Organic anion transporting polypeptide 1A2 mediates fentanyl uptake in cultured cells

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Received January 14, 2019; Accepted June 4, 2019

DOI: 10.3892/mmr.2019.10796

Abstract. Individual differences in the response to fentanyl, which may be caused by different concentrations of the drug in the central nervous system, can complicate analgesic treatment. It has been reported that the organic anion transporting polypeptide (OATP) at the blood-brain barrier (BBB) in Sprague-Dawley rats may serve an important role in the transport of fentanyl across the BBB. However, whether human OATP can transport fentanyl has thus far not been reported. The present study aimed to establish a 293 cell line stably overexpressing OATP1A2, and to determine whether OATP1A2 is able to transport fentanyl across the plasma membrane. Initially, 293 cells were transfected with an OATP1A2-expressing plasmid (referred to as 293-OATP1A2 cells), and single colonies were selected and characterized following geneticin treatment. Subsequently, reverse transcription-quantitative polymerase chain reaction and western blot analyses were conducted to verify the transfection efficiency. Furthermore, treatment of 293-OATP1A2 cells with different concentrations of fexofenadine (FEX) and fentanyl was performed to investigate the transport function of OATP1A2 in 293 cells. FEX and fentanyl uptake experiments were also performed with naringenin, an inhibitor of OATP1A2. The results indicated that FEX and fentanyl uptake was significantly increased in 293-OATP1A2 cells compared with that in the control-transfected cells. The 293-OATP1A2-mediated uptake of

FEX at concentration of 100 nM FEX was ~10-fold higher than that of 293-VC cells. The 293-OATP1A2-mediated uptake of fentanyl (100 nM) was 5.1-fold higher compared with that in 293-VC cells. In 293-OATP1A2 cells, the uptake of FEX without OATP1A2 inhibitor naringenin (100 µg/ml) was 2.8-fold higher compared with that in the presence of naringenin, and the uptake of fentanyl without naringenin was 7.3-fold higher compared with that in the presence of naringenin (100 µg/ml). In conclusion, 293 cells that overexpressed OATP1A2 were successfully constructed, and OATP1A2 was revealed to mediate fentanyl uptake in the cultured cells.

Introduction

Fentanyl is one of the most commonly used opioids in clinical anesthesia and pain treatment. Clinical studies have reported that the minimum effective analgesic concentration of fentanyl ranges between 0.2 and 2.0 ng/ml, with a potential 10-fold difference between individuals (1). Large individual differences in dosage requirements affect the clinical efficacy of fentanyl, while also increasing the risk of severe adverse reactions, such as respiratory depression and coma.

The effective concentration of fentanyl in the central nervous system (CNS) is the key to determine its analgesic and side effects (2). As early as 1999, Henthorn *et al* (3) reported that the process of fentanyl uptake into bovine brain microvascular endothelial cells was primarily through active transport rather than passive diffusion; however, the authors did not further clarify which carrier mediated fentanyl transport. More recently, in a study by Elkiwari *et al* (4), Sprague-Dawley rats were given an intravenous injection of fentanyl, verapamil or pravastatin prior to the quantification of fentanyl in the brain and plasma using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). It was revealed that the brain/plasma partition coefficient of fentanyl decreased slightly (57%) following the administration of verapamil, a competitive substrate of P-glycoprotein. However, treatment with pravastatin, the competitive substrate of organic anion transporting polypeptide (OATP), resulted in a 4-fold reduction in the brain/plasma partition coefficient of fentanyl, suggesting that OATP may serve an important role in fentanyl transport across the blood-brain barrier (BBB) (4).

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Abbreviations: CNS, central nervous system; SLCO, solute carrier organic anion; OATP, organic anion transporting polypeptide; BBB, blood-brain barrier; FEX, fexofenadine; DMEM, Dulbecco's modified Eagle's medium

Key words: organic anion transporting polypeptide 1A2, fentanyl, substrate, transport function, overexpression

Nevertheless, whether human OATP is also capable of transporting fentanyl has not yet been reported.

OATPs belong to the solute carrier organic anion transporter (SLCO) subfamily. Gao *et al.* (5) demonstrated that human OATP-A (previously known as OATP) was localized to the BBB, and that OATP is able to mediate the transport of the analgesic opioid peptides DPDPE and deltorphin II across the BBB. To date, 11 OATP subtypes have been identified in humans (6), among which only the expression of OATP1A2 and OATP2B1 has been confirmed at the BBB (7-9). The expression of the SLCO family member 1A2 (SLCO1A2) mRNA is most abundant in the brain (6), while OATP1A2 is predominantly expressed on the apical side of capillary endothelial cells at the BBB (10,11). OATP1A2 has the most extensive substrate spectrum and can transport anionic, neutral and cationic compounds, including the δ -opioid receptor agonist d-penicillamine 2,5-enkephalin (DPDPE), deltorphin II, bile acids and steroids (12-15). Furthermore, *in vitro* experiments have demonstrated that OATP1A2 serves an important role in the transport of numerous drugs across the BBB and into the CNS (7).

Therefore, in the present study, it was speculated that OATP1A2 may serve an important role in the translocation and distribution of fentanyl across the BBB. The study intended to establish a 293 cell line that stably overexpressed OATP1A2, and to determine whether OATP1A2 was able to transport fentanyl across the plasma membrane of these cells.

Materials and methods

Materials. Standard grade fentanyl was gifted by the Institute of Pharmacology, Central South University (Changsha, China). Internal standard fentanyl-D5 and antibiotic geneticin (G418) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Fexofenadine (FEX) hydrochloride was purchased from the National Institutes for Food and Drug Control (Beijing, China). Naringenin was obtained from Dalian Meilun Company (Dalian, China), while methanol, acetonitrile and formic acid (all of analytical grade) were from Huihong Reagent Co. Ltd (Hunan, China). Fetal bovine serum (FBS), high-glucose Dulbecco's modified Eagle's medium (DMEM), low-serum transfection medium OPTI-MEM and Lipofectamine™ 2000 were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). ReverTra Ace qPCR RT Master Mix was purchased from Toyobo Life Science. PBS, penicillin-streptomycin and trypsin were obtained from Hyclone (GE Healthcare Life Sciences, Little Chalfont, UK). The OATP1A2 antibody was provided by Abcam (ab110392), while the mouse anti- β -actin antibody (KGAA001) was from Nanjing KeyGen Biotech Co., Ltd. and goat anti-rabbit IgG/HRP secondary antibody (ZB-2301) from OriGene Technologies, Inc. The RNA extraction kit was purchased from Omega Bio-Tek, Inc. (Norcross, GA, USA), while the BCA protein assay kit and radioimmunoprecipitation assay (RIPA) buffer were purchased from Beyotime Institute of Biotechnology (Haimen, China). The recombinant plasmid pIRES2-ZsGreen1-OATP1A2 and control plasmid pIRES2-ZsGreen1 were constructed by Changsha Yingrun Biotechnologies Inc., (Changsha, China), and OATP1A2 was amplified from the cDNA library. The primer sequences were

as follows: OATP1A2 forward, 5'-CTAGCTAGCGCCACC ATGGGAGAACTGAGAAAAG-3', and reverse, 5'-CCG CTCGAGTTACAATTTAGTTTTCATT-3'. The full-length coding sequence of OATP1A2 was amplified, digested with endonucleases, inserted into pIRES2-ZsGreen1 plasmid and recombinant plasmid pIRES2-ZsGreen1-OATP1A2 was obtained. The 293 cell line was supplied by the Cell Bank of Xiangya School of Medicine, Central South University (Changsha, China).

Cell culture. The 293 cells were cultured in high-glucose DMEM supplemented with 10% FBS and 1% penicillin (100 U/ml)/streptomycin (100 μ g/ml) at 37°C in an atmosphere consisting of 5% CO₂ and 95% humidity. Cells were passaged at a 1:3 ratio using 0.25% trypsin.

Determination of the optimal G418 concentration for screening. Prior to transfection, dose-response analysis of 293 cells was performed to determine the lowest lethal dose of G418. A total of 100 μ l cell suspension (5×10^6 cells/l) was added to each well of a 12-well plate, in addition to 900 μ l DMEM (10% FBS). To construct a concentration gradient increasing by 100 μ g/ml increments, each well was supplemented with 0-11 μ l G418 stock solution (100 mg/ml) to a final concentration of 0-1,100 μ g/ml, and the plates were incubated in a 37°C incubator with 5% CO₂. The medium was replaced every 3 days with fresh medium containing G418 at the corresponding concentration. After 10 days, the screening concentration of G418 was identified as 600 μ g/ml, which was the minimum concentration for complete cell death.

Transfection. On the day prior to transfection, cells in the exponential growth phase were harvested, and seeded in 12-well culture plates at an initial density of 1×10^4 cells/well in 1 ml DMEM (10% FBS). The cells were cultured at 37°C (5% CO₂, 95% humidity) until 70-80% confluence was reached. According to the manufacturer's protocol of the Lipofectamine™ 2000 reagent, liquid A was prepared by diluting 1.6 μ g purified pIRES2-ZsGreen1-OATP1A2 or pIRES2-ZsGreen1 plasmid in 100 μ l low serum OPTI-MEM. Liquid B was then prepared by diluting 4 μ l Lipofectamine™ 2000 in 100 μ l OPTI-MEM. Liquids A and B were gently mixed and incubated for 20 min at room temperature to form a liposome/DNA complex (liquid AB). Subsequently, the cells were washed three times with 2 ml serum-free DMEM, the liquid AB was slowly added to each well, and the plates were shaken well and incubated for 4 h at 37°C with 5% CO₂. Next, 1 ml DMEM (10% FBS) was added to each well, and the plates were further incubated for 24 h at 37°C and 5% CO₂ for screening.

Screening of transfected 293 cells. On day 2 post-transfection, the cells were trypsinized (0.25%; 100 μ l/well), and cells from one of the wells of the 12-well culture plate were transferred into two 90-mm culture dishes. On day 3 after transfection, 600 μ g/ml G418 was added to the appropriate wells, and the medium was replaced every 3 days (DMEM with 10% FBS). Untransfected cells were used as the control group and were cultured for 2 weeks with G418 treatment as described earlier. When all control cells had died, G418-resistant clones were

Table I. Nucleotide sequences of primers used in quantitative polymerase chain reaction.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')	Product length
OATP1A2	TACGTGGAATGGGTGAACTC	CACAGAATGATGCCAACAAAA	153 bp
β -actin	CATCCTGCGTCTGGACCTGG	TAATGTACACGCACGATTTC	116 bp

OATP1A2, organic anion transporting polypeptide 1A2.

isolated from the transfection groups. In the transfected cell groups, 24 colonies were randomly transferred into 12-well plates. The expression of green fluorescent protein (GFP) was then observed under a fluorescence microscope, and the survival rate of stable OATP1A2-overexpressing cells was determined.

Verification of OATP1A2 mRNA expression using RT-quantitative polymerase chain reaction (RT-qPCR). The plasmid construction was verified by PCR and sequencing comparison. The OATP1A2 sequence of the recombinant plasmid was first verified by PCR amplification using the aforementioned primers. Stable OATP1A2-transfected 293 cells (referred to as 293-OATP1A2), cells transfected with empty plasmid serving as the control group [293-vector control (VC) cells] and con-293 cells (negative control group without treatment) were harvested, and the EZNA Total RNA Kit I was used to extract total RNA, of which 2 μ g was obtained, measured by a BioPhotometer *plus* (Eppendorf). cDNA was synthesized using an RT kit, and qPCR was subsequently conducted with the OATP1A2 primers and with β -actin serving as an internal control (Table I). The 25- μ l reaction consisted of 0.5 μ l cDNA, 7.5 μ l SYBR[®] Green PCR Master Mix (Thermo Fisher Scientific, Inc.) and 75 nM each of the forward and reverse primers. The PCR amplification procedure was as follows: Pre-denaturation at 95°C for 5 min; denaturation at 94°C for 1 min; annealing at 55°C for 1 min; extension at 72°C for 1 min, for 35 cycles; and final extension at 72°C for 10 min. The cycle quantification (Cq) value of OATP1A2 was first normalized to the internal control ($\Delta Cq = Cq_{OATP1A2} - Cq_{\beta-actin}$), and the $2^{-\Delta\Delta Cq}$ method was then used to calculate the relative expression levels (16). The difference between the OATP1A2 gene expression levels of the experimental group (293-OATP1A2) and the control group (con-293 or 293-VC) was calculated using the formula $2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq = \Delta Cq_{Exp} - \Delta Cq_{Control}$. The experiment was repeated three times.

Identification of OATP1A2 protein expression by Western blot analysis. Total protein was extracted from the 293-OATP1A2, 293-VC and con-293 cells using RIPA lysis buffer, and protein concentration was quantified with the BCA protein assay kit, according to the manufacturer's protocol. Equal amounts of protein per sample (2 μ g) were loaded and separated by 8% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were then blocked with blocking solution (BSA, Beyotime Institute of Biotechnology) for 2 h at room temperature (~25°C). After three washes of 10 min each with TBST, the membranes were incubated with primary antibody overnight

at 4°C. On the second day, the membranes were incubated with secondary antibody at 37°C for 1 h, and proteins were visualized using an enhanced chemiluminescence solution (Beyotime Institute of Biotechnology) with a chemiluminescence imaging system (Quant350; GE Healthcare, Chicago, IL, USA). The gray value was detected by ImageJ v1.8.0 software (National Institutes of Health) to compare the protein expression among groups. The experiment was repeated for three times.

Transport and absorption experiments. According to the methods of Cvetkovic *et al* (17), Shimizu *et al* (18) and Glaeser *et al* (19), the transport and absorption efficiency of the 293-OATP1A2 cells was assessed using FEX ($K_m=6$), a known substrate of OATP1A2 (10). Briefly, the 293-OATP1A2 and control (293-VC) groups were seeded into 12-well plates (5×10^5 cells/well), and concentration assays were performed three times with 1, 10 and 100 nM FEX. When cells reached a density of 1×10^6 cells/well, they were rinsed with 500 μ l culture medium (PBS + 2% FBS). The cytotoxicity of fentanyl was determined using an up-and-down sequential method with 4-6 groups. The experiment was initiated at the dose closest to the median lethal dose (LD50). Cells were seeded in 96-well plates, and concentrations of 5, 10, 100, 150 and 300 μ M fentanyl were used according to those of commonly used OATP1A2 substrates. To rule out the toxic effects of fentanyl on 293 cells, subsequent experiments were conducted using a concentration of fentanyl below the LD50. Under microscopic observation, the median lethal dose (LD50) of fentanyl (indicating the dose at which almost 50% of cells were killed) was determined to be between 100 and 150 μ M. The cells were subjected to uptake experiments for 5, 10, 15, 20 and 25 min, and it was found the optimum duration was 15 min (with the maximum uptake value in the linear relationship), so 15 min was selected as the most suitable absorption time used for all absorption experiments. The cells were then subjected to uptake experiments by adding 100 μ l medium containing 1, 10 and 100 nM FEX. Next, cells were incubated in a rotary shaker (450 rpm) at 37°C for 15 min. The spent medium was collected into EP tubes in triplicate for each concentration, and the cells were washed three times with 1 ml ice-cold PBS. Subsequent to adding 500 μ l methanol to each well, the cells were collected into EP tubes and homogenized using a cell grinder. The homogenate was centrifuged at 1,000 x g at 4°C for 10 min, and the supernatant was stored at -20°C until analysis. Furthermore, the OATP1A2-specific inhibitor naringenin (100 μ g/ml) was added to the absorption solution containing 100 nM FEX or 100 nM fentanyl, and a further set of absorption experiments was performed according to the

Figure 1. Verification of pIRES2-ZsGreen1-OATP1A2 construction using the PCR method. (A) Electrophoresis shows the molecular marker (left band) and the OATP1A2 plasmid PCR product (right band) of ~2,013 bp, consistent with the size of the OATP1A2 coding sequence. (B) Molecular marker (left band) and PCR products following restriction endonuclease digestion (right band), which were consistent with the sizes of the plasmid and the respective gene. OATP1A2, organic anion transporting polypeptide 1A2; PCR, polymerase chain reaction.

forementioned method. The experiments were repeated three times for each group, and the entire course of experiments was repeated twice to obtain six samples for each group.

Determination of drug concentration using HPLC-MS/MS. The concentration of fentanyl and FEX was determined using the methods reported by Verplaetse and Tytgat (20), and Mandery *et al* (21). Samples were processed by deproteinization, and a double volume of methanol containing the internal standard was added. Chromatographic separation was performed using a Phenomenex analytical column [Synergi™ Polar-RP 80A; 150x2.0 mm, 4 μ m; Phenomenex, Inc., Torrance, CA, USA], with the following specifications: Mobile phase, methanol; Ammonium acetate (containing 0.2% formic acid)=9:1 (v/v); column temperature, 40°C; flow rate, 200 μ l/min. The injection volume was 2 μ l, and each sample was run for 5 min. The mass spectrometry technique used was electrospray ionization, the detection method involved positive ion mode and multi-ion reaction monitoring. The ions used for quantitative analysis were fentanyl (m/z 337.1→188.2) and FEX (m/z 502.5→466.3), as well as D5-fentanyl (m/z 342.2→188.2), which served as the internal standard for fentanyl and FEX. The standard curve range of fentanyl and FEX was 5–1,000 ng/ml, the intra-day precision (relative standard deviation; RSD) of the quality control (QC) sample at each concentration level was <15%, the daytime precision (RSD) was <15%, and the accuracy was between 85 and 115%. Analyst software (version 1.5.1; Shanghai AB SCIEX Analytical Instrument Trading Co., Shanghai, China) was used to analyze the obtained data.

Statistical analysis. Comparison of the percentage of transporter-mediated uptake between the fentanyl and FEX-treated groups was performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). The net transporter-mediated uptake was obtained by subtracting the uptake into 293-VC cells from that into transporter-expressing cells. Two-way analysis of variance and Dunnett's multiple comparisons test were conducted to

determine the statistical significance of fentanyl and FEX accumulation between 293-OATP1A2 and 293-VC cells. The impact of naringenin on transporter-mediated uptake was analyzed using the unpaired t-test with Welch's correction. All data are presented as the mean $\pm$ standard error of the mean, and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Verification of pIRES2-ZsGreen1-OATP1A2 plasmid construction. The plasmid PCR product of pIRES2-ZsGreen1-OATP1A2 was subjected to 1.0% agarose gel electrophoresis, and the results revealed a specific band of 2,013 bp, which was consistent with the SLCO1A2 gene (Fig. 1A). The recombinant plasmid was digested using *Nhe*I and *Xho*I endonucleases, which yielded a ~2,013 bp fragment corresponding to OATP1A2, and a vector fragment of ~5,300 bp (Fig. 1B). At the same time, pIRES2-ZsGreen1-OATP1A2 was sequence-aligned in NCBI. Gene sequences of OATP were retrieved in PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>) and compared with PubMed Nucleotide BLAST online software. The results demonstrated that the constructed sequence was identical to the base sequence of the target gene, and that the amino acid sequence was 100% correct, indicating that pIRES2-ZsGreen1-OATP1A2 was successfully constructed.

Establishment and verification of 293 cells stably-over-expressing OATP1A2. According to the methods of König *et al* (12) and Taub *et al* (22), 293 cells were liposomally transfected with pIRES2-ZsGreen1-OATP1A2 and the corresponding empty vector, and overexpressed clonal cells were selected. The cells were then screened with geneticin (G418) to obtain 293-OATP1A2 cells that were overexpressing OATP1A2 protein, and 293-VC cells that were expressing only GFP. Fluorescence microscopy revealed that the two groups of cells expressed GFP, confirming that stably-transfected cell lines had been successfully constructed (Fig. 2).

Figure 2. 293 cells were transfected with pIRES2-ZsGreen1 and pIRES2-ZsGreen1-OATP1A2 using the liposomal method. After 48 h, the 293-VC cells (left panels) and 293-OATP1A2 cells (right panels) were observed using white and excitation light under a fluorescence microscope (magnification, $\times 4$; scale bar, $10\ \mu\text{m}$). The transfection efficiency, as determined by the GFP expression, was $>80\%$. 293-OATP1A2, 293 cells that stably overexpressed OATP1A2; OATP1A2, organic anion transporting polypeptide 1A2; VC, vector control.

Figure 3. Characterization of the 293-OATP1A2 cell line. (A) Increased expression of OATP1A2 mRNA in 293-OATP1A2 cells compared with that in 293-VC cells. The relative expression for con-293 was set to 1 for each polymerase chain reaction experiment. (B) Western blot analysis of OATP1A2 protein expression in 293-OATP1A2, 293-VC and con-293 cells. At $\sim 74\ \text{kDa}$, a prominent band was detected in 293-OATP1A2 cells, while weak signals were detectable in 293-VC and con-293 cells. (C) The relative density of the 293-OATP1A2 group was significantly higher than that of the control group. The experiment was repeated three times, and the results are presented as the mean $\pm$ standard error of the mean. * $P < 0.05$ and ** $P < 0.01$, vs. 293-VC and con-293 groups. 293-OATP1A2 cells, 293 cells that stably overexpressed OATP1A2; OATP1A2, organic anion transporting polypeptide 1A2; VC, vector control.

To further determine whether the target gene was overexpressed, OATP1A2 mRNA expression was assessed in 293-OATP1A2, 293-VC and con-293 cells using RT-qPCR. The results indicated that the relative expression of OATP1A2 mRNA in 293-OATP1A2 cells was significantly higher compared with that in the other two cell lines, suggesting that OATP1A2 mRNA was successfully overexpressed in the experimental group (Fig. 3A). Western blot analysis was subsequently used to detect the expression of OATP1A2 protein in the three groups. The results revealed a prominent OATP1A2 protein band in the 293-OATP1A2 group (Fig. 3B). Analysis using ImageJ software indicated that the gray value

of the 293-OATP1A2 group was significantly higher compared with the other two groups, indicating that 293 cells stably overexpressing OATP1A2 had been successfully established (Fig. 3C). There was no significant difference in the OATP1A2 mRNA and protein expression levels between the 293-VC and con-293 groups, suggesting that the empty vector had no significant effect on gene expression in 293 cells.

Functional verification of 293-OATP1A2 cells. The function of OATP1A2 was verified using its known substrate, FEX, as a probe drug. The results of the uptake experiments demonstrated significantly increased uptake of FEX into 293-OATP1A2

Figure 4. Functional verification of 293-OATP1A2 cells using FEX. (A) Percentage uptake of FEX into 293-OATP1A2 and 293-VC cells. Y-axis represents the ratio of intracellular FEX concentration (from mass spectrometry data) to extracellular FEX concentration. The 293-OATP1A2 cells exhibited a significantly higher uptake (~10-fold) of FEX compared with the 293-VC cells ($F=36$). (B) The uptake of FEX was significantly reduced in the presence of the OATP1A2 inhibitor naringenin (100 $\mu\text{g/ml}$). The uptake of FEX without naringenin was 2.8-fold higher compared with that in the presence of naringenin. The experiment was repeated three times, and the results are presented as the mean $\pm$ standard error of the mean. * $P<0.05$; # $P<0.05$ vs. FEX + naringenin group. 293-OATP1A2, 293 cells that stably overexpressed OATP1A2; OATP1A2, organic anion transporting polypeptide 1A2; FEX, fexofenadine; VC, vector control.

Figure 5. Transport of fentanyl. (A) Percentage uptake of fentanyl into 293-OATP1A2 and 293-VC cells. Y-axis represents the ratio of intracellular fentanyl concentration (from mass spectrometry data) to extracellular fentanyl concentration. The 293-OATP1A2 cells exhibited a significantly increased uptake of fentanyl (5.1-fold higher at 100 nM fentanyl; $P=0.042$; $F=8.7$) compared with the 293-VC cells. (B) The uptake of fentanyl was significantly reduced in 293-OATP1A2 cells treated with naringenin compared with that in cells without naringenin. The uptake of fentanyl without naringenin was 7.3-fold higher compared with that in the presence of naringenin. Experiments were repeated three times for each group, and the entire course of experiments was repeated twice to obtain six samples for each group. * $P<0.05$; # $P<0.05$ vs. fentanyl + naringenin group. 293-OATP1A2, 293 cells that stably overexpressed OATP1A2; OATP1A2, organic anion transporting polypeptide 1A2; VC, vector control.

cells compared with the uptake into 293-VC cells ($P<0.05$; $F=36$). In addition, the 293-OATP1A2-mediated uptake of FEX was ~10-fold higher than that of 293-VC cells according to mass spectrometry data, at a concentration of 100 nM FEX (Fig. 4A). The results of the inhibition experiments revealed that the uptake of FEX in the experimental group (treated with 100 $\mu\text{g/ml}$ naringenin) was 12.03 ± 0.14 nmol/ 1×10^6 cells, whilst that in the control group (without naringenin) was 36.24 ± 0.28 nmol/ 1×10^6 cells (2.8-fold higher), representing a statistically significant difference ($P<0.05$; Fig. 4B). This confirmed that the 293-OATP1A2 cell line successfully exhibited its transport function.

Transport of fentanyl. To verify whether fentanyl was a substrate for OATP1A2, 293-OATP1A2 and 293-VC cells were used to conduct uptake experiments using a fentanyl concentration gradient. According to the methods used by

Ziesenitz *et al.* (23), 1, 10, 100 and 1,000 nM fentanyl was used. Preliminary experiments revealed that the absorption time was linearly correlated within 15 min; thus, the absorption time was determined to be 15 min (Fig. S1). The results of the present study revealed the 293-OATP1A2-mediated uptake of fentanyl (100 nM) was 5.1-fold higher compared with that in 293-VC cells (Fig. 5A), according to mass spectrometry data, with statistically significant difference ($P<0.05$).

In order to observe the uptake of fentanyl subsequent to inhibition of OATP1A2 expression, a combination of 100 nM fentanyl and 40 μM naringenin (100 $\mu\text{g/ml}$ each) was used for inhibition experiments with 293-OATP1A2 cells. The uptake of fentanyl was 2.62 ± 0.06 nmol/ 1×10^6 cells in the inhibitor group (fentanyl + naringenin) and 19.39 ± 0.23 nmol/ 1×10^6 cells in the fentanyl group (7.3-fold higher than the inhibitor group), with a statistically significant difference detected between the two groups ($P<0.05$; Fig. 5B).

Discussion

OATP/Oatp isoforms are known to be involved in blood-to-brain transport of opioid analgesic peptides such as deltorphin II and DPDPE (5,24,25). To date, 11 OATP subtypes have been identified in humans (6). Among these subtypes, the most frequently studied subtype with high mutation rate was OATP1B1. OATP1B1 is mainly located at the sinusoidal membrane of hepatocytes (23). Ziesenitz *et al* (23) conducted uptake assays with fentanyl in cells overexpressing OATP1B1 and compared the intracellular concentrations with the corresponding cell line without OATP1B1, and they found that fentanyl is not transported by human OATP1B1. OATP1A2, mainly localized at the blood-brain barrier, can transport anionic, neutral and cationic compounds (13-15). Furthermore, *in vitro* functional assessment revealed that the A516C and A404T variants of OATP1A2 had markedly reduced capacity for mediating the cellular uptake of OATP1A2 substrates, estrone 3-sulfate and two delta-opioid receptor agonists, deltorphin II, and DPDPE (7). Due to high expression at the BBB and an extensive substrate spectrum of OATP1A2, it was hypothesized that OATP1A2 may mediate fentanyl transport. According to the recommendations of the International Transporter Consortium, transporter substrates (including OATPs) are characterized by a significantly greater uptake into transporter-expressing cells compared with control cells, while this higher uptake level should be decreased by known inhibitors (19).

The focus of the current research is whether OATP1A2 can transport fentanyl. Cells in the luminal membrane of the BBB express OATP1A2, as well as numerous other transporters, and thus a blank control was needed in the present study. The 293 cell line has been widely used as an *in vitro* model for studying the association between drug transporters and their substrates (26). This cell line is easier to obtain and expresses fewer transporters, including OATP1A2; therefore, it can function as a better tool cell for overexpressing this transporter in order to study its function (27). Furthermore, 293 cells are widely used in cell biology research with reliable growth and propensity for transfection, and they can be used in biotechnology to produce therapeutic proteins for gene therapy (28). In order to confirm our hypothesis, a 293 cell line stably overexpressing OATP1A2 was first established in the present study, and the expression of GFP (the vector marker) in the obtained 293-OATP1A2 and 293-VC cells was examined by fluorescence microscopy. The results indicated that the transfection rates of the two cell groups were >80%. RT-qPCR analysis further revealed that the expression of OATP1A2 mRNA in the 293-OATP1A2 group was significantly higher in comparison with that in the 293-VC group. There was also a statistically significant difference in the expression of the target protein between the 293-OATP1A2 and 293-VC groups, as determined by Western blot analysis. These observations confirmed the successful establishment of a cell line stably overexpressing OATP1A2.

Using FEX (a known substrate of OATP1A2) as a probe drug, the current study verified the transport function of the 293-OATP1A2 cell line. Subsequently, FEX uptake analyses were performed in the 293-OATP1A2 and 293-VC groups. Uptake experiments were also performed with the addition

of the OATP1A2 inhibitor naringenin, and the intracellular FEX concentration was determined by HPLC-MS/MS. The results demonstrated a statistically significant difference between the FEX/naringenin and FEX groups, which was consistent with the findings of Shimizu *et al* (18), indicating that 293-OATP1A2 cells exhibited a normal transport function. Finally, a fentanyl concentration gradient was constructed using the 293-OATP1A2 cell line, and the concentration of intracellular fentanyl was determined using HPLC-MS/MS. The results revealed that the concentration of fentanyl in 293-OATP1A2 cells was significantly higher as compared with that in 293-VC cells, and that this increased uptake was inhibited by naringenin, indicating that fentanyl is a substrate for OATP1A2.

In conclusion, the present study demonstrated for the first time the OATP1A2-mediated transport of fentanyl *in vitro*, while it was also observed that naringenin, a specific inhibitor of OATP1A2, was able to reduce fentanyl uptake into the cells. Since the present study was restricted to *in vitro* cell line experiments, the findings may not fully represent the internal environment of the BBB. Whether the OATP1A2-mediated uptake of fentanyl is affected by other factors remains to be clarified. Nevertheless, the current findings provide a basis for the further investigation of the mechanism of fentanyl, with important significance for its use in targeted therapy and individualized treatments plans.

Acknowledgements

The authors would like to thank Professor Zhi-rong Tan and Professor Dong-sheng Ouyang (Institute of Pharmacology, Central South University) for their gift of standard grade fentanyl.

Funding

The present study was supported by a grant from the Natural Science Foundation of Hunan Province (grant no. 16JJ2158) and the New Xiangya Talent Projects of the Third Xiangya hospital of Central South University.

Availability of data and materials

The datasets analyzed and/or generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XCH and HWY performed the experiments, analyzed the data and wrote the manuscript. LHH guided the experiments, designed the primers and vectors, analyzed the data and revised the manuscript. FZ contributed to the conception and design of the study. ZYP assisted in the experiments. QL conceived and designed the study, and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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