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The inhibition mechanism of the uptake of lamivudine *via* human organic anion transporter 1 by *Stellera chamaejasme* L. extracts

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[ABSTRACT] *Stellera chamaejasme* L. is a traditional Chinese medicine with a long history to treat stubborn skin ulcer, and it also has antiviral and antitumor effects. Neochamaejasmine B (NCB), Neochamaejasmine A (NCA) and Chamaechromone (CMC) are the major components in dried roots of *Stellera chamaejasme* L.. Our studies suggested that NCB, NCA and CMC are inhibitors of Organic anion transporter 1 (OAT1). OAT1 is encoded by solute carrier family 22 member 6 gene (*SLC22A6*) in humans and plays a critical role in the organic anion drug uptake and excretion in the kidney. Lamivudine is the typical substrate of OAT1 and is frequently used in combination with other antiviral drugs in clinical antiviral treatments. The aim of this study is to investigate the interaction and its mechanism between these bi-flavone components in *Stellera chamaejasme* L. and lamivudine *via* OAT1 both *in vitro* and *in vivo*. *In vitro*, the uptake studies in Madin-Darby canine kidney (MDCK) cells overexpressing OAT1 suggested that NCB inhibited the uptake of 6-CFL and lamivudine.Similar results were obtained for NCA and CMC. NCB was a noncompetitive and competitive inhibitor interaction with OAT1. IC_{50} values of NCB, NCA and CMC for inhibiting OAT1-mediated lamivudine transport were 2.46, 8.35 and 0.61 μmol·L^{-1}, respectively. *In vivo*, the pharmacokinetic results of lamivudine in rats showed that the mean area under the plasma concentration-time curve ($AUC_{0-\infty}$) and maximal plasma concentration (C_{max}) of lamivudine after co-administration is increased 2.94-fold and 1.87-fold, respectively, compared to lamivudine administration alone. The results of interactions between lamivudine and these bi-flavone components in *Stellera chamaejasme* L. extracts *via* OAT1 *in vivo* are consistent with studies *in vitro*. The inhibition of OAT1-mediated uptake of lamivudine by NCB, NCA and CMC is the possible mechanism for *Stellera chamaejasme* L. extracts improving the oral bioavailability of lamivudine in rats.

[KEY WORDS] Lamivudine; Neochamaejasmine B; Neochamaejasmine A; Chamaechromone; OAT1; Inhibition

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Introduction

Stellera chamaejasme L. is a traditional Chinese herb to treat stubborn skin ulcers, and it is also reported having antiviral and antitumor bioactivity [1-2]. Neochamaejasmine B (NCB), Neochamaejasmine A (NCA) and Chamaechromone (CMC) are the major bi-flavone components in dried roots of *Stellera chamaejasme* L. [3-5]. Drug transporters play an important role in pharmacodynamics and pharmacokinetics [5-6]. In our previous study, we observed that the bioavailability of CMC was low in rat [7]. We also found that NCB increased the bioavailability of CMC in rats, and the inhibition of breast cancer resistance protein (BCRP) and multidrug resistance protein 2 (MRP2)-mediated efflux of CMC by NCB was suggested as the mechanism [8-9].

OAT1 is encoded by solute carrier family 22 member 6 gene (SLC22A6) in humans [10]. It is an OAT family proteins member and a trans-membrane protein that is expressed in the

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basolateral membrane of proximal tubular cells of the kidney, brain, placenta, etc. The OAT family plays an important role in the processing of common drugs including antivirals such as lamivudine, toxins such as aristolochic acid, and nutrients such as flavonoids [11]. Moreover, lamivudine is a typical substrate of OAT1 [12] and widely applied in clinical antiviral treatments [13]. *Stellera chamaejasme* L. also has an antiviral effect on HBV and HIV [14-17]. In general, antiviral therapy is commonly used in combination. However, the potential herb-drug interactions of *Stellera chamaejasme* L. extracts with antiretroviral drugs remain unknown. It is intrigue for us to investigate the interaction mechanism between these bi-flavone components in *Stellera chamaejasme* L. and lamivudine *via* OAT1.

Materials and Methods

Materials

Stellera chamaejasme L. extract was prepared in our lab [7]. Briefly, the dried roots powder of the herb was extracted 5 times with 95% ethanol, and the extraction solvents were collected then evaporated to non-alcohol odor by vacum distillation. NCB, NCA and CMC were isolated and purified from *Stellera chamaejasme* L. in our laboratory. Their chemical structures were identified by ^{1}H NMR and ^{13}C NMR that were consistent with reference data [8]. HPLC grade methanol and formic acid were bought from TEDIA Inc. (Fairfield, USA). An ELGA-purelab Ultra system (High Wycombe, UK) was used throughout the study to obtain Ultra-pure water (18.2 MΩ). Standard compounds such as lamivudine, probenecid, 6-carboxyfluorescein (6-CFL), dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). Tris, glycerin, sodium dodecyl sulphate (SDS) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents were analytical grade and obtained from the chemical reagent company of Ludu, Shanghai. Fetal bovine serum, Dulbecco's modified eagle's medium (DMEM, high-glucose), nonessential amino acids, 0.25% trypsin-EDTA solution and antibiotic-antimycotic were obtained from GIBCO (Grand Island, NY, USA).

Evaluation of lamivudine by LC-MS/MS

Sample treatment

Standard solutions of lamivudine at concentrations of 0.02, 0.22, 0.44, 2.18, 4.37, 21.83, 43.67 μmol·L^{-1} were acquired in methanol as calibration and quality control (QC) samples.

The plasma samples (80 μL) were precipitated with three fold of methanol. After vortex-mixing for 4min and centrifuging at 13 000 r·min^{-1} for 10 min, 10 μL of supernatant was injected for LC-MS/MS analysis [18]. The samples of cell lysate of 0.1% SDS (140 μL) or HBSS solution (200 μL) were prepared by the same procedure.

LC-MS/MS system and conditions

The UPLC system (Agilent 1290 series) was furnished with a binary pump, an auto sampler and a column oven and a ZORBAX Eclipse Plus C_{18} column (Agilent, 2.1 mm × 50 mm, 2.2 μm, Stockport, UK) at 25 °C. Besides, 0.1% (*V*/*V*) formic acid in water and methanol were A and B mobile phases, respectively. The flow rate was 150 μL·min^{-1}. The chromatographic method was set a linear gradient as follows: 0–5.0 min, 15% B to 100% B, after each run, a 0.5-min equilibration was performed with the initial mobile phase composition. Analytes were assayed by MS/MS with an electrospray ionization (ESI)-interface in positive multiple reaction monitoring (MRM)-mode. Mass transitions of lamivudine (*m/z* 230→112) were optimized. The parameters of the mass spectrometer were optimized and set as follows: HV capillary was fixed at 3500 V, nebulizer was fixed at 45 psi, and drying gas flow rate was fixed at 8 L·min^{-1} with 300 °C temperature. The collision energy and cell accelerator voltage were set at 8 units and 3 V, respectively, with a dwell time of 200 s.

Cellular uptake assay

Cell culture

MDCK-OAT1 cells and their MOCK cells were prepared in our laboratory as described previously [19]. Briefly, cells were cultured in DMEM supplemented with 10% FBS (fetal bovine serum). All cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. When cells had reached 60% confluence, a solution of 0.25% trypsin-EDTA was added to detach the cells. Cells were seeded into 96-well plates (Corning, Bedford, MA), 48-well plates (Corning, Bedford, MA) for further experiments and cell culture flasks (Corning, Bedford, MA) were used for cell passage cultivation.

6-CFL and lamivudine uptake assay

The uptake of 6-CFL and lamivudine was conducted in MDCK-OAT1 cells. Cells were seeded in 48, 96-well culture plates at a density of 1 × 10^5 cells/well. For 6-CFL, 96-well culture plates were applied. For lamivudine, 48-well culture plates were used. After incubating for 48 h, cells were washed twice with preheated HBSS, and then were pre-incubated in HBSS for 20 min at 37 °C (negative control), and 1 mmol·L^{-1} probenecid, and a variety of concentrations of NCB, NCA, CMC (0, 5, 10, 20, 40, and 60 μmol·L^{-1}) as inhibitors. We used 6-CFL and lamivudine as the substrates of OAT1. Cells were incubated with 6-CFL in the presence of each inhibitor for 4 min at 37 °C. The final 6-CFL concentration was 2.5, 5.0, 8.0, 10.0 μmol·L^{-1} [20] and the concentration of lamivudine was 50.0 μmol·L^{-1}. The experiment was terminated by three washes in ice-cold PBS buffer. Cells were lysed with 0.1% (*V*/*V*) SDS for 15 min at 37 °C. The fluorescence of the lysate was recorded at 490 nm (excitation wavelength) and 525 nm (emission wavelength). Lamivudine concentrations were measured by LC-MS/MS, and the sample treatment procedure and LC-MS/MS condition were listed in 2.2 section.

Uptake data analysis

The data of 6-CFL uptake assay were imported to calculate K_i and K_i' with the following formula:

$$\frac{1}{V}=\frac{K_m}{V_m}\times\left(1+\frac{[I]}{K_i}\right)\times\frac{1}{[S]}+\frac{1}{V_m}\times\left(1+\frac{[I]}{K_i'}\right) \quad \text{(Equation 1)}$$

Here, the relating reaction rate is expressed as V to [S], the concentration of the substrate is expressed as S, V_m represents the maximum rate at maximum (saturating) substrate concentrations. The Michaelis constant K_m is the substrate concentration at which the reaction rate is half of V_m [21].

After the samples` protein concentration was determined by BCA protein assay kit. The IC_{50} of NCB was determined by constructing a dose-response curve and examining the effect of different concentrations of NCB on uptake assay. IC_{50} values was calculated for NCB by determining the concentration of NCB needed to inhibit half of the maximum OAT1 inhibition response to lamivudine. The other two inhibition constants K_i and K_i` were calculated by Equation 1. The type of NCB interaction with OAT1 was determined through comparing the value of K_i and K_i', since double-reciprocal plots were useful for differentiating between noncompetitive and competitive inhibitors. IC_{50} values of NCA and CMC for inhibiting OAT1-mediated lamivudine transport were gotten in the same manner as NCB.

Pharmacokinetic studies

Animals

Ten Sprague-Dawley rats (5 male, 5 female, 200–220 g) were obtained from the Animal Center of Zhejiang Academy of Medical Sciences (Hangzhou, China). All experimental animals had free access to water and food. After one week of acclimation, animals were fasted overnight before starting any treatment. All procedures were approved by the Animal Ethics Committee of Zhejiang University.

Pharmacokinetic studies

The rats were randomly divided into two groups, one group was composed of three male and two female rats, the other group was composed of two male and three female rats. Lamivudine (15 mg·kg^{-1}) and *Stellera chamaejasme* L. extract administrations (234.5 mg·kg^{-1}, containing 33.3 mg·kg^{-1} of NCB, 66.8 mg·kg^{-1} of CMC, 34.9 mg·kg^{-1} NCA) were suspended in 0.5% carboxymethyl cellulose sodium solution (CMC-Na). After an oral administration of lamivudine alone or with the extracts, blood samples were collected. The collecting time points were before, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 11, and 24 h after medication. Then samples were immediately centrifuged for the separation of plasma. Plasma samples were kept frozen at –20 °C until analysis using LC-MS/MS.

Data analysis

Pharmacokinetic parameters were determined by a non-compartmental analysis using DAS2.0 software (Chinese Pharmacologic Society, Beijing, China). And parameters underwent further analysis using standard student's *t*-test. SAS (v8.2; SAS Institute, Inc., Cary, NC) was used for statistical data analysis. Differences were considered significant at $P < 0.05$. For each data group, results were expressed as mean ± SD.

Results

LC-MS/MS method validation

For analysis of lamivudine both in cell and plasma samples, LC-MS/MS was applied. The developed methods reached required specificity, linearity range, precision, sensitivity, recovery, accuracy and sample stability. There was no endogenous interference from the blank lysate and plasma. The calibration curves of lamivudine were linearity within the range of 5.0 to 1000 ng·mL^{-1}, and the correlation coefficients were higher than 0.998. The lower limit of quantification (LLOQ) of lamivudine was 5.0 ng·mL^{-1} of each method. The inter-day and intra-day precisions (RSD%) were lower than 7.4%. The accuracies (RE%) ranged from –0.2% to +6.7% and the extraction recoveries of lamivudine ranged from 93.2% to 98.4% for low, medium and high concentrations QC samples. The QC samples at low, medium, and high concentrations were stable at room temperature for 12 h, and for 30 d at –80 °C with 3 freeze-thaw cycles.

Uptake assay

NCB inhibition of OAT1

After 6-CFL exposure to NCB (40 μmol·L^{-1}), the cell uptake of 6-CFL reduced to 17.6% compared to the negative control exposure. And the uptake of 6-CFL decreased to 27.5% when incubated with the positive control inhibitor probenecid, indicating that NCB is a potential OAT1 inhibitor (Fig. 1A). In the dose-response curve, 6-CFL uptake amounts in MDCK- OAT1 cells decreased in a dose-depended manner

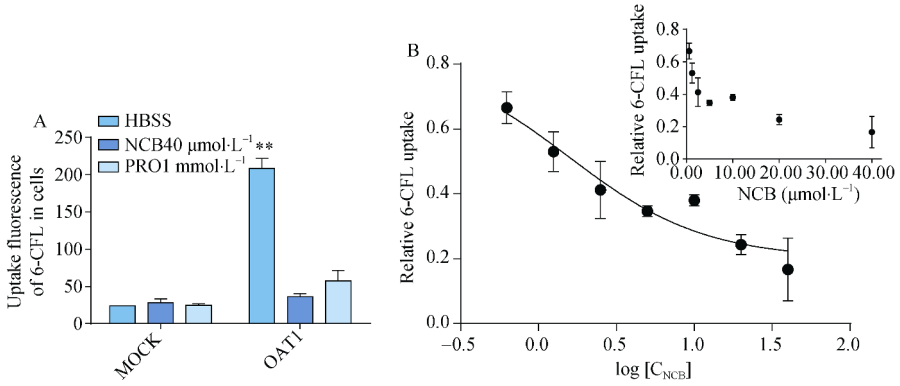

Fig. 1 Inhibition of NCB on 6-CFL transport *via* OAT1 in cells. (A) 6-CFL uptake in MDCK-OAT1 cells; (B) IC_{50} determined in cells. OAT1, MOCK refers to MDCK-OAT1, MDCK-MOCK cells, respectively. Data represents the mean ± SD of triplicate determinations. $^{**}P < 0.01$ means significantly difference from other groups

when incubated with NCB (0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 μmol·L^{-1}). NCB inhibited the uptake of 5μM 6-CFL with IC_{50} of 1.57 μmol·L^{-1} (Fig. 1B). 6-CFL uptake velocities in cells incubated with different concentrations of NCB were calculated (Fig. 2A). When used for determining the type of the inhibition, the Lineweaver-Burk plot can distinguish competitive, non-competitive, uncompetitive and mixed-type inhibitors. Lineweaver-Burk plot of 1/*V* against 1/[S] was obtained (Fig. 2B). K_i of 7.87 μmol·L^{-1} was determined graphically from the intercept on the ordinate of Fig. 2C by plotting the slope values from Fig. 2B against different concentrations of NCB. Meanwhile K_i' of 31.52 μmol·L^{-1} was obtained graphically from the intercept on the ordinate of Fig. 2D, in which the y-intercept values from Fig. 2B were plotted against different concentrations of NCB. Since K_i` was much higher than K_i in our result, NCB was likely a mixed-type inhibitor with both competitive and non-competitive features when interacting with OAT1.

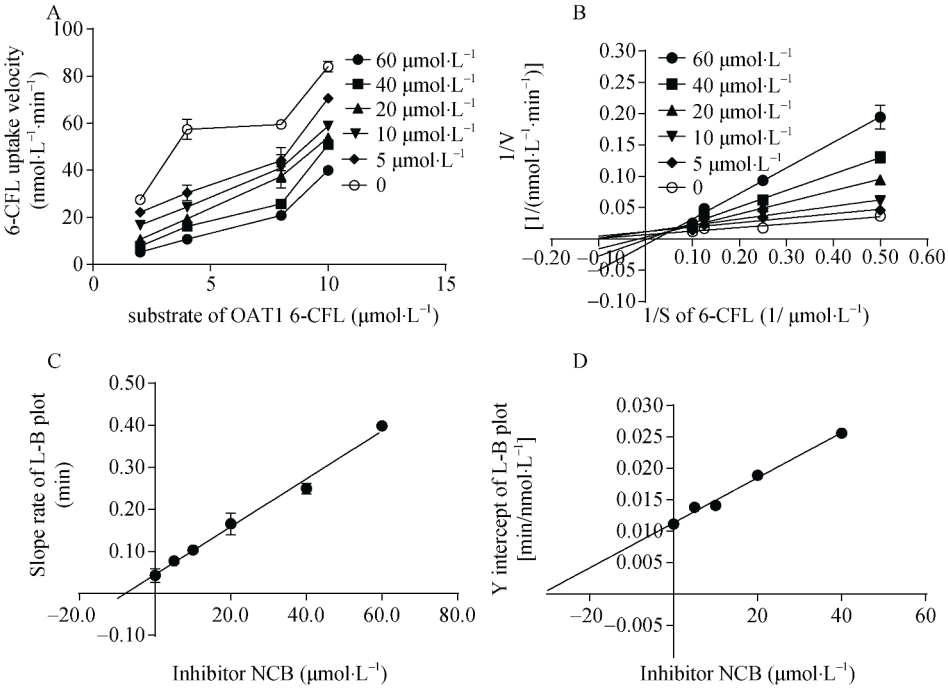

Fig. 2 The inhibitor type of NCB when inhibiting 6-CFL transport *via* OAT1 in cells. (A) 6-CFL uptake in cells incubated with NCB. (B) Lineweaver-Burk plot. (C) *Ki*. (D) *Ki*`. Data represents mean ± SD of triplicate concentrations

Furthermore, time course of OAT1-mediated lamivudine uptake was verified, suggesting that OAT1 had high activity (Fig. 3A). After incubation with NCB (40 μmol·L^{-1}), the cell uptake of lamivudine decreased to 45.2% compared to the buffer control, suggesting that NCB blocked the lamivudine uptake into cells by inhibiting OAT1 (Fig. 3B). The amount of lamivudine uptake decreased in a dose-depended manner with NCB (Fig. 3C). The IC_{50} value of NCB was 2.46 μmol·L^{-1} when co-incubated with 50 μmol·L^{-1} lamivudine (Fig. 3D). All these results confirmed that NCBwas an inhibitor of OAT1.

NCA and CMC inhibition of OAT1

Inhibition results were acquired when MDCK-OAT1 cells exposed to NCA or CMC. After co-incubation with 40μM of NCA or CMC, the cell uptake of lamivudine reduced to 10.8% and 10.1%, compared to HBSS, respectively, indicating that NCA and CMC were OAT1 inhibitors (Fig. 4A and 5A). The amount of lamivudine uptake decreased in a dose-depended manner with NCA and CMC, or in another word, the inhibition of OAT1 was in a dose-depended manner (Fig. 4B and 5B). The IC_{50} values of NCA and CMC were 8.35 and 0.61 μmol·L^{-1}, respectively, when co-incubated with 50 μmol·L^{-1} lamivudine (Fig. 4C and 5C).

Pharmacokinetic studies

Mean plasma concentration *versus* time profiles of lamivudine following a co-administration of lamivudine (15 mg·kg^{-1}) and *Stellera chamaejasme* L. extracts (234.5 mg·kg^{-1}, containing NCB, CMC, NCA) were presented in Fig. 6. The corresponding pharmacokinetic parameters were listed in Table 1. According to the Akaike information criterion (AIC) minimum rule, lamivudine pharmacokinetics after co-administration fit the one compartment model, whereas single administration fit the two-compartment model. The $AUC_{0-\infty}$ of lamivudine was increased by 2.94 folds (4783.4 ± 1112.1 *vs* 1623.7 ± 1200.3 μg·L^{-1}·h^{-1}, $P < 0.05$), and C_{max} of lamivudine was increased by 1.87 folds (2182.6 ± 460.4 *vs* 1029.0 ± 395.0 μg·L^{-1}, $P < 0.05$).

Discussion

Lamivudine is a nucleoside analog reverse transcriptase inhibitor (NRTI) with a long history of use in human immune deficiency virus (HIV)-infected persons [22-23], and it was also

the first oral nucleoside analogue approved by the US Food and Drug Administration [24]. *Stellera chamaejasme* L. has been used as a remedy for stubborn skin ulcers with antiviral activities [14, 17]. *Stellera chamaejasme* L. also has an antiviral effect on HBV and HIV [14-15, 17]. In this study, lamivudine absorption was substantially improved with a 2.94-fold $AUC_{0-\infty}$ increase when co-administered with *Stellera chamaejasme* L. extracts. The pharmacological effects of lamivudine expects to be enhanced when co-administrated with *Stellera chamaejasme* L. extracts.

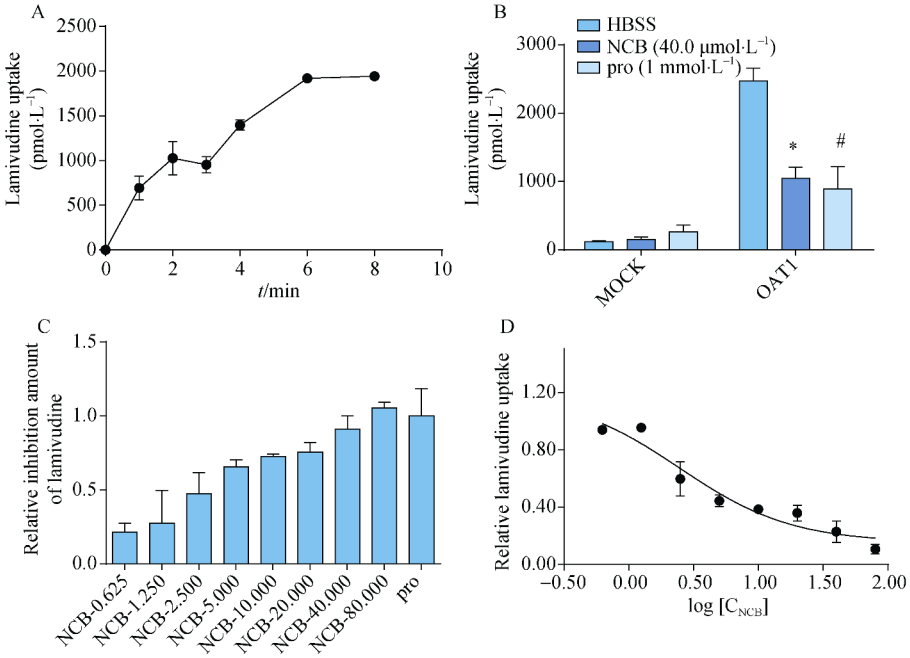

Fig. 3 Inhibition of NCB on lamivudine transport *via* OAT1 in cells. (A)Time course of OAT1-mediated lamivudine uptake. (B) Inhibition of OAT1-mediated 50 μmol·L^{-1} lamivudine uptake. (C)Concentration–dependent inhibition of lamivudine by NCB. (D) IC_{50} for inhibition of OAT1 by NCB. Data represents the mean ± SD of triplicate determinations. $^{*}P < 0.05$ *vs* HBSS group; $^{\#}P < 0.05$ *vs* HBSS group

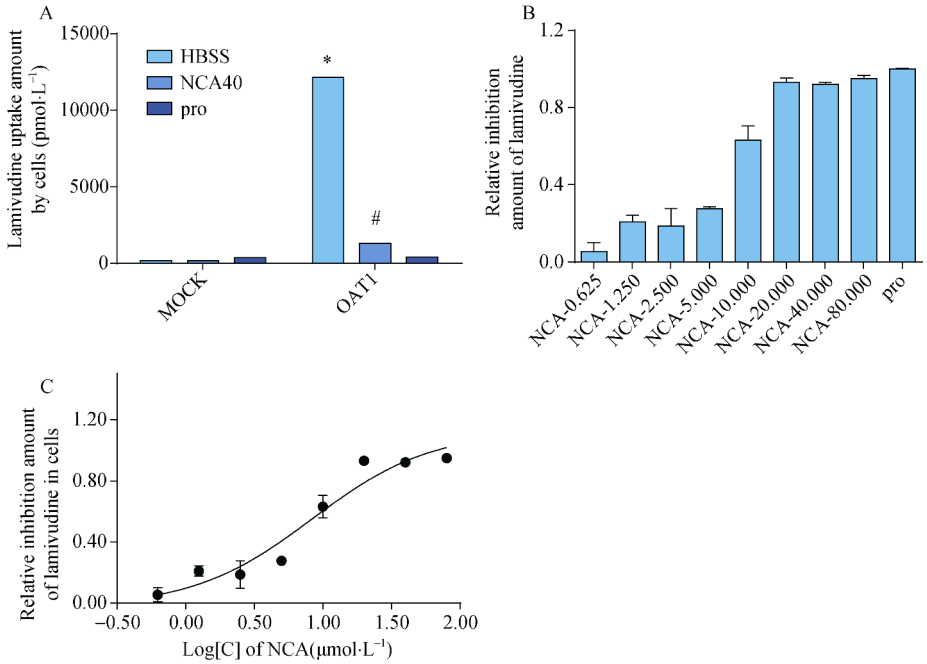

Fig. 4 Inhibition of NCA on OAT1-mediated lamivudine transport in cells. (A) Uptake of lamivudine in cells when in presence or absence of NCA or positive control (probenecid). (B) Relative cells uptake of lamivudine in the presence of different concentrations of NCA or probenecid. Data represents the mean ± SD of triplicate determinations. (C) IC_{50} calculation. $^{*}P < 0.05$, significantly different from other groups

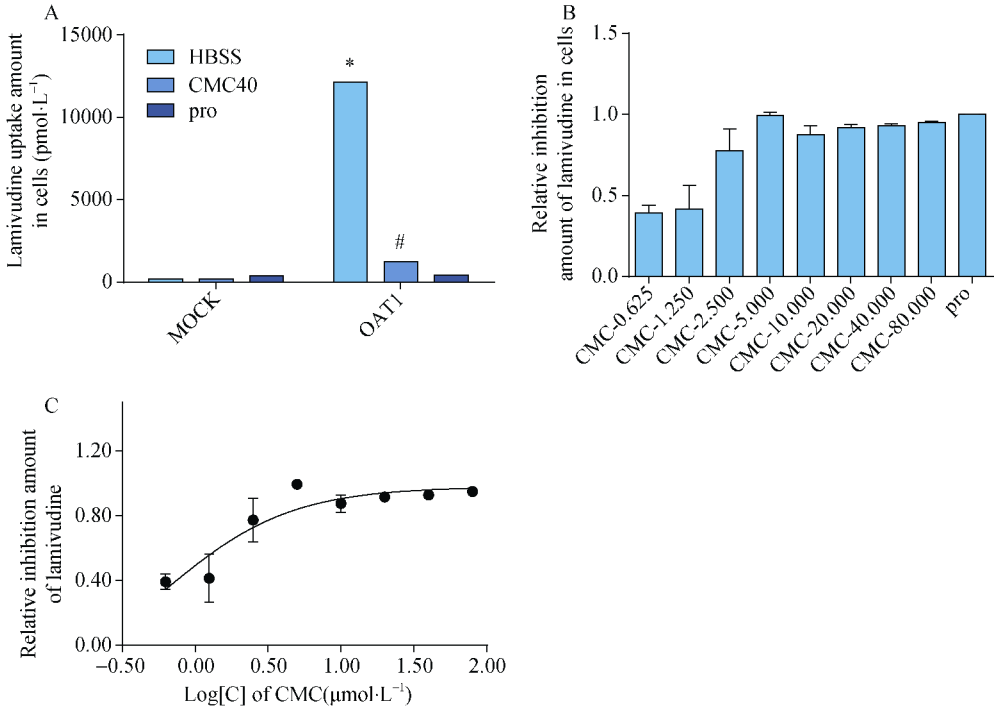

Fig. 5 Inhibition of CMC on OAT1-mediated lamivudine transport in cells. (A) Cell uptake of lamivudine in presence or absence of CMC or positive control (probenecid). (B) Relative cells uptake of lamivudine in the presence of different concentrations of CMC or probenecid. Data represents the mean ± SD of triplicate determinations. (C) IC_{50} calculation. $^{*}P < 0.05$, significantly different from other groups. $^{\#}P < 0.05$, significantly different from other groups

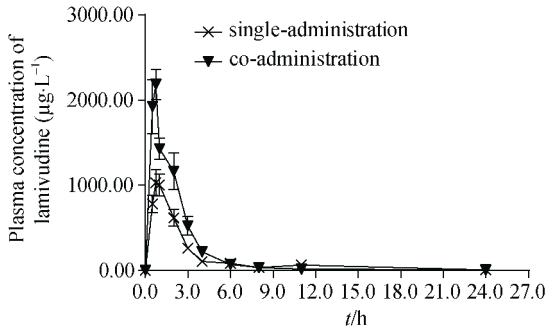

Fig. 6 Mean plasma concentration *versus* time profiles of lamivudine following a single-administration of lamivudine (15 mg·kg^{-1}), co-administration of lamivudine (15 mg·kg^{-1}) and *Stellera chamaejasme* L. extracts (234.5 mg·kg^{-1}, containing NCB, NCA, CMC). Data are shown as mean ± SD (n = 5). The best fit pharmacokinetic model of co-administration is two compartments model with weight of 1/C^2, while the single-administration pharmacokinetic model is one compartments model with weight of 1/C^2

The single-dose pharmacokinetic parameters of lamivudine in rats in this study were as follows: C_{max} was 1029.0 ± 395.0 μg·L^{-1}, T_{max} was 0.75 h and $AUC_{0-\infty}$ was 1623.7 ± 1200.3 μg·L^{-1}·h^{-1}, which was consistent with previous reports [25]. Further, the intracellular lamivudine of Caco-2 cells revealed a good correlation with the *AUC* in healthy volunteers and rabbits[26]. In this study, the lamivudine uptake data inhibited by NCB, CMC, and NCA *in vitro* perfectly explained the increase of C_{max}, T_{max}, $AUC_{0-\infty}$ of lamivudine in rats. The data showed a good *in vitro-in vivo* consistency. *In vitro*, NCB, CMC, and NCA blocked the lamivudine uptake into cells by inhibiting OAT1. *In vivo*, the *Stellera chamaejasme* L. extracts significantly increased plasma pharmacokinetics of lamivudine ($P < 0.05$ for C_{max}, T_{max}, $AUC_{0-\infty}$) *via* inhibition of OAT1-mediated lamivudine uptake from the blood to the kidney.

Recently, *in vitro-in vivo* extrapolation is commonly used to predict the risk of *in vivo* clinical drug-drug interaction (DDI) or herb-drug interaction (HDI) involving transporters using *in vitro* inhibition assays [27]. FDA issued a draft DDI guideline recommending to perform clinical DDI studies when [I] / $IC_{50} \geq 0.1$. In our study, [I] was the unbound plasma concentration of NCB, CMC, and NCA at the highest clinical dose. And the IC_{50} values of NCB, NCA and CMC for inhibiting OAT1-mediated lamivudine transport were 2.46, 8.35 and 0.61 μmol·L^{-1}, respectively. We could speculate that the concentration of NCB, NCA and CMC were limited under 0.246, 0.835, 0.061 μmol·L^{-1} to avoid the DDI in clinical application. For the three compounds, CMC seemed more likely to occur the HDI in clinical application.

It has been reported that lamivudine was also the substrate of organic cation transporters (OCTs) [28], multidrug and toxin extrusion 1 (MATE1), and multidrug and toxin extrusion (MATE2-K) [29]. OATs, OCT2, and MATE mRNA were mainly expressed in the proximal tubules, and OAT1

plays an important role in reabsorption and secretion regulation of compounds [30-31]. Except OCT2 mRNA expression, mRNA expression levels of OAT1 are much higher than that of MATE1, and MATE2-K [32]. Moreover, MATE1-mediated efflux of lamivudine seemed to be a low affinity process, with no observable transporter-driven efflux of lamivudine in MDCK-MDR1, MDCK-MRP2 and MDCK-BCRP monolayers[33]. While for OCT2, our previous results showed that NCB, NCA and CMC were not the OCTs` inhibitors, in addition, OCT2 had no effect on transcellular lamivudine transport [29]. All of these results support that OAT1 plays a more important role than other drug transporters.

Table 1 Pharmacokinetic parameters of lamivudine following a single-administration of lamivudine (15 mg·kg^{-1}), co-administration of lamivudine(15 mg·kg^{-1}) and *Stellera chamaejasme* L. extracts (234.5 mg·kg^{-1}, containing NCB, CMC, NCA) (n = 5)

single-administration		co-administration	
Parameters	Mean ± SD	Parameters	Mean ± SD
C_{max} (μg·L^{-1})	1029.0 ± 395.0	C_{max} (μg·L^{-1})	2182.6 ± 460.4
$t_{1/2\alpha}$ (h)	1.60 ± 1.18	$t_{1/2}$(h)	0.77 ± 0.56
$t_{1/2\beta}$ (h)	3.69 ± 0.52	K_e(1/h)	7.44 ± 3.57
CL/F(L·h^{-1}·kg^{-1})	15.63 ±13.84	CL/F(L·h^{-1}·kg^{-1})	3.24 ± 0.62
$AUC_{0-\infty}$(μg·L^{-1}·h^{-1})	1623.7 ± 1200.3	$AUC_{0-\infty}$ (μg·L^{-1}·h^{-1})	4783.4 ± 1112.1

Data are expressed as arithmetic mean ± standard deviation (median)

We could conclude that the inhibition of OAT1 by these three compounds is the primary contribution to an increase of lamivudine bioavailability. To differentiate the relative contributions among NCB, NCA and CMC in the *Stellera chamaejasme* L. extracts, further studies are needed.

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