major

enzyme in urate production, did not interact with zotepine or chlorprothixene. This is the first study showing that drugs that have the structure of a tricyclic compound interact with URAT1. Elucidation of these interactions may contribute to the development of new anti-hyperuricemic agents by clarifying the structure of the substrate-binding site of URAT1.

Introduction

In humans, urate (uric acid) is the end-product of purine metabolism as a result of genetic loss of uricase^{1,2)}. Generation of urate occurs primarily in the liver, while the excretion of urate is mainly from the kidney. Therefore, hyperuricemia results from the overproduction of urate and/or from insufficient renal elimination³⁾. An elevated serum urate level plays a pivotal role not only in gout but also lifestyle-related diseases, including hypertension, insulin resistance, diabetes, chronic renal disease, diabetic renal disease, and cardiovascular disease^{4,5)}. Thus, urate-lowering therapy is necessary for successful long-term hyperuricemia management to achieve a sustained reduction in urate, and common therapeutic targets are urate transporters in the kidney and the production

Received August 5, 2015, Accepted October 12, 2015

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Key words : Urate, Zotepine, Chlorprothixene, URAT1, Xanthine Oxidase

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pathways of urate in the liver⁶⁾.

In addition to anti-hyperuricemic agents, drugs that lower serum urate levels such as losartan and salicylic acid have also been reported⁷⁾. Zotepine and chlorprothixene are antipsychotic drugs that have a broad therapeutic effect in patients with schizophrenia⁸⁻¹⁰⁾. Table 1 illustrates the similar chemical structures of zotepine and chlorprothixene. They also have been reported to decrease serum urate levels in several clinical studies¹¹⁻¹⁶⁾. The oral administration of 40 mg of zotepine reduced serum uric acid levels by a mean of 2.71 mg/dL¹²⁾, and the oral administration of a mean of 272.7 mg of chlorprothixene reduced serum uric acid levels by 2.64 mg/dL¹⁴⁾. In addition, zotepine and chlorprothixene have been reported to increase the renal clearance of urate^{11,14)}. However, the molecular mechanism(s) of these effects remains unknown. Previous studies showed that many drugs such as probenecid, benzbromarone, and losartan can decrease the serum urate level by their inhibitory effects on URAT1¹⁷⁾. Human serum urate levels are mostly determined by urate reabsorption and secretion in the kidney. Renal

urate reabsorption is mainly controlled via the proximal tubular urate transporter URAT1 (SLC22A12)⁷⁾. Therefore, we examined the effects of zotepine and chlorprothixene on urate transporters involved in the regulation of the serum urate level.

The rate-limiting step of urate production is an enzymatic reaction of the xanthine dehydrogenase/xanthine oxidase (XDH/XO) enzyme that oxidizes hypoxanthine-xanthine into urate. Human XDH/XO is expressed in the liver and small intestine of XDH/XO-rich parenchyma cells, and the urate production rate is based on the amount of substrate and/or XO activity¹⁷⁾. However, the effects on urate production of zotepine and chlorprothixene have not been examined clinically. Consequently, we determined the XO inhibitory effects of these two antipsychotic drugs that reflect urate production.

Methods

1. Materials

[¹⁴C]urate (57.8 mCi/mmol) was purchased from Moravек Biochemicals (Brea, CA, USA). Zotepine, chlorprothixene, and benzbromarone were obtained

Table 1 Structures and molecular weights of zotepine and chlorprothixene

Drug	Structure	Molecular weight
zotepine		331.86
chlorprothixene		351.861

from Sigma Aldrich (St Louis, MO, USA). Allopurinol was obtained from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum was obtained from Biowest (Nuaillé, France), and Dulbecco's modified eagle's medium (DMEM), trypsin, and geneticin were purchased from Sigma Aldrich.

2. Cell culture

HEK-hURAT1 cells were established as previously described¹⁸. The cells were grown in a humidified incubator at 37° C and in 5% CO₂ using minimum essential medium containing 10% fetal bovine serum and 500 µg/mL of geneticin.

3. Urate transport activity assay

The kinetic behavior of urate transport was examined by performing uptake studies using confluent HEK-hURAT1 and HEK-mock cells. Both cell lines were cultured at a density of 1.5×10^5 cells/well on a 24-well plate coated with poly-D-lysine (Corning, NY, USA) for 48 hours before the uptake studies were performed. Immediately before the experiment, the medium was replaced by the uptake solution, which was chloride-free Hanks' balanced salt solution (HBSS) containing the following in mM: 125 Na gluconate, 4.8 K gluconate, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.3 Ca gluconate, 5.6 glucose, and 25 HEPES, pH 7.4. After pre-incubation in the same solution in a water bath at 37° C for 10 min, the cells were incubated in a solution containing 5 µmol/L of [¹⁴C]urate at 37° C for the indicated time. After 2 minutes, uptake was stopped by washing two times with ice-cold incubation medium. The cells in each well were lysed with 0.5 mL of 0.1 N sodium hydroxide and 2.5 mL of the liquid scintillation cocktail Insta-Gel Plus (Perkin Elmer, Waltham, MA, USA), and radioactivity was determined using a β -scintillation counter, LSC-7200 (Hitachi Aloka Medical, Tokyo, Japan). Uptake measured in HEK-mock cells was subtracted from the corresponding uptake measured in HEK-hURAT1

cells to calculate cDNA-specific uptake, after it had been calibrated using the average protein content of other wells in the same 24-well plate as that used in the uptake study, with the BCA protein assay reagent (Pierce, Rockford, IL, USA). To evaluate the inhibitory effects of zotepine, chlorprothixene, and benzbromarone on urate uptake via URAT1, HEK-hURAT1 cells were incubated in a solution containing 5 µmol/L [¹⁴C]urate in the absence or presence of various concentrations (0.01, 0.1, 1, 3, 10, 30, and 100 µM) of drugs at 37° C for 2 min. Drugs were dissolved in dimethylsulfoxide (Wako Pure Chemical Industries). The final concentration of dimethylsulfoxide in the incubation medium was adjusted to less than 0.5%.

4. Xanthine oxidase activity assay

We measured the activity of human XO to determine whether zotepine and chlorprothixene have inhibitory effects on urate production. In the presence of 1, 10, 50, 100, and 300 µM zotepine or chlorprothixene, the fluorescence of resorufin, which is produced during uric metabolism by XO, was analyzed using the Xanthine oxidase fluorometric assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the instructions of the manufacturer and was normalized to the protein concentration. XO produces H₂O₂ during the oxidation of hypoxanthine. In the presence of horseradish peroxidase in the product, H₂O₂ reacts with ADHP (10-acetyl-3,7-dihydroxyphenoxazine) in a 1:1 stoichiometry to produce the highly fluorescent compound resorufin, which can be easily analyzed¹⁹.

5. Statistical analyses

Uptake experiments were conducted three times, and each uptake experiment was performed in triplicate. All of the data are shown as means ± S.E.M. Statistical differences were determined using one-way ANOVA with Dunnett' s post-hoc test. A value of P < 0.05 was considered to be significant.

Results

1. Urate transport activity assay

To investigate the interaction of zotepine and chlorprothixene with URAT1, the uptake of [14 C]urate was measured in chloride-free HBSS in the presence of zotepine or chlorprothixene ($50\mu\text{M}$) or in the presence of benzbromarone ($50\mu\text{M}$), which has long been used as a uricosuric drug. As shown in Fig. 1, zotepine and chlorprothixene inhibited URAT1-mediated urate transport ($45 \pm 3\%$ for zotepine and 35

$\pm 3\%$ for chlorprothixene, respectively; $P < 0.01$). We then checked the concentration dependency of these interactions. Fig. 2 shows the dependence of inhibition on the zotepine (A) and chlorprothixene (B) concentrations. The percentage of urate uptake relative to the control (no inhibitor) level decreased dose-dependently from 0.01 to $100\mu\text{M}$ (IC_{50} values of $14.52\mu\text{M}$ for zotepine and $6.08\mu\text{M}$ for chlorprothixene). These findings suggest that these drugs have inhibitory action against URAT1.

Fig. 1 Inhibition of human URAT1-mediated uric acid uptake by zotepine and chlorprothixene.

Human URAT1 cDNA was expressed in HEK293 (HEK-hURAT1) cells. HEK293 cells transfected with pcDNA3.1 lacking an insert (HEK-mock) were used to determine endogenous transport. The uptake of [14 C]uric acid ($5\mu\text{M}$) was measured in chloride-free Hanks' balanced salt solution (HBSS) in the presence of zotepine, chlorprothixene ($50\mu\text{M}$), or benzbromarone ($50\mu\text{M}$). The uptake measured in HEK-mock cells was subtracted from the corresponding uptake measured in cells transfected with cDNA to calculate cDNA-specific uptake. The results are expressed as a percentage of the control uptake (100%) measured in the absence of drugs. The degree of statistical significance for uric acid uptake measured in the presence of zotepine and chlorprothixene compared to uric acid uptake measured in the absence of the drugs in hURAT1 cDNA-transfected cells is indicated by ** $P < 0.01$.

Fig. 2 Concentration-dependent inhibition of urate uptake via URAT1 by zotepine/chlorprothixene (0.01 – $100\mu\text{M}$).

URAT1-mediated uptake is expressed as a percentage of the no-inhibitor control (absence of drugs). The uptake of [14 C]uric acid ($5\mu\text{M}$) was measured in the presence of increasing concentrations of zotepine (A) or chlorprothixene (B). The uptake measured in HEK-mock cells was subtracted from the corresponding uptake measured in cells transfected with cDNA to calculate cDNA-specific uptake. The results are expressed as a percentage of the control uptake (100%) measured in the absence of drugs. The 50% inhibitory concentration (IC_{50}) values were generated using a sigmoidal dose-response relationship.

2. Xanthine oxidase activity assay

The fluorescence of resorufin, which is produced during uric metabolism by XO, was analyzed in the presence of 50 μ M of zotepine and chlorprothixene and was normalized to the protein concentration (Fig. 3). Allopurinol (10 μ M), which has long been used as an XO inhibitor, was also examined for comparison with the inhibitory effects of zotepine and chlorprothixene. Compared with the control, 50 μ M of zotepine and chlorprothixene did not significantly inhibit XO activity. Further inhibition study showed no concentration dependence (Fig. 4).

Discussion

To elucidate the mechanism of the urate-lowering effects of the two anti-psychotic drugs zotepine and chlorprothixene, the urate transport activity assays and the XO activity assay were conducted. In the uptake study, zotepine and chlorprothixene inhibited urate transport via URAT1 in a concentration-dependent manner. Since no XO inhibitory activity was observed in the XO activity assay, the urate-lowering effects of these drugs seem to be largely due to URAT1 inhibition.

Fig. 3 Effects of zotepine and chlorprothixene (50 μ M) on xanthine oxidase (XO) activity.

The fluorescence of resorufin, which is produced during uric metabolism by XO, was analyzed in the presence of 50 μ M of zotepine/chlorprothixene. Allopurinol (10 μ M) was also examined for comparison with the XO inhibitory effect of zotepine/chlorprothixene. The results are expressed as a percentage of the control activity (100%) measured in the absence of the drugs. No significant difference was observed between XO activity measured in the presence of zotepine/chlorprothixene and in the absence of the drugs. The degree of significance in the presence of allopurinol is indicated by ** $P < 0.01$.

Fig. 4 Effects of zotepine and chlorprothixene (1-300 μ M) on xanthine oxidase (XO) activity.

The fluorescence of resorufin was measured in the presence of increasing concentrations of zotepine (A) or chlorprothixene (B). The results are expressed as a percentage of the control activity (100%) measured in the absence of the drugs. No concentration dependence was observed.

In addition to benzbromarone, the angiotensin II receptor blocker (ARB) losartan and salicylic acid have been reported to be URAT1 inhibitors. However, there has been no study on the urate-lowering effects of drugs that have the structure of a tricyclic compound such as zotepine and chlorprothixene²⁰⁾. As shown in Fig. 2, the inhibitory effects of zotepine and chlorprothixene on urate transport via URAT1 were concentration-dependent, and IC₅₀s of zotepine and chlorprothixene were 14.52 and 6.08 μ M, respectively. The maximum plasma concentration (C_{max}) of zotepine after oral administration (50 mg) was approximately 0.02 μ M (interview form), and the serum concentration of chlorprothixene was 0.04-0.22 μ M after oral administration (100 mg)²¹⁾. Considering that the protein-binding rates of both drugs are over 95%, it is possible that the intratubular concentrations of these drugs in humans are much lower than the IC₅₀s. Thus, the inhibition of renal uric acid reabsorption from the luminal side seems to be unlikely at the therapeutically relevant plasma concentrations of these drugs.

However, we revealed that human sodium phosphate transporter 4 (hNPT4/SLC17A3) is a multispecific organic anion efflux transporter at the apical side of renal tubules, indicating the possibility that drugs, such as loop diuretics, can be excreted via this secretory pathway²²⁾. The amount of a drug excreted into the urine via a secretory pathway, such as NPT4, may be correlated with the urate-lowering effect of the drug. Besides, the drug concentration most likely increases in the tubules owing to the volume reduction after water reabsorption. In the present study, we could not examine the interaction of these drugs because there are currently no commercially available metabolites. In addition, benzbromarone, which has long been used as a uricosuric drug, is thought to inhibit urate reabsorption from the apical side of the proximal tubule; however, benzbromarone itself is not

excreted into urine. Thus, it is predicted that 6-hydroxybenzbromarone, a major hydroxylated metabolite of benzbromarone, inhibits urate reabsorption mediated by URAT1²⁰⁾. Furthermore, drugs such as chlorpromazine that have structures similar to those of zotepine and chlorprothixene are not involved in the regulation of serum urate levels. By focusing on the effects of similar substances including their metabolites, it may be possible to identify the important structure(s) associated with URAT1.

In summary, we found that zotepine and chlorprothixene have inhibitory effects on urate transport via URAT1. Although IC₅₀s of these two drugs are higher than their plasma concentrations, this is the first study showing that drugs that have the structure of a tricyclic compound interact with URAT1. Our findings may contribute to the development of new anti-hyperuricemic agents by clarifying the structure of the substrate-binding site of URAT1.

Acknowledgments

We thank Ms. Mio Maekawa and Mrs. Sawako Nakadate for technical assistance.

Financial Disclosure

The authors declare no conflict of interest associated with this manuscript.

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