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ORIGINAL ARTICLE

High affinity of 4-(4-(dimethylamino) styryl)-*N*-methylpyridinium transport for assessing organic cation drugs in hepatocellular carcinoma cells

Metee Jinakote[a,b], Atcharaporn Ontawong[c], Sunhapas Soodvilai[d], Jeerawat Pimta[d], Tipthida Pasachan[b], Varanuj Chatsudthipong[d], Chutima Srimaroeng[b]*

[a]*Faculty of Oriental Medicine, Chiang Rai College, Chiang Rai 57000, Thailand*
[b]*Department of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*
[c]*Unit of Excellence of Coffee, Division of Physiology, School of Medical Sciences, University of Phayao, Phayao 56000, Thailand*
[d]*Research Center of Transport Protein for Medical Innovation, Department of Physiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

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*Correspondence and reprints: chutima.srimaroeng@cmu.ac.th

ABSTRACT

Human organic cation transporter 1 (hOCT1) and human organic cation transporter 3 (hOCT3) are highly expressed in hepatocytes and play important roles in cationic drug absorption, distribution, and elimination. A previous study demonstrated that downregulation of hOCT1 and hOCT3 mRNA was related to hepatocellular carcinoma (HepG2) prognosis and severity. Whether these transporters expressed in HepG2 cells serve for cationic drug delivery has not been investigated. Besides radioactive transport, options for assessing hOCTs in hepatocytes are limited. This study clarified the significant roles of hOCTs in HepG2 by comparing cationic fluorescent 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium (ASP^+) with traditional [^{3}H]-1-methyl-4-phenylpyridinium (MPP^+). The results showed ASP^+ was preferably transported into HepG2 compared to [^{3}H]-MPP^+ with high affinity and a high maximal transport rate. Selective transport of ASP^+ mediated by hOCTs was influenced by extracellular pH, temperature, and membrane depolarization, corresponding to hOCT1 and hOCT3 expressions. Furthermore, transport of cationic drugs, metformin, and paclitaxel in HepG2 cells was blunted by OCT inhibitors, suggesting that hOCT1 and hOCT3 expressed in HepG2 cells exhibit notable impacts on cationic drug actions. The fluorescent ASP^+-based in vitro model may also provide a rapid and powerful analytical tool for further screening of cationic drug actions and interactions with hOCTs, particularly hOCT1 and hOCT3 in hepatocellular carcinoma.

INTRODUCTION

Human organic cation transporter isoform 1 (hOCT1), human organic cation transporter isoform 2 (hOCT2), and human organic cation transporter isoform 3 (hOCT3) have been cloned, characterized, and known to play crucial roles in absorption, distribution, and elimination of several cationic compounds [1–3]. Among OCTs, hOCT1 is highly expressed in liver; hOCT2 is found mainly in kidney, while hOCT3 is ubiquitous being expressed in several tissues including intestine, placenta, and liver [4,5]. Therefore, hOCT1

and hOCT3 are likely to impact hepatocellular drug uptake and elimination. The major transport mechanism of organic cations (OC^+) – including acetylcholine, dopamine, acyclovir, metformin, and typical substrates, for example, 1-methyl-4-phenylpyridinium (MPP^+) and tetraethylammonium (TEA) – by hOCTs has been identified as electrogenic/uniport driven by inside negative charge at physiological pH [6]. Moreover, hOCTs transport substrates bi-directionally by facilitated diffusion or electroneutral OC^+/OC^+ exchanger mechanisms [7,8].

Recent study has shown that hOCT1 is responsible for hepatic drug actions and being a target for drug interactions [9]. For instance, metformin, the anti-diabetic drug, inhibited hepatic gluconeogenesis and lipogenesis by phosphorylation of 5′ AMP-activated protein kinase (AMPK) in primary rat and human hepatocytes similar to that human hepatocellular carcinoma (HepG2) cells [10–12]. When lacking Oct1 ($Oct1^{-/-}$), intracellular metformin accumulation decreased 30-fold in mouse liver [13]. Likewise, irinotecan and paclitaxel, anti-cancer drugs, exhibited cytotoxic effects, partly mediated by hOCT1 in lymphoma cells [14] while several neurotransmitters, for example, histamine, epinephrine, and norepinephrine, were taken up by hOCT3 into HEK-293 cells [15]. At present, nine percent of pharmakon drug library have been extensively identified as hOCT1 inhibitor using in silico and in vitro assessments [16]. Thus, hOCT1 and/or hOCT3 functions are significantly associated with pharmacokinetics and pharmacodynamics of these cationic drugs, and potential adverse drug–drug interactions (DDIs) by these transporters require intensive investigation.

Currently, traditional cationic radio-labels, for example, [^{3}H]-1-methyl-4-phenylpyridinium (MPP^+) or [^{14}C]-TEA, are widely used to assess hOCTs functions due to their high affinities [17,18]. However, challenges include safe handling and disposal, the sensitivity of dynamic detection, and the limited availability of these radioactive compounds to single or manual application. Non-radioactive fluorescent organic cation-based assay is an alternative assessment tool for hOCTs functions. Previously, endogenous hOCTs function mediated fluorescent 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium (ASP^+) transport were demonstrated in human kidney epithelial cell line [19], freshly isolated human proximal tubule [20], and human respiratory epithelial cells – A549 and NCI-H441 [21,22]. Moreover, the K_m of ASP^+ for hOCT1 was comparable to hOCT3 with values of 2.3 ± 0.3 μM and 1.1 ± 0.1 μM in hOCT1- and hOCT3-overexpressing HEK cells, respectively [23,24]. Thus, transport of fluorescent ASP^+, mediated by hOCTs, could illuminate transport properties in a comparable manner to traditional radioactive assay.

Human hepatocellular carcinoma cell line derived from HepG2 has been widely used as an in vitro model for drug toxicity screening and the regulation of drug-metabolizing enzymes due to reproducibility, ease of handling, and commercial availability [25,26]. Upregulated efflux transporter hABCB1, also known as multidrug resistance protein 1 (MDR1), in hypoxic condition reversed apoptosis induced by doxorubicin in HepG2 cells [27]. Others have noted low expression of hOCT1 mRNA was shown in both primary hepatocellular carcinoma (HCC) and HepG2 cells [28–30]. Additionally, hOCT3 expression was downregulated in HCC with either no change or low expression in HepG2 cells relative to normal human tissues [28,29]. Whether hOCT1 and hOCT3 have precise roles in and significant responsibility for organic drug uptake in HepG2 cells, however, remains unknown. Therefore, the aims of this study were (i) to characterize the functionally significant roles of hOCT1 and hOCT3 in hepatocellular carcinoma (HepG2) using cationic fluorescence ASP^+ transport compared to traditional [^{3}H]-MPP^+ and (ii) to demonstrate the beneficial role of hOCT1 and hOCT3 in transport of cationic drug substrates, metformin, and paclitaxel, in HepG2 cells.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin solution, trypsin–EDTA, and 4-(4-(dimethylamino)styryl)-*N*-methyl-pyridinium (4-Di-1-ASP or ASP^+) were purchased from Life Technologies (Eugene, OR, USA). Phosphate-buffered saline (PBS) was purchased from Biochrom AG (Berlin, Germany). [^{3}H]-1-methyl-4-phenylpyridinium ([^{3}H]-MPP^+; specific activity 80 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). MEM non-essential amino acid solution, tetrapentylammonium (TPeA), TEA, paclitaxel, clonidine, unlabeled MPP^+, and *p*-aminohippuric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) while metformin was purchased from MP Biomedicals (Solon, OH, USA). All other chemicals with high purity were obtained from commercial sources.

Cell culture

Human hepatocellular carcinoma cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The cells in the passage of 25th–55th were grown in DMEM containing 4.5 g/L of D-glucose, 3.7 g/L of $NaHCO_3$ supplemented with 20% FBS, 1× MEM non-essential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin solution in humidified incubator with 37°C and 5% CO_2. The cells were sub-cultured in PBS containing 0.05% trypsin–EDTA and seeded at a cell density of 2×10^5 and 4×10^4 cells/well into 24- or 96-well plates, respectively. After culturing for 2–3 days, the cells were 80–90% confluent and ready for subsequent experiments.

Uptake of fluorescent ASP^+ and [^{3}H]-MPP^+ into HepG2 cells

To determine dynamic transport of fluorescence ASP^+, the cells were pre-incubated with D-PBS (in mM: 137 NaCl, 3 KCl, 8 Na_2HPO_4, 1 KH_2PO4, 0.5 $MgCl_2$, 1 $CaCl_2$ and 5.6 D-Glucose) at 37°C for 10 min as previously described [31]. Subsequently, the cells were incubated in D-PBS containing 10 μM of ASP^+ in the presence or absence of 0.01–1 mM of TPeA, a potent OCTs inhibitor, or 0.1 and 1 mM of test compounds (see figure legends in detail) in a dark room. The inhibitory constant (IC_{50}) value of TPeA for ASP^+ mediated endogenous hOCTs transport was calculated from sigmoidal dose–response analysis using GraphPad Prism version 4.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). For kinetic studies, cells were incubated in D-PBS containing ASP^+ at various concentrations (5–80 μM) for 10 min at both 37°C and 4°C. The mediated component of ASP^+ uptake was calculated by subtracting the uptake at 37°C from 4°C. The data were plotted as a Lineweaver–Burk plot ($1/[ASP^+]$ vs. $1/ASP^+$ uptake), and the K_m (Michaelis–Menten constant) was estimated from the *x*-axis intercept while the maximal rate of ASP^+ uptake (V_{max}) mediated by hOCTs was estimated from the *y*-axis intercept. Moreover, assessments of the influences of extracellular pH, temperature, and membrane depolarization on ASP^+ uptake were also performed (see figure legends in detail). The uptake was stopped by ice-cold D-PBS, and the fluorescence intensity in each well was detected in fluorescence unit (a.u.) at excitation/emission of 485/590 nm using Synergy™ HT microplate reader (Biotek, VT, USA). The total protein concentration of each sample was also measured using commercial Bradford protein assay for colorimetric detection (Bio-Rad, Hercules, CA, USA). The ASP^+ transport activity was then normalized by mg of protein.

To determine the actual transport of [^{3}H]-MPP^+ uptake, cells were pre-incubated with D-PBS for 10 min at 37°C. Subsequently, cells were incubated in D-PBS containing 1.25 nM [^{3}H]-MPP^+ in the presence or absence of 0.1 and 1 mM of TPeA or test compounds (see figure legends in detail). For kinetic studies, cells were incubated in D-PBS containing various concentrations of [^{3}H]-MPP^+ (final concentrations of 50–1 000 μM derived from 1.25 to 2.5 nM of [^{3}H]-MPP^+ and unlabeled MPP^+) at both 37°C and 4°C. The mediated component of [^{3}H]-MPP^+ uptake was calculated by subtracting the uptake at 37°C from 4°C. The kinetics were plotted, and the influences of extracellular pH, temperature, and membrane depolarization on [^{3}H]-MPP^+ uptake were analyzed in the similar manner as to the ASP^+ assay (see figure legends in detail). The total accumulation of [^{3}H]-MPP^+ was detected using liquid scintillation counters (Perkin Elmer, Waltham, MA, USA), and the intracellular [^{3}H]-MPP^+ uptake was normalized by mg of protein.

The hOCTs mRNA expression in HepG2 cells

Total RNA extracts of each sample were isolated and purified from HepG2 cells cultured for 3 days in 4–5 different passages using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The first-strand complementary DNA was subsequently synthesized using iScript cDNA synthesis kit (Bio-Rad). The quantitative real-time PCR (qPCR) reaction was performed using SYBR green real-time PCR master mix (Bioline, London, UK) on an ABI 7500 (Life Technologies, Grand Island, NY, USA). Specific primer sets were designed or used according to published sequences [14,32,33] as shown in *Table I*, and purchased from Bio Basic, Inc. (Amherst, NY, USA). The relative quantification of hOCTs and hMATEs mRNA expression was normalized to GAPDH mRNA level. The data were reported as $2^{(-\Delta Ct)}$, and the qPCR amplification was performed in duplicates for each cDNA.

Human OCT1, hOCT3, and AMPK protein expressions in HepG2 cells

HepG2 cells were lysed by CelLytic mammalian tissue lysis/extraction reagent (Sigma-Aldrich) containing 1% protease inhibitor cocktail (Sigma-Aldrich). The samples were disrupted by homogenizer and subsequently centrifuged at 5 000 ***g*** for 10 min at 4°C. The supernatant was designated as *whole cell lysate*. For membrane

Table I Details giving primer sequences and expected product sizes for the gene amplification.

cDNA	Genbank accession no.	Forward primers5′ to 3′	Reverse primers5′ to 3′	PCRProduct size (bp)
hOCT1	NM_003057	CATTTTGTTTGCGGTGTTGGG	CGTGTTTTCTTTGGGCTTTGC	136
hOCT2	NM003058	AGTCTGCCTGGTCAAT GCT	AGGAATGGCGTGATGATGC	105
hOCT3	XM_ 017011203	GGCGAACCAACGTTTGAAGG	AGATGTTGCTCTTTGCCCCA	137
hOCTN1	NM003059	CAGACAGGTTTGGCAG GAAGA	GCCCACGATGACAAATAACACA	122
hOCTN2	NM003060	TTACTTCATCCGAGAC TGG	CTGCTTCTTGGAACTTAGG	229
hMATE1	NM018242	TCTTCAGGCAGGACCC AGAT	AACAGATAGTTGGCGAGGGC	187
hMATE2	NM152908	ACTGCTGCCTTTGTTGC TTATACT	TCTCAGGCCCAGGTCT GGTT	127
hGAPDH	NM002046	AGCCTTCTCCATGGTGGTGAAGAC	CGGAGTCAACGGATTTGGTCG	307

fraction, the whole cells were subsequently centrifuged at 100 000 ***g*** for 120 min at 4°C and the pellet from the spin was specified as *membrane-rich fraction*. The samples (100 μg/lane) from 3 to 4 different cell passages were resolved in 4× Laemmli solution and separated on 10% sodium dodecyl sulfate polyacrylamide gel. The primary antibodies against hOCT1, hOCT3 (Sigma-Aldrich), AMPKα2, and phosphorylated Thr172 AMPKα (*p*-AMPKα) (Merck, Boston, MA, USA) were incubated overnight. Goat anti-mouse or rabbit IgG horseradish peroxidase-conjugated secondary antibody was subsequently incubated for 1 h at RT. Anti-β-actin and Anti-Na^+/K^+ ATPase (Abcam, Cambridge, MA, USA) were used as loading control and membrane marker, respectively. Target proteins were detected by enhanced chemiluminescence kit (Bio-Rad), and the specific band density was analyzed using ImageJ program from RSB of the NIMH/NIH (Bethesda, MD, USA).

Actions of metformin and paclitaxel mediated by hOCTs in HepG2 cells

As metformin is a low affinity substrate for hOCTs/hMATEs compared to that of high affinity substrates, for example, MPP^+ and ASP^+ as shown previously using [^{14}C]-metformin [34,35], the alternative/indirect approach for identifying whether metformin is transported by hOCTs into HepG2 cells was performed through the activation of AMPK and inhibition by organic cation inhibitors, TPeA, and clonidine. HepG2 cells were cultured for 2 days and, subsequently, incubated with 2 mM of metformin similarly to a previous study [12] in the presence or absence of either 1 or 10 μM of TPeA or 100 μM of clonidine for another 24 h. The cells were harvested and purified as *whole cell lysate* as mentioned above. The expressions of AMPKα2 and *p*-AMPKα (Thr172) were determined using Western blot analysis.

The existing direct measurement of [^{3}H]-paclitaxel transport faced limitation due to it adhered to the external surface of the cells and plastic ware as previously seen [14]. Therefore, indirect approach of paclitaxel transport mediated by hOCTs, and whether its action was reversed by organic cation inhibitors, TPeA, and clonidine, were carried out using cytotoxic study. Two-day-cultured cells were pre-incubated in 10 μM of paclitaxel in the presence or absence of TPeA, clonidine, and metformin at different concentrations (see figure legends in detail) for 24 h at 37°C. Subsequently, the cell viability was investigated using MTT assay. Briefly, the cells were washed and incubated with phenol red free-DMEM (Biochrom AG) containing 0.5 mg/mL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) (Amresco, Solon, OH, USA) for 4 h. The reaction was stopped by ice-cold PBS, and then, the cells were lysed by dimethyl sulfoxide (DMSO) for 30 min at 37°C. The absorbance of dissolved formazan was measured at the wavelength of 570 nm with a reference wavelength of 680 nm using a microplate reader. The data were analyzed and expressed as percentage of control (absence of test compound).

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). The statistical analysis was performed using one-way ANOVA followed by Fisher's LSD or Dunnett's multiple comparison using GraphPad prism 4.0 (GraphPad Software Inc.). Statistical significance was considered when $P < 0.05$.

RESULTS

Functional transports of ASP^+ and [^{3}H]-MPP^+ mediated by hOCTs in HepG2 cells

To determine the functional transport of organic cation substrates mediated by hOCTs in HepG2 cells, uptake of fluorescent ASP^+ and [^{3}H]-MPP^+ was analyzed and compared. As shown in *Figure 1a*, total ASP^+

accumulation in HepG2 cells rapidly increased up to 10 min and the rate gradually reached saturation at 60 min while the addition of TPeA, a potent OCTs inhibitor, significantly reduced the rate of ASP^+ transport linearly over time. Like ASP^+, [^{3}H]-MPP^+ uptake increased linearly up to 2 min and reached saturation at 10 min. Additionally, TPeA linearly increased [^{3}H]-MPP^+ uptake into HepG2 cells up to 10 min (*Figure 1b*). These data indicated ASP^+ was transported into HepG2 cells and was sensitive to TPeA, similar to a typical and well-known OCTs substrate, [^{3}H]-MPP^+.

Kinetic parameters of ASP^+ and [^{3}H]-MPP^+ transports into HepG2 cells

Transport of ASP^+ and [^{3}H]-MPP^+ into HepG2 cells as mediated by hOCTs was determined using varying concentration of both ASP^+ and [^{3}H]-MPP^+. The results showed total ASP^+ uptake into HepG2 cells at 37°C increased linearly up to 20 μM and reached saturation at 40 and 80 μM while the uptake of ASP^+ at 4°C was minimal, indicating ASP^+ was selectively taken up by hOCTs rather than by simple diffusion (*Figure 2a*). In addition, the calculated kinetic parameters from the Lineweaver–Burk plot revealed a K_m of 76.7 ± 14.3 μM and V_{max} was 11.1 ± 2.9 nmol/mg protein/min for ASP^+ (*Figure 2b*). Similarly, [^{3}H]-MPP^+ uptake at 37°C showed linearity from 50 to 200 μM and reached saturation after 500 μM whereas the uptake of [^{3}H]-MPP^+ at 4°C increased linearly but more slowly (*Figure 2c*). The calculated K_m of [^{3}H]-MPP^+ mediated by hOCTs was 252.1 ± 34.8 μM, and the V_{max} was 1.4 ± 0.3 pmol/mg protein/min (*Figure 2d*). These findings suggested ASP^+ and [^{3}H]-MPP^+ were transported into HepG2 cells as mediated by endogenous hOCTs. Moreover, ASP^+ was a more highly preferred hOCTs substrate than [^{3}H]-MPP^+.

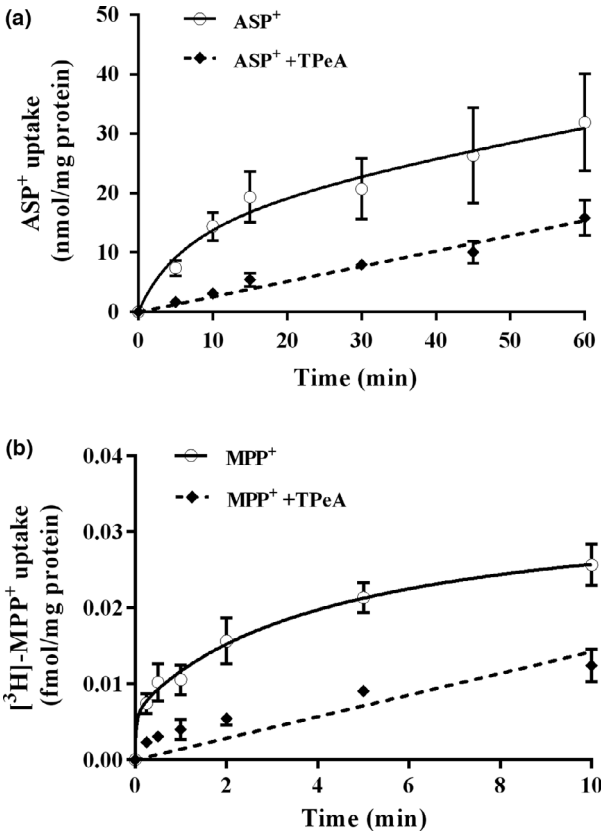

Figure 1 Time course of ASP^+ (a) and [^{3}H]-MPP^+ (b) uptake into HepG2 cells. (a) The cells were incubated in 10 μM of ASP^+ in the presence or absence of 1 mM of tetrapentylammonium (TPeA). (b) The cells were incubated in 1.25 nM [^{3}H]-MPP^+ at different time points in the presence or absence of 1 mM of TPeA. The data are shown as mean ± SD from three separate experiments.

ASP^+ and [^{3}H]-MPP^+ transport into HepG2 cells depends on extracellular pH, temperature, and membrane depolarization

This study further characterized the influences of extracellular pH, temperature, and membrane depolarization on ASP^+ and [^{3}H]-MPP^+ uptake mediated by hOCTs into HepG2 cells. The results showed ASP^+ and [^{3}H]-MPP^+ uptake was increased with increased extracellular pH. Nevertheless, adding TPeA diminished this effect (*Figure 3a,b*). Furthermore, ASP^+ and [^{3}H]-MPP^+ uptake at 4°C significantly decreased compared with respective controls at 37°C, implying the transport of both substrates into HepG2 cells depends on temperature (*Figure 3c,d*). In addition, membrane depolarization induced by high K^+ significantly reduced ASP^+ uptake compared to NaCl. In the [^{3}H]-MPP^+ uptake condition, this effect did not occur (*Figure 3e,f*), suggesting membrane depolarization specifically interfered with organic cation transport, and ASP^+ was profoundly sensitive to inside negatively charged in HepG2 cells.

Interactions of ASP^+ and [^{3}H]-MPP^+ with various organic cations in HepG2 cells

To confirm the specificity of ASP^+ and [^{3}H]-MPP^+ transport mediated by hOCTs into HepG2 cells, *cis*-inhibition of both substrates with various organic cation compounds was carried out. The results showed ASP^+ uptake significantly decreased in the presence of TPeA,

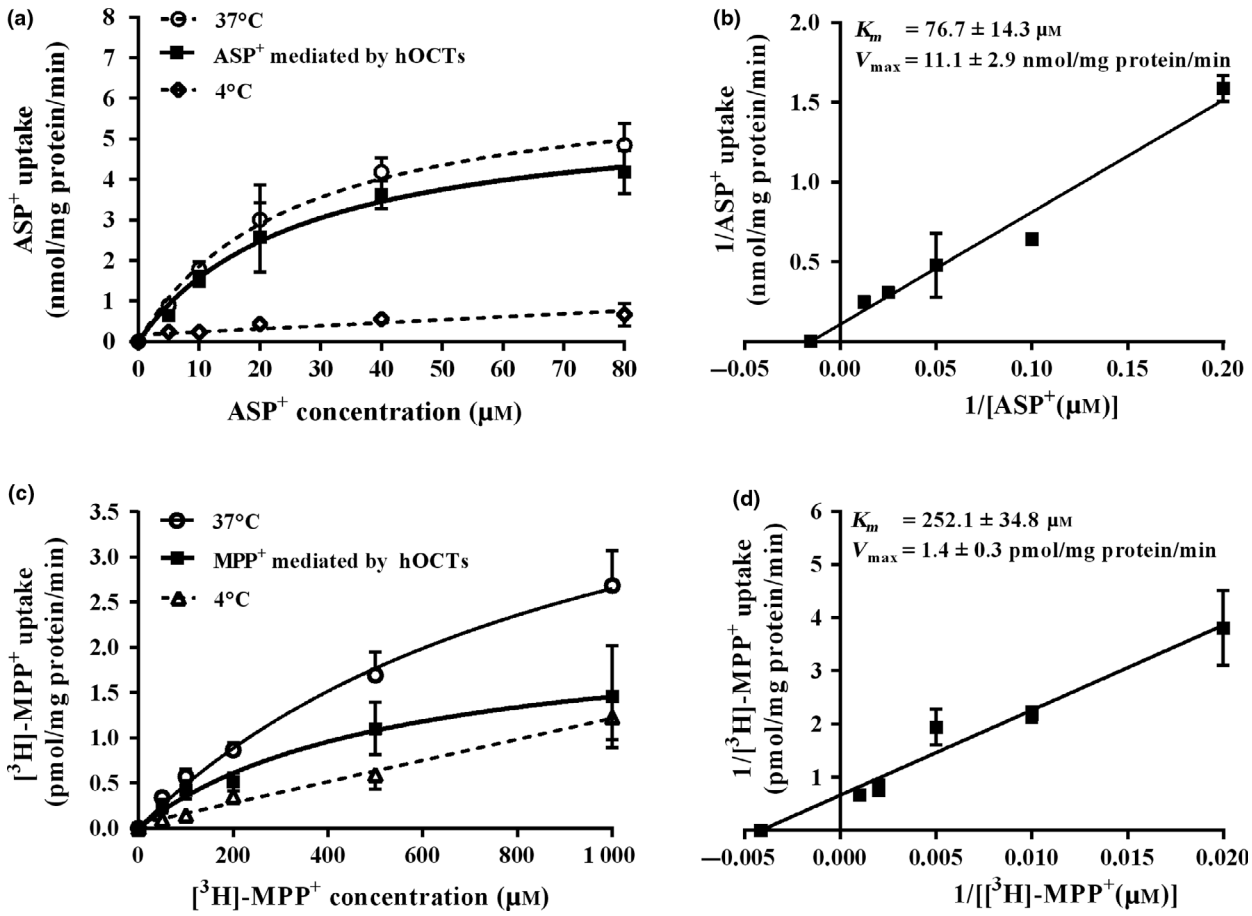

Figure 2 Concentration-dependent and kinetic parameters of ASP^+ and [^{3}H]-MPP^+ transport into HepG2 cells. (a) The cells were incubated in buffer containing 5–80 μM of ASP^+. The data are shown as mean ± SD from three separate experiments. (b) The ASP^+-mediated transport component was used to calculate kinetic parameters (K_m and V_{max}) by Lineweaver–Burk plot, and representative data are shown. (c) The cells were incubated in 50–1 000 μM of [^{3}H]-MPP^+ at 37°C and 4°C. The data are shown as mean ± SD from three separate experiments. (d) The [^{3}H]-MPP^+-mediated component was used to calculate kinetic parameters (K_m and V_{max}) using double reciprocal plot and presented as the Lineweaver–Burk plot, and representative data are shown.

a typical inhibitor of OCTs, in a dose-dependent manner with the calculated IC_{50} of 109.60 ± 1.84 μM for endogenous hOCTs transport function (*Figure 4a*). Moreover, a potent OCT1 inhibitor, clonidine, and unlabeled MPP^+ also inhibited ASP^+ transport by approximately 56%. In contrast, low affinity hOCT substrates – including metformin, TEA, and typical organic anion, PAH – did not inhibit ASP^+ uptake (*Figure 4b*). Consistently, uptake of [^{3}H]-MPP^+ was remarkably inhibited by metformin, unlabeled MPP^+, clonidine, TPeA, and ASP^+, whereas TEA and PAH did not show any inhibitory effect on MPP^+ transport in HepG2 cells (*Figure 4c*). Thus, OCT substrates and/or inhibitors interacted with endogenous hOCTs in HepG2 cells.

Human OCTs mRNA and protein expression in HepG2 cells

Since several OCTs mRNAs were previously detectable in HepG2 cells [28,29], hOCTs and hMATEs mRNA expression were also confirmed as the transporters involved in ASP^+ and [^{3}H]-MPP^+ transport in HepG2 cells using quantitative real-time PCR (qPCR). The mRNA expression of hMATE1, an efflux transporter, was most highly expressed in HepG2 cells followed by the influx transporters: hOCTN2/hOCT3, hOCT1, and hOCTN1, respectively. Expression of hOCT2 and hMATE2 was undetectable in HepG2 cells (*Figure 5a*). Human OCT1 and hOCT3 protein expression was found in whole cell lysate and membrane-rich fraction of HepG2 cells (*Figure 5b,c*), suggesting influx

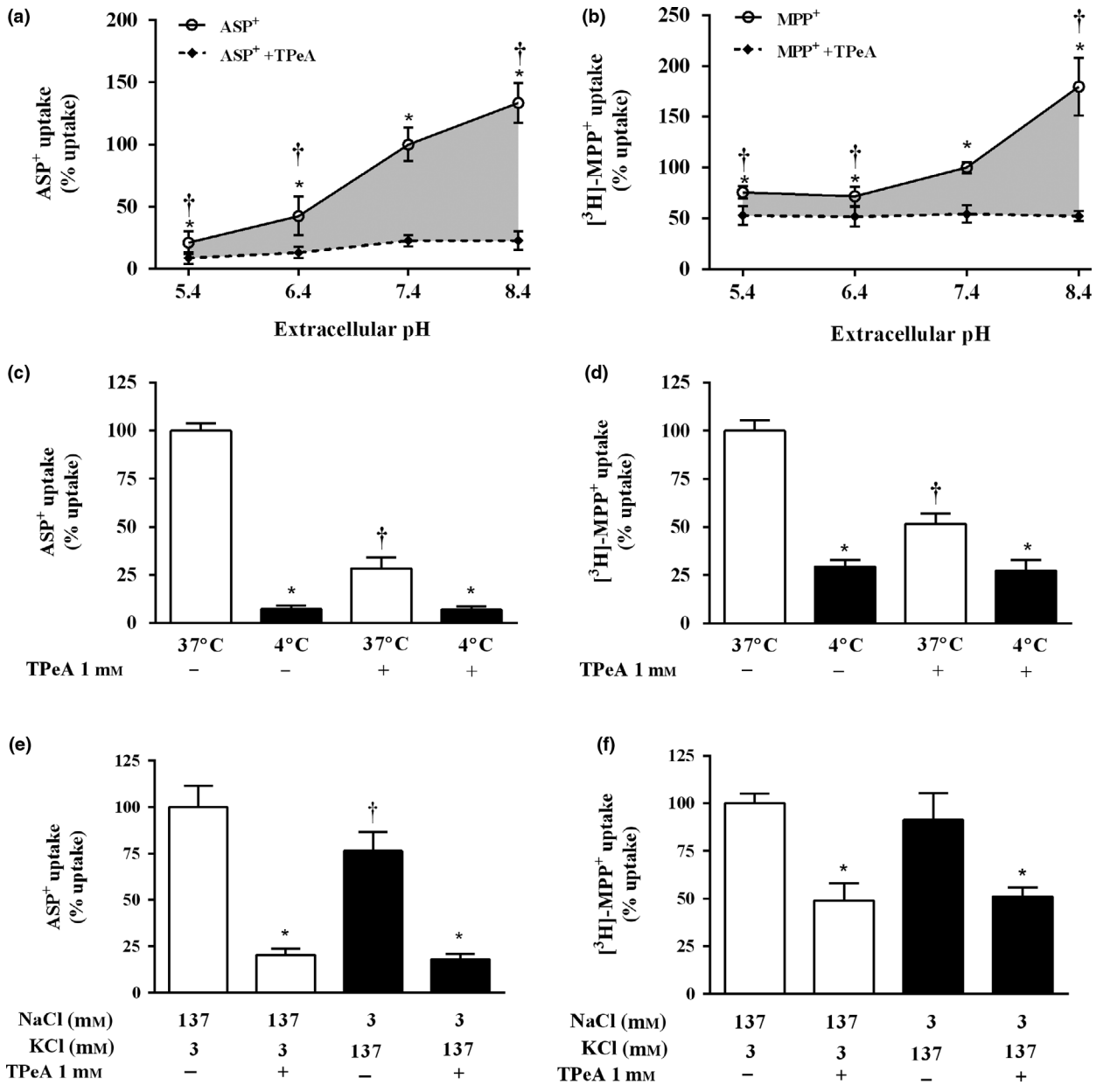

Figure 3 Influences of pH, temperature, and membrane potential on ASP^+ and $[^3H]$-MPP^+ transport in HepG2 cells. (a, b) The cells were incubated in 10 μM of ASP^+ or 100 nM of $[^3H]$-MPP^+ in the presence or absence of 1 mM of tetrapentylammonium (TPeA) at different pHs. †$P < 0.05$ indicates significant differences from pH 7.4, and *$P < 0.05$ indicates significant differences from ASP^+ or $[^3H]$-MPP^+ alone. The data are shown as mean ± SD from 3 to 4 separate experiments. (c, d) The cells were incubated in either 10 μM of ASP^+ or 100 nM of $[^3H]$-MPP^+ in the presence or absence of 1 mM of TPeA at 37°C and 4°C. †$P < 0.05$ indicates significant differences from control at 37°C, and *$P < 0.05$ indicates significant differences from respective control at 37°C. The data are shown as mean ± SD from three separate experiments. (e, f) The cells were pre-incubated in 137 mM of NaCl for 10 min and then incubated in D-PBS containing 137 mM of either NaCl or KCl with 10 μM of ASP^+ for another 10 min or 100 nM of $[^3H]$-MPP^+ for 2 min in the presence or absence of 1 mM of TPeA. †$P < 0.05$ indicates significant differences from control with Na^+ buffer, and *$P < 0.05$ indicates significant differences from respective control. The data are shown as mean ± SD from three separate experiments.

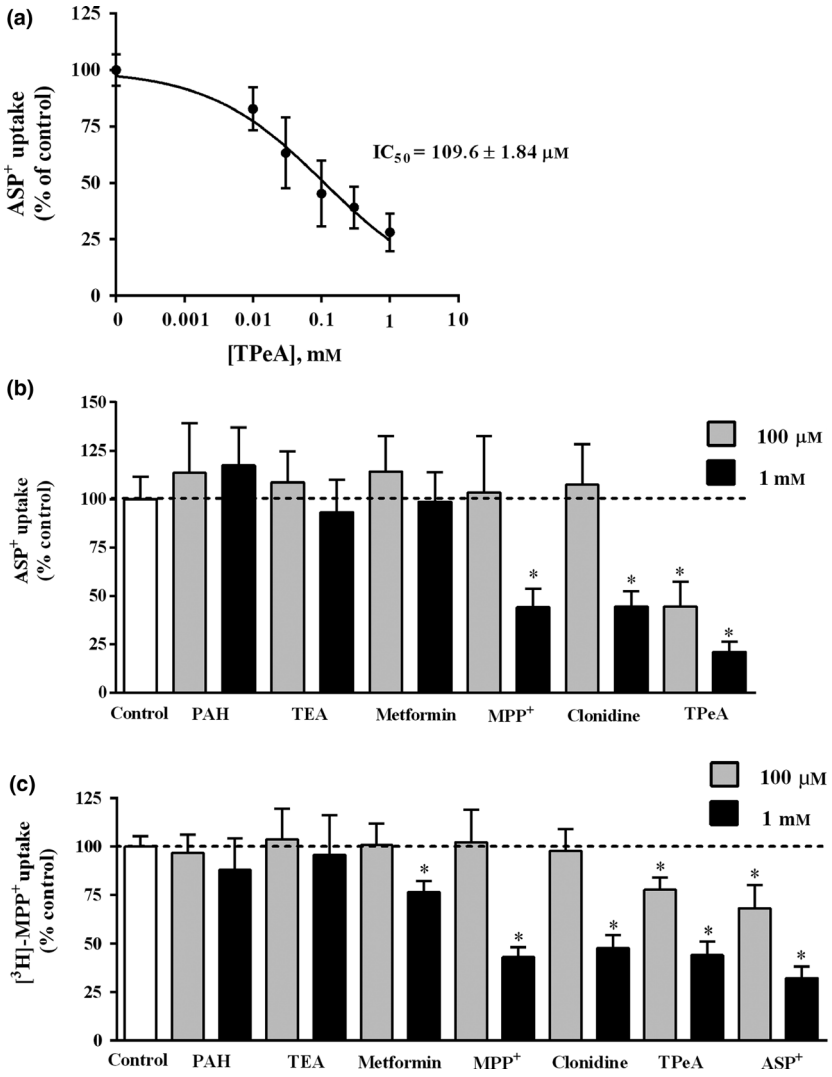

Figure 4 Interactions of various organic cations with hOCTs mediated transport of ASP^+ and [^{3}H]-MPP^+ in HepG2 cells. The cells were incubated in 10 μM of ASP^+ in the presence or absence of various concentrations of (a) tetrapentylammonium (TPeA) with the calculated IC_{50} value, and (b) 100 μM or 1 mM of TPeA, TEA, clonidine, metformin, MPP^+, and organic anion, PAH. (c) The cells were incubated in 100 nM of [^{3}H]-MPP^+ for 2 min in the presence or absence of 100 μM or 1 mM of ASP^+, TPeA, TEA, clonidine, metformin, MPP^+, and PAH. *$P < 0.05$ indicates significant differences from control. The data are shown as mean ± SD from three to six separate experiments.

transporters, hOCT1 and hOCT3, exist and are involved in ASP^+ and [^{3}H]-MPP^+ transport in HepG2 cells.

Actions of metformin and paclitaxel transport mediated by hOCT1 and hOCT3 were blunted by other organic cations in HepG2 cells

To investigate the impact of hOCT1 and hOCT3 transport of metformin, a known substrate of both hOCT1 and hOCT3 in hepatocytes, phosphorylated AMPK was determined using Western blot analysis as previously described [12]. As shown in *Figure 6a*, total AMPKα2 was not different among experimental groups. However, AMPKα phosphorylation (*p*-AMPKα) significantly increased when HepG2 cells were treated with 2 mM metformin, and *p*-AMPKα expression was markedly reduced to normal levels when HepG2 cells were exposed to a combination of 2 mM metformin with either 1 and 10 μM TPeA, or 100 μM clonidine (*Figure 6b*). Thus, this result indicated that metformin was selectively transported via hOCTs – in particular, hOCT1 and hOCT3 – and exerted its action by phosphorylation of AMPK in HepG2 cells. Likewise, exposure to 10 μM of paclitaxel, hOCT1 substrate, significantly decreased cell viability compared with vehicle control (0.2% DMSO), whereas 1 and 10 μM TPeA, 10 and 100 μM of clonidine, and 1 and 2 mM of metformin alone did not affect cell viability. Similar to metformin action, the combination of paclitaxel with either 100 μM clonidine or 2 mM of metformin markedly reversed paclitaxel-induced cytotoxic action in HepG2 cells. Neither 1 nor 10 μM of TPeA could reverse HepG2 cell viability compared with paclitaxel-

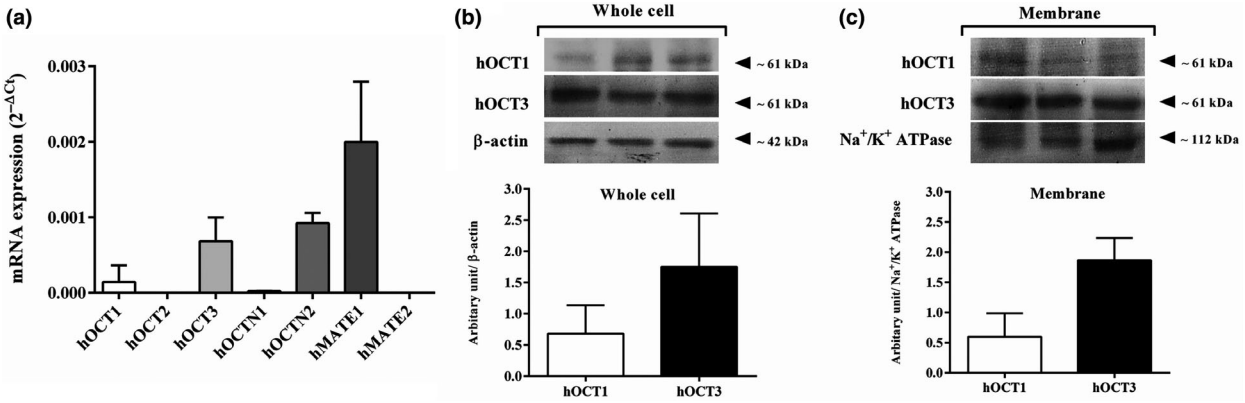

Figure 5 The mRNA and protein expression of hOCTs in HepG2 cells. (a) hOCT1, hOCT2, hOCT3, hOCTN1 and hOCTN2, hMATE1 and hMATE2 mRNA expression determined using quantitative real-time PCR. The data are expressed as mean ± SD from 4 to 5 different cell passages (b, c). The quantification of hOCT1 and three protein levels in *whole cell lysate* were normalized by β-actin while hOCT1 and hOCT3 expressed in *membrane-rich fractions* were normalized by Na^+/K^+ ATPase. The data represent as mean ± SD from four different cell passages.

treated alone, suggesting cytotoxic action of paclitaxel involved hOCT1 and/or hOCT3 (*Figure 7*).

DISCUSSION

The present study demonstrated high affinity transport of fluorescent organic cation ASP^+ mediated by functional endogenous hOCTs in HepG2 cells relative to traditional [^{3}H]-MPP^+. In addition, the study showed cationic drugs, metformin and paclitaxel, were transported partly by hOCT1 and/or hOCT3 which suggests transport of organic cations into hepatocellular carcinoma requires endogenous hOCTs.

Previously, HepG2 cell line has been employed to show broad range expressions of drug-metabolizing enzymes – for example, cytochromes P450 (CYP) 1A2 (CYP1A2), CYP2B6, and CYP3A4 [36,37], cytokines, and drug transporters [38]. Thus, this cell line has been widely used as a tool for drug metabolism, toxicity, and drug resistance screening [37,39]. Previous study found the presence of expression of rat Oct1 in diethylnitrosamine-induced hepatocarcinoma rats and in rat hepatoma cell line, Fao. On the other hand, downregulation of rat Oct1 was demonstrated in other rat hepatoma cell lines: H5, Faza, HTC, and RHC1 [40]. In addition, it was shown that the mRNA expression of influx transporter hOCT1 was higher than that of hOCT3 and hMATE1, an efflux transporter, in normal human liver tissues [35,41]. Additionally, the hOCTN1 was detectable only in fetal liver, but not in adult liver [42] and hOCTN2 plays a major role in taking up carnitine and its derivatives for liver metabolism through carnitine biosynthesis [43]. Although the present study did not quantitatively compare the expression levels of these transporters to that of normal hepatocyte cells, several studies found that hOCT1/hOCT3 and hOCT1/hMATE1 were downregulated in human highly differentiated hepatoma HepaRG cells, cholestasis, hepatocellular carcinoma (HCC), and hepatoma HuH-7 cells when compared with normal human hepatocytes [28,35,41,44]. Consistent with this study, low expression levels of hOCT1 and hOCT3 in HepG2 cells were shown when compared with normal liver tissues using qPCR [29]. In addition, relatively low expression of influx transporter hOCT1/hOCT3 to high expression of efflux transport hMATE1 in HepG2 cells was observed in this study, suggesting that the expression of drug transporters under hepatopathogenesis could be subjected to alteration and the variability of specific organic drug-targeting to hepatocytes need to be crucially considered.

Regarding the affinity of ASP^+ to transporters, the K_m value of hOCT1 and hOCT3 was much higher than that of hMATE1 (1–2 μM vs. 34 μM) [23,24,45]. Thus, the uptake of ASP^+ in this study might be preferably transported by hOCT1 and in particularly by hOCT3. Although hOCTN2, an influx transporter, was highly expressed in HepG2 cells, hOCTN2 did not transport ASP^+ as previously reported [21]. Therefore, the confounding factors of hMATE1 and hOCTN2 on ASP^+ transport into HepG2 cells in this study were negligible. A fluorescent ASP^+-based method has been used to

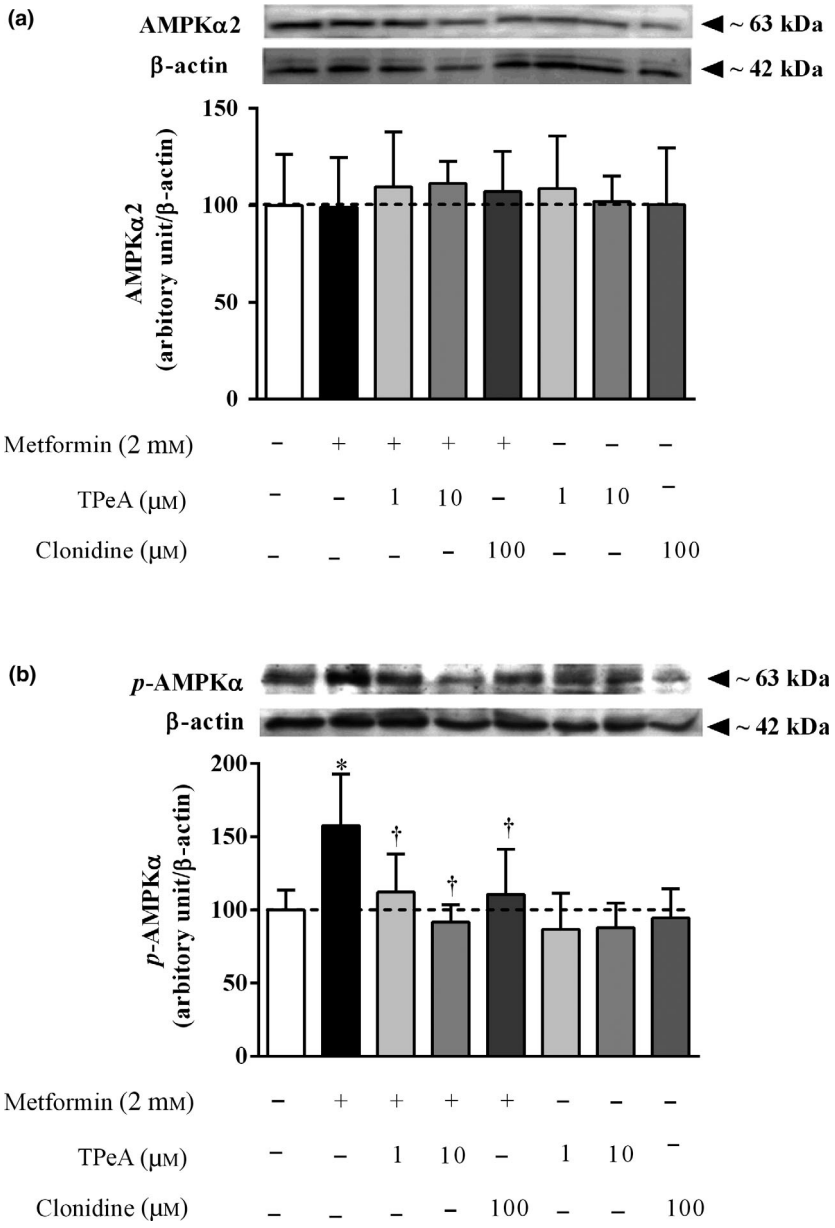

Figure 6 AMPKα2 phosphorylation by metformin transported by hOCTs in HepG2 cells. The cells were incubated with 2 mM of metformin in the presence or absence of either 1, 10 μM of tetrapentylammonium (TPeA) or 100 μM clonidine for 24 h. Subsequently, the expressions of (a) AMPKα2 (1 μg/mL) and (b) *p*-AMPKα (2 μg/mL) were determined using Western blotting. $^{*}P < 0.05$ indicates significant differences from control. $^{\dagger}P < 0.05$ indicates significant differences from metformin-activated AMPK alone. The data are expressed as mean ± SD from three different cell passages. Representative blots of AMPKα2 and *p*-AMPKα protein expression are shown above.

investigate hOCTs function in HEK-293 overexpressed hOCT1 and hOCT3 with the K_m of 2.3 and 1.1 μM, respectively [23,24]. Moreover, endogenous hOCTs transported ASP^+ into freshly isolated human proximal tubules [19], in which hOCT2 was highly expressed [20]. Similarly, human respiratory epithelial cell line A549 highly expresses hOCT2 and hOCT3 and transports ASP^+ with low and high affinities (K_m were 12.5 ± 4 and 456.9 ± 164.5 μM, respectively) [21]. Consistently, the present study has also demonstrated that ASP^+ transport mediated by hOCTs, particularly hOCT1 and hOCT3, in HepG2 cells has a higher affinity than traditional [^{3}H]-MPP^+, implying ASP^+ could be employed in investigation of organic cations mediated by endogenous hOCTs in hepatocellular carcinoma cells. Nonetheless, besides membrane transporters, organelle trapping also contributes to intracellular pool for the uptake, distribution, and response of several drugs and substances in the cells. Previous study demonstrated that ASP^+ – a nontoxic, low hydrophobic fluorescent organic cation – could rapidly accumulate into acidic intracellular organelles including

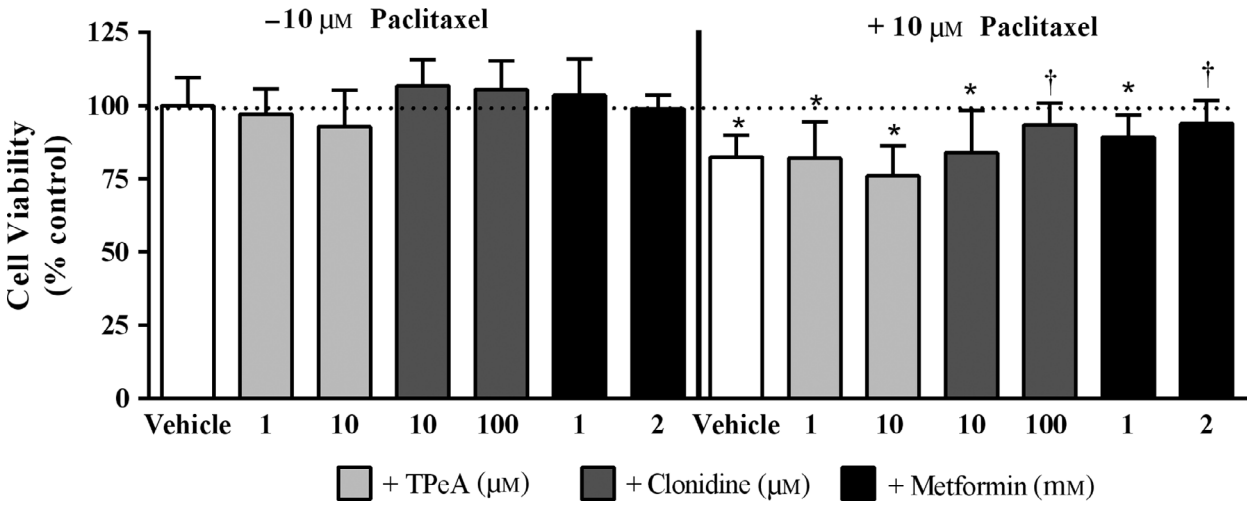

Figure 7 Cytotoxic effect induced by paclitaxel transported by hOCTs in HepG2 cells. The cells were incubated with 10 μM of paclitaxel in the presence or absence of 1 or 10 μM tetrapentylammonium (TPeA), 10 and 100 μM of clonidine, and 1 mM, and 2 mM of metformin for 24 h. $^{*}P < 0.05$ indicates significant differences from vehicle. $^{\dagger}P < 0.05$ indicates significant differences from paclitaxel-induced cytotoxic effect alone. The data are expressed as mean ± SD from three to four separate experiments.

mitochondria in rat renal cortex [46]. Likewise, TEA, a typical substrate for OCTs, is taken up into lysosomal-rich rat liver tissues [47]. The lysosomal trapping also takes part for intracellular accumulation of propranolol, an anti-hypertensive drug and inhibitor for OCTs, in immortalized hepatocyte cells [48]. Consistently, acidic lysosomal sequestration of imatinib, an anti-cancer drug and a substrate for OCT1, is the determinant factor for cellular accumulation of imatinib [49]. Despite this study differentiated the transport of ASP^+ mediated by hOCTs from passive diffusion using TPeA, the potential trapping of ASP^+ into either lysosome or mitochondria might implicate a possibility of high level of intracellular accumulation into HepG2 cells.

Since HepG2 cell line is derived from HepG2 cells, the pathophysiological condition might alter cellular properties and/or functional characteristics of membrane transporters, including pH, temperature, and membrane depolarization. Hence, characteristics of endogenous hOCTs were examined. Previously, increasing [^{14}C]-TEA transport mediated by rOct1, rOct2, and rOct3 was demonstrated with increasing extracellular pH [50,51]. Similarly, increased ASP^+ and [^{3}H]-MPP^+ transport mediated by hOCTs in HepG2 cells was demonstrated under high extracellular pH. On the other hand, a low pH condition markedly reduced transport of both substrates. One possible explanation is a high external positive charge of H^+ generating a small potential difference, decreasing hOCTs mediated ASP^+ and [^{3}H]-MPP^+ transport and vice versa. Similar to previous study, decreased extracellular pH resulted in decreased K^+ outward conductance, and subsequently, generated membrane depolarization in primary cultured rat hepatocyte [52]. Another possible explanation relates to hMATE1 which are also expressed in HepG2 cells and are known pH-dependent transporters [53]. Perhaps these transporters were partially responsible for transport of ASP^+ and [^{3}H]-MPP^+ into HepG2 cells. In addition, hOCT1 and hOCT3 were operated properly in HepG2 cells under physiological temperature condition. Similarly, a previous study showed TEA transport mediated by hOCT1 was decreased at 4°C compared to room temperature in HeLa cells overexpressing hOCT1 [17]. Therefore, optimal membrane fluidity could directly influence membrane permeability and dynamic function of membrane proteins, including hOCTs/hMATE1 that was expressed in HepG2 cells. Regarding membrane depolarization, reduction of ASP^+ transport mediated by hOCTs was observed after K^+-induced membrane depolarization. However, this effect was blunted when [^{3}H]-MPP^+ transport studies were conducted. As the value of resting membrane potential of HepG2 cells was higher than normal hepatocytes (-9.8 ± 0.5 mV vs. -25.1 ± 1.5 mV) [54], the former may exhibit smaller electrical difference than the latter and subsequently drive relatively lower [^{3}H]-MPP^+ transport.

Additionally, [^{3}H]-MPP^+ may be taken up through other facilitated cationic transporters expressed in HepG2 cells, such as hMATE1, as previously seen in normal human hepatocytes [4].

As TPeA interacts with both hOCT1- and hOCT3-mediated ASP^+ uptake in HEK-293 overexpressing cells with the IC_{50s} of 5.5 and 28 μM, respectively [24,55], the relatively higher figure of calculated IC_{50} value of TPeA on ASP^+ mediated endogenous hOCTs in HepG2 cells was presented. In addition, MPP^+ and clonidine, organic cations, interacted with endogenous hOCTs, mediating high affinity ASP^+ transport, in HepG2 cells. Since drug–hOCT1 interaction has been emerged for potential evaluation in drug development [9], endogenous hOCTs expressed in HepG2 cells could be beneficial tools for in vitro DDIs screening and clinical studies, particularly anti-cancer drugs. Finding lower mRNA and protein expression of hOCT1 than hOCT3 in HepG2 cells, like a previous study showing mRNA expression of hOCT1, was less than hOCT3 [29,30]. Human OCT3 might be the primary transporter in cationic uptake in hepatocellular carcinoma. Moreover, since metformin action was reversed by TPeA, and clonidine and paclitaxel action were reduced by clonidine and metformin, transport of metformin and paclitaxel into hepatocellular carcinoma cells likely requires hOCTs – particularly, hOCT1 and hOCT3. As the combination of sitagliptin and metformin in HepG2 cells blunted metformin action [56] while paclitaxel-induced cytotoxicity was reversed by either TEA or corticosterone, another OCTs substrate, in CHO cells overexpressing hOCT1 [14], the current findings align neatly. Accordingly, the major target sites for cationic drugs entering HepG2 cells appear to be hOCT1 and hOCT3, meaning these transporters have a role in drug actions in hepatocellular carcinoma cells. The contribution ratio for endogenous hOCT1- and hOCT3-mediated uptake of ASP^+, MPP^+, or other organic cations in hepatocellular carcinoma cells was not differentiated in this study yet; however, 40% knockdown of hOCT3 expression by silencing interference RNA (siRNA) technique reduced its activity by more than 60% [57], and plasma membrane monoamine transporter (PMAT)-knockdown in $CaCo_2$ cells could provide a contribution of PMAT-mediated metformin absorption in intestinal cells [58]. Therefore, specific knockdown of either hOCT1 or hOCT3 by siRNA might be a tool for further delineation.

In conclusion, the functional characteristics of endogenous hOCT1 and hOCT3 expressed in HepG2 cells using florescent organic cation ASP^+ revealed higher affinity and a higher maximal transport rate compared to traditional radioactive [^{3}H]-MPP^+. In addition, ASP^+ was transported mainly by endogenous hOCT1 and hOCT3 expressed in HepG2 cells, which play an important role in cationic drug uptake including anti-cancer drugs in HepG2 cells. The rapid and optimal fluorescence-based assay could be an optional in vitro model for the assessment of organic cation drug screening and interactions with hOCTs in hepatocellular carcinoma cells and also provide beneficial information for drug discovery and development.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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ABBREVIATIONS

A549 – adenocarcinomic human alveolar basal epithelial
AMPK – 5' adenosine monophosphate-activated protein kinase
ASP^+ – 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium
DDIs – drug–drug interactions
HepG2 – human hepatocellular carcinoma cell line
hOCT1 – human organic cation transporter isoform 1
hOCT3 – human organic cation transporter isoform 3
hOCTs – human organic cation transporters
MPP^+ – 1-methyl-4-phenylpyridinium
TEA – tetraethylammonium

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