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Genetic variants of organic cation transporter 1 (OCT1) and OCT2 significantly reduce lamivudine uptake

Min-Koo Choi[a] and Im-Sook Song[b,c,*]
[a]*College of Pharmacy, Dankook University, Cheon-an 330-714, Korea*
[b]*Department of Pharmacology and Pharmacogenomics Research Center, Inje University, College of Medicine, Busan, 614-735, Korea*
[c]*College of Pharmacy, Kyungpook National University, Daegu, 702-701, Korea*

ABSTRACT: The study sought to investigate the effect of genetic variants of OCT1 (OCT1-P283L and -P341L) and OCT2 (OCT2-T199I, -T201M and -A270S), which were identified in a Korean population, on the transport of lamivudine *in vitro* and to compare the substrate dependent effects of OCT1 and OCT2 variants with 1-methyl-4-phenylpyridinium (MPP^+), tetraethyl ammonium (TEA), metformin and lamivudine as substrates for these transporters. When the transport kinetics of lamivudine uptake in oocytes overexpressing OCT1 and OCT2 wild-type (WT) and variant proteins were measured, lamivudine uptake mediated by OCT1-WT was saturable, and uptake was decreased in oocytes expressing OCT1-P283L and -P341L variants compared with that in OCT1-WT. The Cl_{int} of lamivudine in oocytes expressing OCT1-P283L was decreased by 85.1% compared with OCT1-WT, whereas it was decreased by 48.7% in oocytes expressing OCT1-P341L. The Cl_{int} of lamivudine in oocytes expressing OCT2-T199I, -T201M and -A270S was decreased by 86.2%, 88.9% and 73.6%, respectively, compared with OCT2-WT. When comparing various substrates such as MPP^+, TEA, metformin and lamivudine, the effects of the OCT1 genetic polymorphisms on their uptake were not identical. However, contrary to the case of OCT1, the uptake of MPP^+, TEA, metformin and lamivudine in oocytes expressing OCT2-T199I, -T201M and -A270S variants was decreased significantly compared with that in oocytes expressing OCT2-WT. In conclusion, the effect of genetic variations of OCT1 and OCT2 on the uptake of MPP^+, TEA, metformin and lamivudine was substrate-dependent. Copyright © 2012 John Wiley & Sons, Ltd.

Key words: genetic polymorphism; OCT1, OCT2; lamivudine; transport activity; substrate-dependence

Introduction

Lamivudine (2′-deoxy-3′-thiacytidine; 3TC) is a dideoxynucleoside analog that undergoes anabolic phosphorylation by intracellular kinases to form lamivudine 5′-triphosphate, the active anabolite that prevents human immunodeficiency virus (HIV) and hepatitis B virus (HBV) replication by competitively inhibiting viral reverse transcriptase and terminating DNA chain extension. Lamivudine is used at a high dose (300 mg/day) in combination with other anti-HIV agents in the treatment of HIV infection and at a low dose (100 mg/day) as a monotherapy in the treatment of HBV infection [1].

Lamivudine is eliminated primarily by urinary excretion without undergoing significant metabolism [1]. As the renal clearance of lamivudine (0.29 ± 0.05 l/kg/h) is 2.99-fold higher than creatinine clearance (0.097 ± 0.02 l/kg/h) [2], the role of renal transporters in the disposition of lamivudine *in vivo* is of great importance. The presence of a specific transporter system in the

*Correspondence to: College of Pharmacy, Kyungpook National University, Daegu 702–701, Korea.
E-mail: issong@inje.ac.kr

uptake of lamivudine has been demonstrated in brush-border membrane vesicles from the rat renal cortex and in a porcine renal epithelial cell line (LLC-PK1) [3,4]. Jung *et al*. [5] demonstrated that lamivudine was a substrate for OCT1 and OCT2 with high transport efficacies. Because OCT2 is highly expressed in the kidney [6], the pharmacokinetics of lamivudine may be changed by functional and expressional variations in OCT2. Jung *et al*. [5] also reported the increased expression (up to 1000-fold) of OCT1 mRNA in the mononuclear cells of the lymph nodes of HIV-infected patients, compared with HIV-negative controls, suggesting increased potential for the accumulation of OCT1 substrates such as lamivudine in lymph nodes.

Recently, several groups have reported polymorphic variants in the OCT families. In fact, 25 single nucleotide polymorphisms (SNPs) of *OCT1* were identified in 57 Caucasians [7], and three (R61C, C88R, G401S) of eight non-synonymous variants showed reduced transport activities. Shu *et al*. [8] reported that four SNPs (R61C, G220V, G401S, G465R) showed reduced transport function, whereas S14F exhibited increased transport function. Two non-synonymous SNPs (P283L, P341L) of *OCT1*, found in a Japanese population and a Korean population with allele frequencies of 1.3% and 16.7%, respectively, showed reduced transport activity [9–11]. Two non-synonymous SNPs of *OCT2* (M165I, R400C), which have been identified with $\geq 1\%$ allele frequency in an African-American population, were reported to confer significantly decreased transporter activity compared with the activity of wild-type (WT) OCT2 [12]. The OCT2-T199I and -T201M variants have been found only in an Asian population with a low allele frequency ($\leq 1\%$) [11,13], whereas the OCT2-A270S variant has been identified as a common SNP with a high allele frequency ($\geq 10\%$) in all ethnic groups examined. These three SNPs showed decreased metformin uptake [11–13].

However, some non-synonymous SNPs (S14F, S189L, P341L, M420del) of *OCT1* showed different functional changes depending on the substrates examined (MPP^+ vs metformin) [6]. It was also previously demonstrated that the effect of genetic variations in organic anion transporting polypeptide 1B1 and Na^+/taurocholate co-transporting polypeptide was substrate dependent according to the transport characteristics of substrates using statins as substrates [14,15]. These results suggested that the pharmacokinetic consequences of genetic variants in transporters may differ depending on the substrate administered and, as a result, the effect of genetic variants of drug transporters should be examined using several substrate drugs with a different structure.

Therefore, the purpose of the present study was to investigate the differential effect of amino acid-substitution SNPs of OCT1 (OCT1-P283L and -P341L) and OCT2 (OCT2-T199I, -T201M and -A270S), which have been identified in Asians, on the transport of MPP^+, TEA, metformin and lamivudine *in vitro*, using an oocyte overexpression system.

Methods and Materials

Chemicals

[^{3}H]Lamivudine (8.4 Ci/mmol) and [^{14}C]metformin (54 mCi/mmol) were purchased from Moraveck Biochemicals Inc. (Brea, CA). [^{14}C]TEA (24 mCi/mmol) and [^{3}H]MPP^+ (81 Ci/mmol) were purchased from Perkin Elmer Life Science (Boston, MA). Unlabeled MPP^+, TEA, metformin and lamivudine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

In vitro transcription of OCT1 and OCT2 WT and their genetic variants

Plasmid DNAs containing WT of OCT1 and OCT2 (pcDNA3.1-OCT1-WT pcDNA3.1-OCT2-WT) were confirmed by full sequencing with forward and reverse sequencing primers to ensure that there were no unwanted mutations [11].

To obtain mutant plasmids that carried single nucleotide substitution mutations in the *OCT1* and *OCT2* gene (pcDNA3.1-OCT1-P283L, pcDNA3.1-OCT1-P341L, pcDNA3.1-OCT2-T199I, pcDNA3.1-OCT2-T201M, pcDNA3.1-OCT2-A270S), mutagenesis reactions were carried out using the Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutations, as well as the fidelity of the remaining DNA, were confirmed by sequencing. The double-stranded oligonucleotides used for site-directed mutagenesis of the *OCT1*

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and *OCT2* gene were as follows: OCT1-P283L, forward 5′-CTACTGGTGTGTGCTGGAGTCCCCTCGG-3′ and reverse 5′-CCGAGGGGACTCCAGCACACCAGTAG-3′; OCT1-P341L, forward 5′-CCTGTTCCGCACGCTGCGCCTGAGGAAG-3′ and reverse 5′-CTTCCTCAGGCGCAGCGTGCGGAACAGG-3′; OCT2-T199I, forward 5′-ATGGCCATTTCCCCAATCTATACGTGGATG-3′ and reverse 5′-CATCCACGTATAGATTGGGGAAATGGCCAT-3′; OCT2-T201M, forward 5′-CATTTCCCCAACCTATATGTGGATGTTAATTTTT-3′ and reverse 5′-AAAAATTAACATCCACATATAGGTTGGGGAAATG-3′; and OCT2-A270S, forward 5′-GTTGCAGTTCACAGTTTCTCTGCCCAACTTCTTC-3′ and reverse 5′-GAAGAAGTTGGGCAGAGAAAACTGTGAACTGCAAC-3′.

Capped cRNAs were synthesized *in vitro* using T7 RNA polymerase with linear plasmid DNAs of OCT1 and OCT2 WT and their genetic variants and the synthesized cRNAs were separated by electrophoresis on 1.5% agarose gels and visualized with ethidium bromide staining.

Uptake of substrates in oocytes expressing OCT1 and OCT2 variants

3Defolliculated oocytes were injected with 50 ng of the capped cRNA and incubated at 18 °C in Barth's solution [88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_2$, 2.4 mM $NaHCO_3$, 10 mM Hepes (pH 7.4)] containing 50 g/ml gentamicin and 2.5 mM pyruvate. After incubation for 2 days, the uptake reaction was initiated by replacing the ND96 solution [96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, (pH 7.4)] containing [^{3}H]MPP^+ (30 nM), [^{14}C]TEA (10 M) [^{14}C]metformin (3.7 M) or [^{3}H]lamivudine (0.5 M), and incubated for 30 min at room temperature. Uptake experiments were terminated by the addition of ice-cold ND96 solution, followed by five washes. Oocytes were solubilized with 10% SDS and the radioactivity in the oocytes was analysed in the MicroBeta TriLux 96-well Scintillation/Luminescence Detector (PerkinElmer).

To measure the concentration dependence in the uptake of lamivudine in oocytes expressing OCT1 and OCT2 WT and their genetic variants, uptake experiments were performed in ND96 solution containing various concentrations of [^{3}H]lamivudine (5–200 M) for 30 min. After washing five times with ice-cold ND96 solution, the oocytes were solubilized with 10% SDS and the radioactivity in the oocytes was analysed.

Data analysis

To estimate the kinetic parameters for the uptake of lamivudine, the transporter-mediated uptake rates were calculated by subtracting the uptake rate of water-injected oocytes from that of transporter-expressing oocytes, followed by fitting to the Michaelis-Menten equation

$$v = \frac{V_{\max} \times [S]}{K_m + [S]}$$

using WinNonlin (version 5.2, Pharsight, Mountain View, CA), where v is the apparent linear initial rate (fmol/min/oocyte) and $V_{\max}$ and K_m represent the maximum transport rate and the Michaelis-Menten constant, respectively. The intrinsic clearance for the transport (Cl_{int}) was obtained from $V_{\max}/K_m$.

Statistical significance was analysed using an unpaired *t*-test, and $p < 0.05$ was deemed to be statistically significant. The reproducibility of the results was confirmed by at least three separate experiments. Data are expressed as mean ± SD.

Results

Uptake of substrates in oocytes expressing OCT1 and OCT2

The size and intactness of the *in vitro* transcribed cRNA from the WT and variants of OCT1 and OCT2 constructs were confirmed by gel electrophoresis. The cRNAs from OCT1 and OCT2-WT and their genetic variants (OCT1-P283L, -P341L; OCT2-T199I, -T201M, -A270S) showed similar band intensities, respectively (Figure 1A, B). Then 10, 50 and 100 ng of OCT1 and OCT2 cRNAs was injected into oocytes and incubated for 1, 2 and 3 days. Uptake experiments were performed using MPP^+ as a representative substrate for both OCT1 and OCT2. The uptake of MPP^+ increased linearly up to 50 ng injection of cRNA, became saturated at 100 ng cRNA injected, and peaked after 2 days of incubation (Figure 1C). Thus, the experimental conditions of injecting 50

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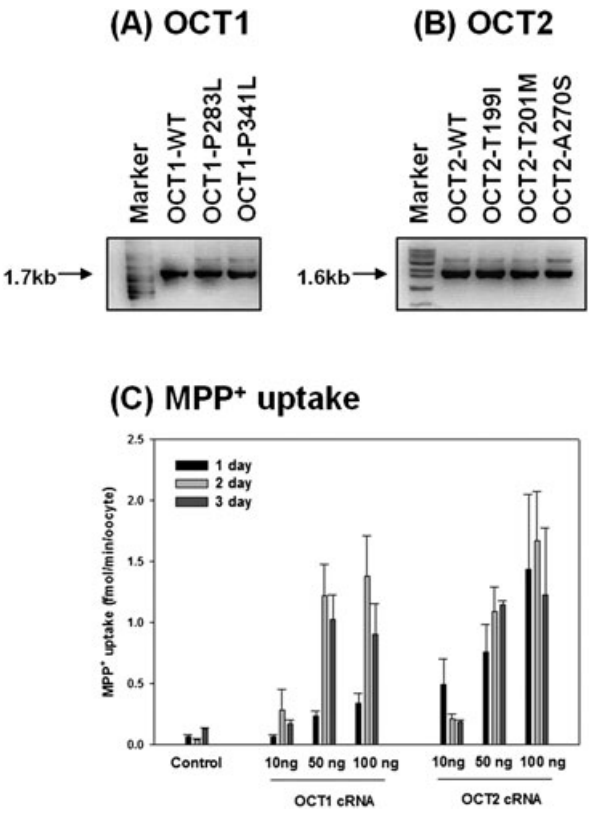

Figure 1. Gel electrophoresis results of the *in vitro* transcribed cRNA of (A) OCT1 WT and variant constructs (OCT1-P283L and -P341L) and (B) OCT2 WT and variant constructs (OCT2-T199I, -T201M and -A270S). (C) Uptake of 30 nM MPP^+ by oocytes overexpressing OCT1 and OCT2 after incubating oocytes that were injected with 10, 50 and 100 ng cRNA of OCT1 and OCT2 WT for 1, 2 and 3 days

ng of OCT1 and OCT2 cRNA and incubating for 2 days were selected for subsequent experiments. The expression of OCT1-WT and variants (P283L, P341L) was previously reported to be similar and localized primarily to the surface of the oocyte membrane [9,10]. Similarly, the expression of OCT2-WT and variants (T199I, T201M, A270S) was comparable and localized to the surface of the oocyte membrane [11].

After expressing the OCT1 and OCT2 protein for 2 days, MPP^+, TEA, metformin and lamivudine uptake was performed for 60 min. The uptake of MPP^+ and metformin increased linearly up to 30 min and the uptake of TEA and lamivudine increased linearly up to 60 min (data not shown). Thus, the uptake rates of MPP^+, TEA, metformin and lamivudine were measured for a 30 min incubation period in all uptake experiments.

Effects of genetic variation in OCT1 and OCT2 on the uptake of its substrates in vitro

The uptake of MPP^+, TEA, metformin and lamivudine in oocytes overexpressing OCT1-WT was greater than that in control oocytes, suggesting all these compounds are substrates for OCT1. The uptake of MPP^+ in oocytes expressing OCT1-P283L variant was significantly decreased, whereas the uptake of MPP^+ in oocytes expressing the OCT1-P341L variant was unchanged compared with that in oocytes expressing OCT1-WT (Figure 2A). The uptake of TEA in oocytes expressing OCT1-P283L and -P341L variants was significantly decreased compared with that in oocytes expressing OCT1-WT (Figure 2B). However, OCT1-P283L and -P341L variants did not show altered metformin uptake compared with that in oocytes expressing OCT1-WT (Figure 2C). In the case of lamivudine, the uptake into oocytes expressing OCT1-P283L and -P341L variants was decreased significantly compared with that in oocytes expressing OCT1-WT (Figure 2D). Taken together, these results suggest that the effects of the OCT1 genetic polymorphism on the uptake of MPP^+, TEA, metformin and lamivudine are not identical, although these compounds are all substrates of OCT1.

The uptake of MPP^+, TEA, metformin and lamivudine in oocytes overexpressing OCT2-WT was greater than that in control oocytes, suggesting that all these compounds are substrates for OCT2. However, in contrast to the case of OCT1, the uptake of MPP^+, TEA, metformin and lamivudine in oocytes expressing OCT2-T199I, -T201M and -A270S variants was decreased significantly compared with that in oocytes expressing OCT2-WT (Figure 3).

Concentration dependence in OCT1 and OCT2-mediated lamivudine uptake

The study determined the transport kinetics of lamivudine uptake in oocytes overexpressing OCT1 and OCT2 WT and variant proteins. Lamivudine uptake mediated by OCT1-WT was saturable, and the uptake was decreased in oocytes expressing OCT1-P283L and -P341L variants, compared with that in OCT1-WT. Similarly,

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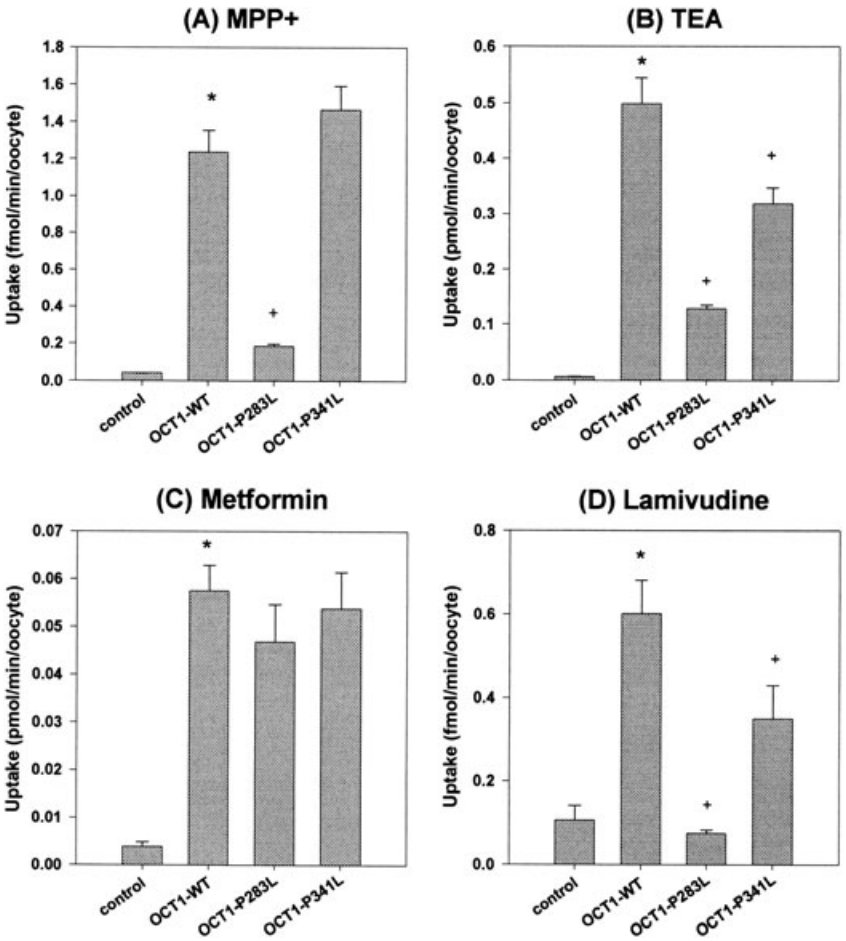

Figure 2. Uptake of (A) 30 nM MPP^+, (B) 10 μM TEA, (C) 3.7 μM metformin, (D) 0.5 μM lamivudine by oocytes overexpressing OCT1 WT and its variants, OCT1-P283L and -P341L. Each data point represents the mean ± SD from eight independent experiments. $^*p < 0.05$, statistically significant compared with the control group; $^+p < 0.05$, statistically significant compared with OCT1-WT

lamivudine uptake mediated by OCT2-WT was saturable, and the uptake was decreased in oocytes expressing OCT2-T199I, -T201M and -A270S variants, compared with that in OCT2-WT oocyte (Figure 4). Kinetic parameters, including K_m, V_{max} and intrinsic clearance (Cl_{int}), are shown in Table 1. The Cl_{int} of lamivudine was decreased by 85.1% in oocytes expressing OCT1-P283L compared with OCT1-WT, whereas the Cl_{int} of lamivudine was decreased by 48.7% in oocytes expressing OCT1-P341L. The Cl_{int} of lamivudine in oocytes expressing OCT2-T199I, -T201M and -A270S was decreased by 86.2%, 91.3% and 73.6%, respectively, compared with OCT2-WT.

Discussion

To gain a better understanding of the genetic basis of variation in the disposition of and the response to lamivudine, the effects of several functionally relevant genetic variants of OCT1 and OCT2 on the cellular accumulation of lamivudine were investigated. Among seven non-synonymous variants of *OCT1* and *OCT2* genes that have been identified in a Korean population, five functional variants of OCT1 and OCT2 (OCT1-P283L, -P341L; OCT2-T199I, -T201M, -A270S) were selected based on previous results [7–9,11,16]. Lamivudine uptake mediated by OCT1-WT was

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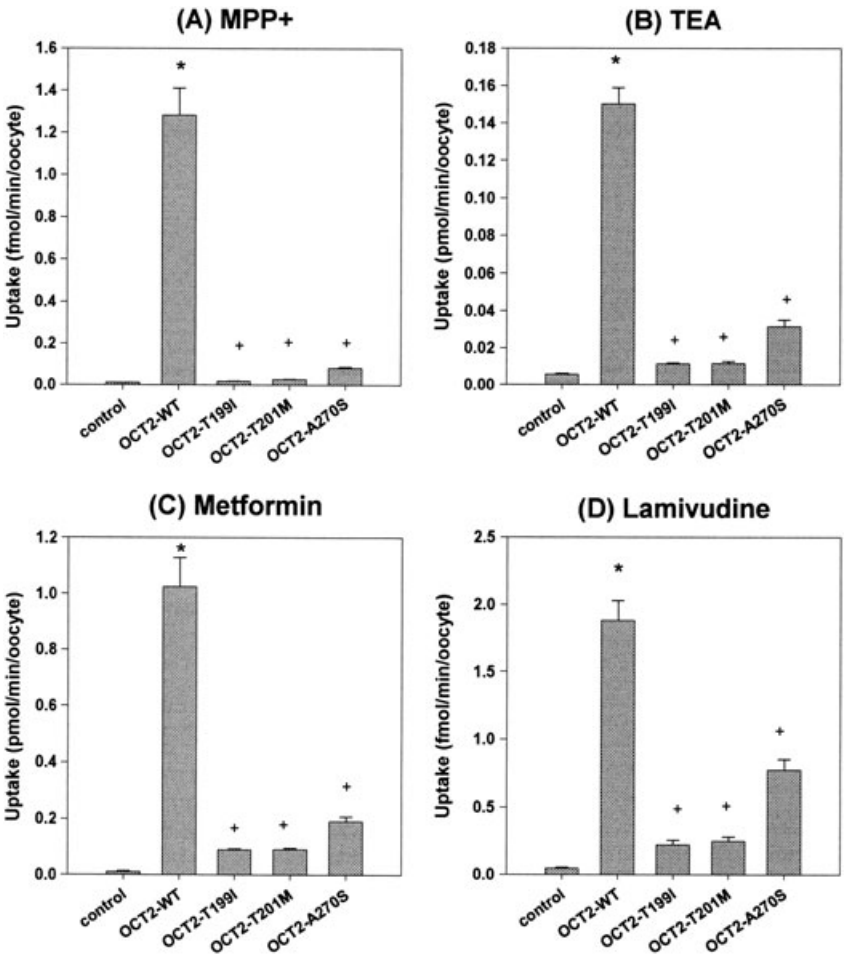

Figure 3. Uptake of (A) 30 nM MPP^+, (B) 10 μM TEA, (C) 3.7 μM metformin, (D) 0.5 μM lamivudine by oocytes overexpressing OCT2 WT and its variants, OCT2-T199I, -T201M and -A270S. Each data point represents the mean ± SD from eight independent experiments. $^*p < 0.05$, statistically significant compared with the control group; $^+p < 0.05$, statistically significant compared with OCT2-WT

saturable, and the uptake was decreased in oocytes expressing OCT1-P283L and -P341L variants, compared with that in OCT1-WT. Similarly, lamivudine uptake mediated by OCT2-WT was saturable, and the uptake was decreased in oocytes expressing OCT2-T199I, -T201M and -A270S variants, compared with that in OCT2-WT (Figure 4; Table 1). Because OCT1 and OCT2 are the major transporters expressed in the liver and kidney, respectively, and lamivudine has a higher affinity for OCT1 and OCT2, the decreased transport activity of lamivudine by five functional variants of OCT1 and OCT2 could reduce the hepatic uptake and renal elimination of lamivudine and may be responsible for the inter-individual variability in the pharmacokinetics and drug response of lamivudine.

When the effect of genetic variation in OCT1 and OCT2 on the uptake of various substrates was investigated, the differential effect of genetic variants on the transport activity of various substrates was confirmed using MPP^+, TEA, metformin and lamivudine as representative substrates for OCT1 and OCT2. Specifically, the P283L and P341L variants of OCT1 were shown to affect the transport function in a substrate-dependent manner, with decreased activity toward TEA and lamivudine, and retained transport activity for metformin. Although the structural consequences of these OCT1-P283L and -P341L genetic variants were not elucidated, these two

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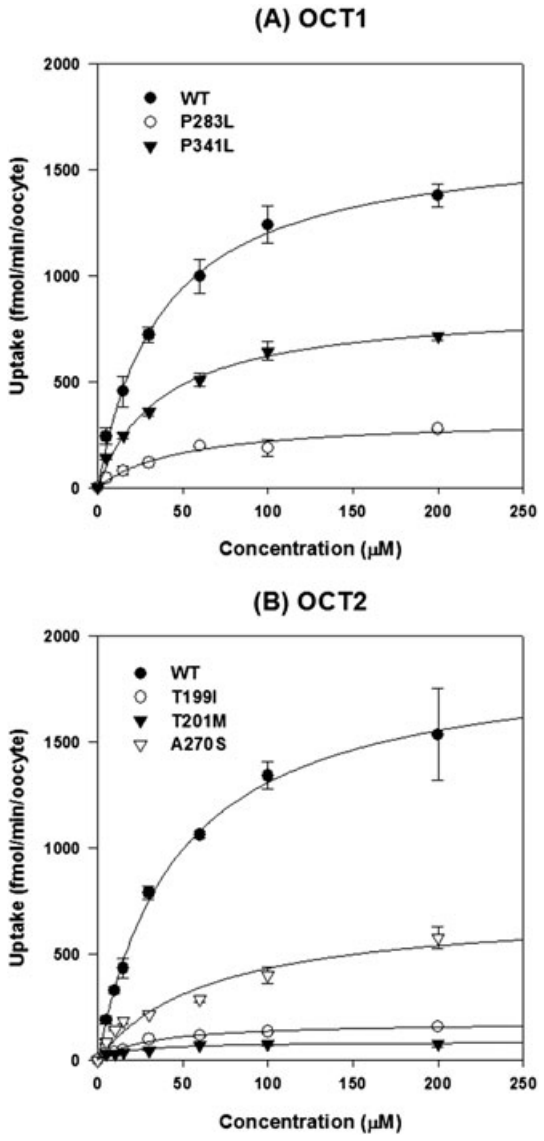

Figure 4. Concentration dependence of the uptake of lamivudine in oocytes overexpressing (A) OCT1 WT and its variants, OCT1-P283L and -P341L or (B) OCT2 WT and its variants, OCT2-T199I, -T201M and -A270S, measured in a concentration range of 1–200 μM of lamivudine. Each data point represents the mean ± SD of at least triplicate measurements of eight independent cRNA injections. The OCT1- or OCT2-mediated transport rate was obtained by subtracting the transport rate in water-injected oocytes from that in oocytes overexpressing OCT1 and OCT2 WT and variant proteins

variants are found in the loops of the protein (i.e. between transmembrane helices (TMH) 6 and 7) according to the presumed transmembrane topology of OCT1 transporters [17], which interact with TMH4 in a model of the outward facing conformation [18]. Considering that several cysteine, proline and arginine residues are thought to maintain the secondary structures of proteins and/or bind charged substrates [19], the two regions in the OCT1 protein in which these variants occur are potentially important for substrate recognition or as functional domains.

It has been demonstrated that organic cations such as choline, TEA, MPP^+ and lamivudine have more than two binding sites with high affinity (picomolar range) and low affinity (millimolar range, similar to K_m values) [18,20,21]. It was reported that ligand binding to high affinity sites of OCTs can mediate an allosteric effect on the low affinity site and/or on transport-related conformational changes, while the low affinity site was suggested to be crucial for translocation [21]. Although the high affinity binding sites have not yet been determined, genetic variations in the high affinity binding sites may cause transport-related conformational changes that could lead to substrate dependent effects on OCTs-mediated transport kinetics, which may account for the decreased V_{max} of lamivudine in OCT1-P283L and -P431L compared with OCT1-WT, without significantly altering the K_m value (Table 1).

It was reported previously that the changes in V_{max} values of the OCT2-T199I, -T201M and -A270S mutants were larger than the changes in the K_m values of the variants when measured using MPP^+ and metformin as substrates [11,22], which are consistent with the present results that V_{max} values of lamivudine in the OCT2-T199I, -T201M and -A270S mutants were significantly decreased without altering the K_m values. However, the degree of reduction varied substantially between the compounds. The Cl_{int} of lamivudine, MPP^+ and metformin in OCT2- A270S was decreased by 73.6%, 95.7% and 39.6%, respectively, compared with OCT2-WT. Altogether, three functional variants of OCT2 (T199I, -T201M, -A270S) showed markedly decreased maximum transport rates with a differential reduction depending on the substrate tested. These findings highlight the need for testing several compounds when *in vitro* functional differences between a reference transporter and its protein sequence variant are characterized. The OCT2-T199I and -T201M variants are located in the short extracellular loop between TMH3 and

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Table 1. Kinetic parameters for the uptake of lamivudine in oocytes expressing OCT1 and OCT2 WT and their genetic variants (OCT1-P283L and -P341L; OCT2-T199I, -T201M and -A270S)

		K_m (μM)	V_{max} (fmol/min/oocyte)	Cl_{int} (nl/min/oocyte)
OCT1	WT	37.8 ± 13	1653 ± 48	43.7 ± 5.7
	P283L	50.3 ± 14	327 ± 36[a]	6.5 ± 2.3[a]
	P431L	38.1 ± 15	855 ± 37[a]	22.4 ± 7.3[a]
OCT2	WT	46.3 ± 13	1912 ± 47	41.3 ± 3.5
	T199I	38.5 ± 15	178 ± 29[a]	5.7 ± 2.1[a]
	T201M	31.6 ± 14	86.0 ± 16[a]	3.6 ± 1.8[a]
	A270S	65.8 ± 21	715 ± 99[a]	10.9 ± 4.2[a]

[a] $p < 0.05$, statistically significant compared with WT.

TMH4, and OCT2-A270S is located in the loop between TMH5 and TMH6 according to the presumed transmembrane topology of OCT2 transporter [12]. It is reported that three amino acids on TMH4 (W217, Y221 and T225), three amino acids on TMH10 (I442, L446 and E448) and one amino acid on TMH11 (C474) formulate one region surrounding a large cleft, which opens to the intracellular side and this region is believed to be the substrate-binding pocket for OCT2 [23,24]. Thus, the amino acid substitutions in OCT2-T199I, -T201M and -A270S mutants might have inhibitory effects on conformational changes of the transporter rather than on substrate binding.

Since OCT2-T199I and -T201M were reported in the Asian population [11], their potential impact on the therapeutic efficacy or disposition of substrate drugs will be crucial in the Asian population. Moreover, the functional consequences of genetic variants in OCT1 and OCT2 (OCT1-P283L and -P341L and OCT2-T199I, -T201M and -A270S) were substrate-specific. Therefore, the clinical relevance of these genetic variants to the therapeutic efficacy or disposition of substrate drugs will require a dedicated genotype-phenotype study. Further studies are needed to clarify the *in vivo* impact of these OCT1 and OCT2 variants.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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