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Screening and verifying potential NTCP inhibitors from herbal medicinal ingredients using the LLC-PK1 cell model stably expressing human NTCP

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[ABSTRACT] NTCP is specifically expressed on the basolateral membrane of hepatocytes, participating in the enterohepatic circulation of bile salts, especially conjugated bile salts, to maintain bile salts homeostasis. In addition, recent studies have found that NTCP is a functional receptor of HBV and HDV. Therefore, it is important to study the interaction between drugs and NTCP and identify the inhibitors/substrates of NTCP. In the present study, a LLC-PK1 cell model stably expressing human NTCP was established, which was simple and suitable for high throughput screening, and utilized to screen and verify the potential inhibitors of NTCP from 102 herbal medicinal ingredients. The results showed that ginkgolic acid (GA) (13 : 0), GA (15 : 1), GA (17 : 1), erythrosine B, silibinin, and emodin have inhibitory effects on NTCP uptake of TCNa in a concentration-dependent manner. Among them, GA (13 : 0) and GA (15 : 1) exhibited the stronger inhibitory effects, with IC_{50} values being less than 8.3 and 13.5 μmol·L^{-1}, respectively, than the classical inhibitor, cyclosporin A (CsA) (IC_{50} = 20.33 μmol·L^{-1}). Further research demonstrated that GA (13 : 0), GA (15 : 1), GA (17 : 1), silibinin, and emodin were not substrates of NTCP. These findings might contribute to a better understanding of the disposition of the herbal ingredients *in vivo*, especially in biliary excretion.

[KEY WORDS] Herbal medicinal ingredients; Human NTCP; Inhibitor; Transport; Biliary excretion

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Introduction

As the largest metabolic organ in human body, the liver plays an important role in the maintenance of homeostasis. One of its important functions is bile formation [1]. In humans, more than 90% of bile salts return to the liver through the passive diffusion or the active transport via the transporters, and then be secreted again into bile, which is called the enterohepatic circulation of bile salts [2]. Transporters which participate in the enterohepatic circulation of bile salts include Na^+/taurocholate co-transporting polypeptide (NTCP), bile salt export pump (BSEP), multidrug resistance protein 3 (MDR3), apical Na^+-dependent bile acid transporter (ASBT), organic solute transporter *α-β*, (OST *α-β*), and organic anion transporting polypeptides (OATPs) [3]. Interrupting the enterohepatic circulation can affect the homeostasis of bile salts, which may cause different degrees of cholestasis, damage to the mitochondria, and liver toxicity [4].

NTCP belongs to the SLC10 family (SLC10A1). It is specifically expressed in the basolateral membrane of hepatocytes, participating in the enterohepatic circulation of bile salts, especially conjugated bile salts, to maintain bile salts homeostasis [5]. NTCP, together with OATPs, is responsible for the uptake of bile salts from plasma into the liver [6]. Although the transport of bile salts by NTCP in the body has compensatory mechanism, the function of NTCP

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can't be entirely replaced by OATPs. Severe clinical symptoms may be emerged when the transport function of NTCP is blocked [7]. As reported by Vaz *et al*. [7] a patient with NTCP deficiency is clinically characterized by mild hypotonia, growth retardation, and delayed motor milestones. Although there were no clinical signs of cholestatic jaundice, pruritis, or liver dysfunction, his total bile salts in plasma are 10 times that of the normal.

Recent studies have found that NTCP is a functional receptor of Hepatitis B virus (HBV) and Hepatitis D virus (HDV), revealing the new role of NTCP [8]. Considering the dissatisfactory results of IFN-based therapies and nucleostide analogs in the treatment of HBV, it is critical for developing new classes of anti-HBV agents to identify cellular factors serving as possible drug targets. With further research, the competition between HBV and classic ligands of NTCP in binding to NTCP has been identified. CsA, a typical inhibitor of human NTCP, is found to prevent HBV infection by targeting NTCP [9, 10]. Iwamoto *et al.* [11] have identified oxysterols, oxidized derivatives of cholesterol, as inhibitors of HBV infection, using HepG2/hNTCP-C4 cell line. These results have demonstrated that the inhibitors or substrates of NTCP may inhibit the transportation of HBV or HDV by NTCP and become a new way for the treatment of HBV. It also reminds us that the interaction between some drugs and NTCP may be one of the mechanisms of their hepatoprotective effects.

With the development of herbal medicines, the effect of herbal medicines on the treatment of diseases has been increasingly recognized and popular. As reported, some herbal medicines even have hepatoprotective effects [12], which implies potential interaction between herbal medicines and NCTP. Therefore, studying the interaction between herbal medicinal ingredients and NTCP may provide a basis for clinical medication and HBV treatment and prevention. However, rare reports have mentioned the interaction between the herbal medicinal ingredients and NTCP so far.

Therefore, it is of significance to establish a cell model stably expressing NTCP for studying the interaction between drugs and NTCP and high-throughput screening of potential inhibitors/substrates of NTCP. In the present study, we developed a LLC-PK1 cell model with stable expression of human NTCP by lentivirus package technique and applied this cell model to screen and verify the potential inhibitor and/or substrate of NTCP from 102 herbal medicinal ingredients.

Materials and Methods

Materials

Sodium taurocholate hydrate (TCNa), CsA, Lithocholic Acid (LCA), Chenodeoxycholic Acid (CDCA), and Ursodeoxycholic Acid (UDCA) were purchased from Aladdin Co., Ltd. (Shanghai, China). Polybrene was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium butyrate was purchased from TCI (Japan). Jujuboside A was purchased from Mansite Co., Ltd. (Chengdu, China). L-Bicuculline, Androstenedione, Isoalantolactone, and Bufalin were purchased from Tongtian Co., Ltd. (Shanghai, China). Apigenin was purchased from Tiancao Co., Ltd. (Hangzhou, China). GA (17 : 1) and GA (15 : 1) were isolated in our laboratory [13, 14]. Other ingredients were obtained from Aladdin Co., Ltd. (Shanghai, China). The purity of all compounds was > 98%. Dulbecco's modified Eagle's medium/ F12 (DMEM/F12) and fetal bovine serum (FBS) were purchased from GIBCO (Invitrogen Life Technologies, Carlsbad, CA, USA). Sodium dodecyl sulfonate (SDS) was gained from Amresco (Solon, OH, USA). All of the chemicals or solvents used for UPLC-MS/MS were commercially available and were of HPLC grade.

Reconstruction of pCDH-CMV

Since there was no suitable double restriction enzyme cutting sites for NTCP connection, *Xho I* restriction site was introduced into the expression plasmid pCDH-CMV. Double strand DNA fragment containing *Xho I* was synthesized using Annealing Buffer for DNA oligos (Beyotime Institute of Biotechnology, Haimen, China) with the forward primer 5'-CGGCTAGCCTCGAGGGGCCCGAATTCCG-3' and the reverse primer 5'-CGGAATTCGGGCCCCTCGAGGCTAG CCG-3'. Then the DNA fragment was inserted into pCDH-CMV and verified by *Xho I* single enzyme digestion.

Construction of pCDH-CMV/NTCP expression plasmid

The expression plasmid pCDH-CMV/NTCP was transformed for overexpressing human NTCP stably. Total RNA from human liver was reverse-transcribed using PrimeScript^{TM} RT reagent Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. NTCP cDNA was first amplified from the reverse-transcription product using PrimSTAR polymerase (Takara Bio Inc., Shiga, Japan) with the forward primer containing *Hind III* site, 5'-CCCAAGCTTTTCCACTGCCTCACAGGAGGAT-3' and the reverse primer containing *Xho I* site, 5'-CCGCTCGA GGAATTGCTTTGGGACCAGAATCC-3' and then ligated into the pMD19-T vector. The full-length NTCP was cut from pMD19-T using *Hind III* and *Xho I* and inserted into pcDNA3.1(+) to construct the plasmid pcDNA3.1(+)-NTCP. Then the full-length NTCP was cut from pcDNA3.1(+)-NTCP using *Hhe I* and *Xho I* and subcloned into the reconstructed lentiviral vector pCDH-CMV to construct the expression plasmid pCDH-CMV/NTCP. After each step of plasmid construction, the inserted cDNA was sequenced and aligned with the reference sequence (NM_003049) to ensure authenticity.

Cell culture and lentivirus package technique

LLC-PK1 and HEK 293T cells were cultured in DMEM/F12 and DMEM, respectively, with 10% FBS, 100 U/mL of penicillinand streptomycin in a humid atmosphere of 5% CO_2 and 95% air at 37 °C. The LLC-PK1/NTCP polyclones were obtained by utilizing lentivirus package technique. When HEK 293T cells reached 90% confluence in