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Proton-Coupled Organic Cation Antiporter Contributes to the Hepatic Uptake of Matrine

Chunyong Wu [1, 2], Xiaomin Sun [1, 2], Chao Feng [1, 2], Xiaoying Liu [1, 2], Hufang Wang [3], Fang Feng [1, 2, *], Junying Zhang [3, *]

[1] Key Laboratory of Drug Quality Control and Pharmacovigilance, Ministry of Education (China Pharmaceutical University), Nanjing 210009, China
[2] Department of Pharmaceutical Analysis, China Pharmaceutical University, Nanjing 210009, China
[3] Department of Pharmaceutics of Traditional Chinese Medicine, China Pharmaceutical University, Nanjing 210009, China

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ABSTRACT

Matrine is the major bioactive alkaloid found in certain *Sophora* plants and has been used for the treatment of liver diseases and protection of liver function. The aim of this study was to investigate the human liver uptake mechanism of matrine by using HepG2 cells as the *in vitro* model. Matrine was transported into HepG2 cells in a time- and temperature-dependent manner. The cellular uptake was saturable and was significantly reduced by the metabolic inhibitors, such as sodium azide and rotenone. Furthermore, the uptake of matrine was found to be regulated by a protonophore (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) and pH, indicating that this influx transporter may be a proton-coupled antiporter. The uptake of matrine was sensitive to inhibition by the cationic drugs including pyrilamine, quinidine, verapamil, amantadine, diphenhydramine, and cimetidine but insensitive to other typical substrates or inhibitors of well-known organic cation transport systems. The present study reveals that, for the first time, in HepG2 cells, the existence of a proton-coupled organic cation antiporter that contributes substantially to the hepatic uptake of matrine.

Introduction

Matrine is the major bioactive alkaloid component found in several famous traditional Chinese medicinal herbs, such as *Sophora flavescens* (kushen), *Sophora subprostrata* (shandougen), and *Sophora alopecuroides* (kudouzi).[1,2] Matrine has been widely used in China for the clinical treatment of viral hepatitis, chronic liver disease, chemotherapy-induced hepatotoxicity, and protection of liver function during chemotherapy.[3-5] Recently, extensive studies indicate that matrine also has great antitumor effects and could be a promising alternative anticancer drug for the treatment of hepatocellular carcinoma.[6-8]

In terms of the therapeutic efficacy against liver diseases, matrine transport into the intracellular space across the sinusoidal membrane of hepatocytes is essential to achieve sufficient drug concentrations in the liver. Rat *in vivo* study has shown significant liver uptake of orally administered matrine with liver-to-plasma partition coefficient of 5.5.[9] However, little is known about the mechanism underlying matrine transport into liver cells.

Systems transporting organic cations, such as organic cation transporters (OCTs), organic cation/carnitine transporters (OCTNs), the plasma membrane monoamine transporter (PMAT), and the multidrug and toxin extrusion (MATE) transporters, have been identified.[10-13] Recently, *in vitro* and *in vivo* studies on the blood-brain barrier, intestine or liver transport of pyrilamine,[14,15] pramipexole,[16] oxycodone,[14,17] clonidine,[18] diphenhydramine,[19,20] nicotine[21] and naloxone[22] led to the discovery of a novel proton-coupled organic cation antiporter at the function level. Although the molecular features of this antiporter have not yet been identified, it is distinct from any of the cloned organic cation transport systems, and all of its substrates have a secondary or tertiary amine moiety as well as a hydrophobic group in their structures.

Matrine has tertiary amine moieties and is positively charged at physiological pH.[5] Thus, we hypothesized that matrine may be actively transported into the liver by the proton-coupled organic cation antiporter. The aim of the present study was, therefore, to

Wu and Sun contributed equally to this work.
The authors declare no conflict of interest.

* *Correspondence to:* Fang Feng (Telephone: +86-25-83271301; Fax: +86-25-83271269) and Junying Zhang (Telephone:+86-25-83271251; Fax: +86-25-83271269).
E-mail addresses: fengfang1@126.com (F. Feng), ivy366300@hotmail.com (J. Zhang).

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elucidate the hepatic transport mechanism by determining the functional characteristics of matrine uptake with human-derived liver HepG2 cells as a model system.

Materials and Methods

Chemicals

Matrine was purchased from National Institutes for Food and Drug Control (Beijing, China). Huperzine A (purity of 98%) was purchased from Nanjing Spring & Autumn Biological Engineering Co. Ltd. (Nanjing, China). Methanol was high performance liquid chromatography (HPLC) grade and purchased from Merck KGaA (Darmstadt, Germany). All cell culture reagents used were purchased from GIBCO (Life Technologies, Grand Island, NY). All other chemicals and solvents were commercially available and of analytical grade.

Cell Culture

The human hepatocellular carcinoma cell line HepG2 cells were cultured as described previously.[23] In brief, the cells were grown in Dulbecco's modified Eagle's medium with 4500 mg/L glucose supplemented with 10% fetal bovine serum, nonessential amino acids, 100-U/mL penicillin, and 100-μg/mL streptomycin. Cells were cultured in an incubator at 37°C under 5% CO_2 and 95% humidity.

Uptake Studies in HepG2 Cells

The *in vitro* uptake study was carried out as described previously with some modifications.[14] Briefly, HepG2 cells were seeded on 12-well plates (Corning, Corning, NY) at a density of 1.5×10^5 cells per well. After the cells reached approximate 80%-90% confluence, the cells were washed 2 times with prewarmed Krebs–Henseleit buffer (142-mM NaCl, 23.8-mM Na_2CO_3, 4.83-mM KCl, 0.96-mM KH_2PO_4, 1.20-mM $MgSO_4$, 12.5-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5-mM glucose, and 1.53-mM $CaCl_2$, pH 7.4 unless otherwise specified) and were preincubated at 37°C for 30 min. After preincubation, the buffer (0.9 mL) containing matrine (0.5 μM unless otherwise specified) was added to initiate the uptake. The cells were incubated at 37°C for a designated time and then washed 3 times with ice-cold buffer (1 mL) to terminate the uptake. The cells were collected and homogenized by sonication in 300 μL of water, and the homogenate was stored at −20°C until analysis by HPLC-tandem mass spectrometry (HPLC-MS/MS) as described in the following section. The cellular protein content was determined by the Lorry method with bovine serum albumin (Thermo Fisher Scientific, Rockford, IL) as a standard. The uptake of matrine was expressed as the cell-to-medium ratio (μL/mg protein) obtained by dividing the uptake amount by the matrine concentration in the incubation buffer.

To estimate the kinetic parameters, the initial matrine uptake of matrine (0.5-400 μM, for 1 min) was determined. The concentration dependence of matrine uptake was also examined at extracellular pH (pH_e) 7.4 or 8.4 after the cells being preincubated in buffer at pH 7.4. The initial uptake was fitted to the following equation by means of nonlinear least-squares regression analysis with Graphpad Prism software (Graphpad, La Jolla, CA):

$$V = \frac{V_{max} \times S}{K_m + S} + P_{dif} \times S$$

where V is the initial matrine uptake rate (nmol/min/mg protein), S is matrine concentration in the medium (μM), P_{dif} is the nonsaturable uptake clearance (mL/min/mg protein), K_m is the Michaelis–Menten constant (μM), and V_{max} is the maximum uptake rate (nmol/min/mg protein).

To examine the energy dependence of matrine accumulation in HepG2 cells, the uptakes were measured under adenosine triphosphate (ATP)-depleted conditions. After the pretreatment of HepG2 cells with 0.1% sodium azide (NaN_3) or 10-μM rotenone for 30 min to reduce metabolic energy, the uptake of matrine was measured in the presence of 0.1% NaN_3 or 10-μM rotenone, respectively. To examine the effect of proton gradient on matrine uptake in HepG2 cells, uptake was measured in the presence or absence of 25-μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a protonophore. To examine the effects of pH_e on matrine uptake, the uptake was measured at medium pH values of 6.4, 7.4, and 8.4. To examine the influence of intracellular pH (pH_i) on matrine uptake, cells were manipulated with NH_4Cl.[24,25] The uptake at alkaline pH_i was measured in the presence of 30-mM NH_4Cl (acute treatment). To measure the matrine uptake at acidic pH_i, extracellular NH_4Cl was removed after preincubating HepG2 cells with 30-mM NH_4Cl. In the inhibition study, the initial uptake of matrine (0.5 μM, for 1 min) was measured in the presence or absence of the selected organic cationic compounds. In the *trans*-stimulation study, HepG2 cells were preloaded with 0.5 mL of pyrilamine (0.5 mM) or diphenhydramine (0.5 mM) for 30 min. Cells were then rinsed 3 times with 1 mL of prewarmed buffer, followed by matrine uptake (0.5 μM, for 1 min).

Analysis of Matrine by HPLC-MS/MS

Fifty microliters of huperzine A solution (0.15 μg/mL) as the internal standard (IS) was added to 50 μL of the cell homogenates. The mixture was vortexed for 3 min and centrifuged at 12,000 rpm for 8 min. Eighty microliters of the supernatant was diluted with 40 μL of 10-mM ammonium acetate solution containing 0.1% formic acid, and then, 20-μL aliquot was injected into a LC-2010 liquid chromatography (Shimadzu, Japan) coupled to an TSQ Quantum Ultra AM triple-quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA) via an electrospray ionization source.

The separation was carried out on a Hanbon Megres C18 analytical column (4.6 mm × 250 mm, 5 μm) at 40°C. The isocratic mobile phase consisted of a mixture of 10-mM ammonium acetate solution containing 0.1% formic acid and methanol (35:65, vol/vol) at a flow rate of 1.0 mL/min. The eluate was split and introduced into the mass spectrometer operated in the positive electrospray ionization mode with selected reaction monitoring of the transitions of m/z 249.08 → 148.03 and 243.06 → 209.79 for matrine and huperzine A (IS), respectively. The spray voltage was 5 kV, and heated capillary temperature was 350°C. The collision energy for insource collision-induced dissociation was set at 8 eV. The nitrogen sheath and auxiliary gas were maintained at 35 psi and 5 units, respectively. The collision energy of 32 eV and 30 eV at a pressure of 1.2 mTorr were used for the collision-activated dissociation of matrine and IS, respectively.

Statistical Analysis

Data were analyzed statistically by unpaired Student t-test for single comparison or 1-way ANOVA followed by Dunnett's test when multiple comparisons were needed. Difference were considered statistically significant at $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Results

Time Course of Matrine Uptake Into HepG2 Cells

Figure 1 shows the time course for the uptake of 0.5-μM matrine in the incubation medium by HepG2 cells. The cellular