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Effect of Pharmaceutical Excipients on Intestinal Absorption of Metformin via Organic Cation-Selective Transporters

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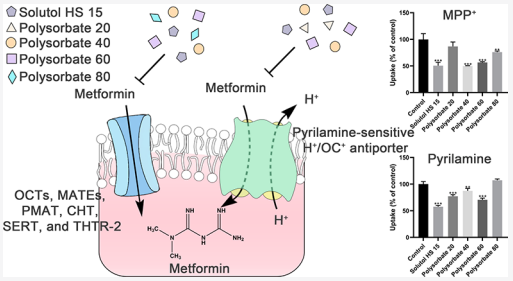
ABSTRACT: Growing evidence has shown that some pharmaceutical excipients can act on drug transporters. The present study was aimed at investigating the effects of 13 commonly used excipients on the intestinal absorption of metformin (MTF) and the underlying mechanisms using Caco-2 cells and an *ex vivo* mouse non-everted gut sac model. First, the uptake of MTF in Caco-2 cells was markedly inhibited by nonionic excipients including Solutol HS 15, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, and crospovidone. Second, transport profile studies showed that MTF was taken up via multiple cation-selective transporters, among which a novel pyrilamine-sensitive proton-coupled organic cation (H^+/OC^+) antiporter played a key role. Third, Solutol HS 15, polysorbate 40, and polysorbate 60 showed *cis*-inhibitory effects on the uptake of either pyrilamine (prototypical substrate of the pyrilamine-sensitive H^+/OC^+ antiporter) or 1-methyl-4-phenylpyridinium (substrate of traditional cation-selective transporters including OCTs, MATEs, PMAT, SERT, and THTR-2), indicating that their suppression on MTF uptake is due to the synergistic inhibition toward multiple influx transporters. Finally, the pH-dependent mouse intestinal absorption of MTF was significantly decreased by Solutol HS 15, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, and pyrilamine. In conclusion, this study revealed that a novel transport process mediated by the pyrilamine-sensitive H^+/OC^+ antiporter contributes to the intestinal absorption of MTF in conjunction with the traditional cation-selective transporters. Mechanistic understanding of the interaction of excipients with cation-selective transporters can improve the formulation design and clinical application of cationic drugs.



KEYWORDS: *pharmaceutical excipients, metformin, intestinal absorption, pyrilamine-sensitive proton-coupled organic cation antiporter, drug−excipient interaction*

INTRODUCTION

The drug−drug interactions (DDIs) arisen from the concomitant administration of multiple drugs could alter the pharmacokinetic property of the drug. It has become a major concern during the drug discovery and development process. Several drugs, such as cerivastatin and terfenadine, were withdrawn from the market because of serious DDIs.[1] The transport of many drugs through cell membranes is controlled by transporters, which play essential roles in drug absorption, distribution, and elimination. Unexpected transporter-mediated DDIs may lead to therapeutic failure or toxic side effects.[2−4]

For many oral formulations, the concentrations of excipients in the intestine can reach hundreds of micromolar to millimolar, which is markedly higher than that of the active pharmaceutical ingredient (API).[5] In addition to pharmaceutical products, many foods contain excipients as additives.[6,7] Although excipients are traditionally recognized as pharmacologically inert, growing evidence has shown that many excipients are active in modulating the activities of biological targets including the transporters.[5,8] Therefore, it is important to evaluate the potential drug−excipient interactions thoroughly when designing formulations of the generic drugs, which require assessment of bioequivalence and clinical multidrug applications.

Currently, most studies are focused on efflux transporters. Some excipients, such as cremophor EL, poly(ethylene glycol) 400, polysorbate 20, and polysorbate 80, have been shown to inhibit the cellular efflux mediated by P-glycoprotein (P-gp),[9−11] breast cancer resistance protein (BCRP),[12−14] and multidrug resistance-associated protein 2 (MRP2).[15−17] On the other hand, the interactions between excipients and drugs, focused on influx transporters, are yet to be disclosed.[8]

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Metformin (MTF), a front-line oral antihyperglycemic drug to treat type 2 diabetes mellitus,[18] is positively charged under physiological conditions (pK_a = 12.4).[19] It is likely to be the substrate of promiscuous cation-sensitive drug transporters, including organic cation transporters (OCTs),[20−22] organic cation/carnitine transporter 1 (OCTN1),[23] multidrug and toxin extrusion transporters (MATEs),[24] plasma membrane monoamine transporter (PMAT),[25,26] serotonin reuptake transporter (SERT),[26] choline high-affinity transporter (CHT),[26] and human thiamine transporter (THTR-2).[27] Recently, studies have shown that a novel pyrilamine-sensitive proton-coupled organic cation (H^+/OC^+) antiporter functions in HepG2 cells,[28] hCMEC/D3 cells,[29,30] Caco-2 cells,[31] and the rat brain.[32] Considering the chemical structure of MTF, we hypothesized that MTF may be also actively absorbed by the pyrilamine-sensitive H^+/OC^+ antiporter.

Patients with type 2 diabetes mellitus are likely to suffer from different diseases and receive multiple therapies, making it imperative to elucidate the potential interactions between MTF and the excipients. To date, the transporter-mediated MTF−excipient interactions remain unclear. In this study, we investigate the interactions of 13 frequently used pharmaceutical excipients with MTF and the underlying mechanisms by using Caco-2 cells and an *ex vivo* mouse non-everted gut sac model.

EXPERIMENTAL SECTION

Chemicals. Metformin hydrochloride (99.9%) was purchased from the National Institutes for Food and Drug Control (China). Phenformin hydrochloride (97%) was gained from the Aladdin Industrial Corporation (China). Pyrilamine maleate (>97%), 1-methyl-4-phenylpyridinium iodide (MPP^+, ≥98%), and diphenhydramine hydrochloride (>98%) were from Wako Pure Chemical (Japan), Sigma-Aldrich (China), and Tokyo Chemical Industry Co., Ltd. (Japan), respectively. Solutol HS 15 was provided by Yunhong Chemical Co., Ltd. (China). Polysorbate 20, polysorbate 40, and polysorbate 60 were from Sigma-Aldrich (China). Polysorbate 80 was purchased from Aladdin (China). Poly(ethylene glycol) (PEG, average M.W. = 2000, 4000, 6000, 20 000) was provided from J & K Scientific Ltd. (China). Poly(ethylene glycol) (PEG, average M.W. = 400) was from Lingfeng Chemical Reagent Co. Ltd. (China). Meglumine, chitosan lactate, and crospovidone (PVPP) were obtained from Libang Pharmaceutical Co., Ltd. (China), AK Biotech Ltd. (China), and Chineway Pharma Tech Co., Ltd. (China), respectively. All of the other solvents and chemicals used were of analytical grade and were commercially available.

Cell Culture. The Caco-2 cells were purchased from the American Type Culture Collection (ATCC) and cultured in the Minimum Essential Medium (MEM) (KeyGen, China) supplemented with 10% fetal bovine serum (Wisent Inc., Canada) and 1% nonessential amino acid (Gibco, USA). The cells were maintained at 37 °C in an incubator (Thermo Fisher Scientific, USA) with 95% humidity and 5% CO_2. The culture medium was changed every 2 days.

Animals. Male ICR mice weighing 18−22 g, purchased from the Comparative Medicine Center of Yangzhou University (China), were housed in a controlled temperature (22 ± 2 °C) and humidity (50 ± 5%) with a 12 h light/dark cycle with *ad libitum* access to food and water. The study was conducted following the protocols approved by the Experimental Animal Ethical Committee of China Pharmaceutical University, in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals of China.

Cytotoxicity in Caco-2 Cells. Cytotoxicity was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, Caco-2 cells were subcultured on the collagen-I-coated 96-well culture plates (Jet Bio-Fil, China) at a density of 1 × 10^4 cells/well. At 80−90% confluence, the cells were exposed to each excipient or inhibitor for 2 h at 37 °C, washed twice with phosphate buffer saline (PBS), and incubated with 100 μL of culture medium and 20 μL of MTT solution (5 mg/mL) for 4 h at 37 °C. After the solution was discarded immediately, 150 μL of DMSO was added to each well to dissolve the formazan crystals. The plate was shaken for 10 min, followed by the detection of absorbance at 570 nm using an Infinite F50 microplate reader (TECAN, Switzerland).

Uptake Studies in Caco-2 Cells. The experiments were performed as described previously,[28,29] with some modifications. Caco-2 cells were subcultured on collagen-I-coated 12-well plates (Jet Bio-Fil, China) at a density of 1 × 10^5 cells/well. At 80−90% confluence, the cells were washed and preincubated with HBSS buffer (1.26 mM $CaCl_2$, 0.493 mM $MgCl_2$, 0.407 mM $MgSO_4$, 5.33 mM KCl, 0.441 mM KH_2PO_4, 137.93 mM NaCl, 0.338 mM Na_2HPO_4, 10 mM HEPES, 4.17 mM $NaHCO_3$, 5.56 mM glucose, and 1 mM Na-pyruvate, pH 7.4, unless otherwise specified) for 30 min at 37 °C. Then, cells were incubated with buffer containing the specific substrate (MTF, 300 μM unless otherwise specified; pyrilamine or MPP^+, 150 μM) in the absence and presence of the individual excipient or transporter inhibitor at 37 °C. The concentrations of each excipient were based on the FDA inactive ingredient database, articles, and cytotoxicity results: Solutol HS 15 = 0.35 mg/mL;[33] polysorbate 40,[33] polysorbate 60,[33] and PVPP = 1 mg/mL; polysorbate 20,[12,34,35] polysorbate 80,[34,35] and chitosan lactate = 0.1 mg/mL; PEG 400, PEG 2000, PEG 4000, PEG 6000, PEG 20 000 = 2 mg/mL;[33] meglumine = 0.2 mg/mL.[33] The concentrations of transporter inhibitors were as follows: pyrilamine, diphenhydramine, amantadine, cimetidine, tetraethylammonium (TEA), MPP^+, L-carnitine = 1 mM; ergothioneine, verapamil = 0.5 mM; pyrimethamine = 0.1 mM. After 90 min, unless otherwise specified, the cells were washed three times with the ice-cold buffer to terminate the uptake and stored at −80 °C until analysis. The cells were homogenized with 300 μL of water containing 10% DMSO, followed by the HPLC analysis. The protein content was analyzed using a BCA protein assay kit (KeyGen, China). The uptake of MTF was expressed as the cell-to-medium ratio (mL/mg of protein) obtained by dividing the uptake amount of MTF (nmol/mg of protein) by the concentration of MTF in the incubation buffer (μM). The uptake (% of control) of each sample was normalized by that of the control.

To study the concentration-dependence of MTF uptake, concentrations of 25−6000 μM was analyzed, and the results were fitted using eq 1 by the nonlinear least-squares regression analysis (Graphpad Prism, USA).

$$V = V_{max1}*S/(K_{m1} + S) + V_{max2}*S/(K_{m2} + S) + PS_{dif}*S \quad (1)$$

where V, V_{max1}, and V_{max2} are the uptake rate and the maximum uptake rate for two saturable components (nmol/min/mg of protein), respectively; S is the MTF concentration in the incubation buffer (μM); K_{m1} and K_{m2} are the Michaelis

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Table 1. Primers for RT-qPCR

target	sense	antisense
β-actin	GACTACCTCATGAAGATCCTCACC	TCTCCTTAATGTCACGCACGATT
OCT1	ACGGTGGCGATCATGTACC	CCCATTCTTTTGAGCGATGTGG
OCT2	AGACAGTGTAGGCGCTACGA	GTTAAACTCGGTGACGATGGAC
OCT3	ATCGTCAGCGAGTTTGACCTT	ACCTGTCTGCTGCATAGCCTA
OCTN1	TGGTAGCCTTCATACTAGGAACA	TGGCAGCAGCATATAGCCAAC
OCTN2	TCCACCATTGTGACCGAGTG	ACCCACGAAGAACAAGGAGATT
MATE1	TCAACCAGGGAATTGTACTGC	CAGAGCCTATCACCCCAAGA
MATE2	TGGGGCATATTTTTACCAATG	GAACTCCGCCATAGACACAAC
PMAT	GCTTTCACGGATACTACATTGGA	ATGTCAAACACGATGGAGGTC
SERT	ACGGAGTTCTACAGAAGGTTGT	ATAGAGTGCCGTGTGTCATCT
CHT	ATCCCAGCCATACTCATT	CAGAAACTGCACCAAGACCA
THTR-2	TTCTCCATGATGAGACCCTC	ATGATGACTGGCTTGTAGCG

Table 2. HPLC Chromatographic Conditions

parameters	MTF	pyrilamine	MPP^+
column	Zorbax 300-SCX (4.6 × 250 mm, 5 μm)	Hedera ODS-2 (4.6 × 250 mm, 5 μm)	Hedera ODS-2 (4.6 × 250 mm, 5 μm)
column temperature (°C)	40	25	35
mobile phase	1.7% ammonium dihydrogen phosphate solution (pH 3.0)−acetonitrile (75:25)	0.1 M potassium dihydrogen phosphate (pH 4.6)−acetonitrile (70:30)	0.1 M acetic acid (adjusted to pH 5.6 with triethylamine)−acetonitrile (10:90)
flow rate (mL/min)	0.8	0.6	0.8
detection wavelength (nm)	240	205	293

constants of two components (μM); PS_{dif} is the passive diffusion clearance (mL/min/mg of protein).

To verify the bidirectional transport of MTF, Caco-2 cells preincubated with MTF (300 μM) for 60 min were washed and exposed to the prewarmed blank buffer (pH 6.4 or 8.4). After 40 min, the cells were washed three times with the ice-cold buffer. In the *trans*-stimulation study, the cells were preincubated with a warm buffer with or without diphenhydramine[29,36] (0.5 mM) for 30 min. Following washing three times, the cells were incubated with MTF (300 μM) for 60 min.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis. The total RNA was isolated from Caco-2 cells using RNAiso Plus (Takara, Japan) and then reverse-transcribed with PrimeScript RT Master Mix (Perfect Real Time) (Takara, Japan). The qPCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., China). The primer sequences listed in Table 1 were purchased from TsingKe Biotech Co., Ltd. (China). The thermal protocol was set to 30 s at 95 °C, 40 cycles of 10 s at 95 °C, and then 30 s at 60 °C. The mRNA level of transporters was normalized with that of β-actin.

***Ex Vivo* Mice Non-everted Gut Sac Study.** The mice non-everted gut sac study was carried out as described previously,[37,38] with some modifications. The mice were fasted but allowed water *ad libitum* for 12 h prior to the experiment. After the mice were anesthetized with pentobarbital (50 mg/kg, i.p.), the duodenum was collected, washed with cold preoxygenated Tyrode buffer (136.9 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 4.2 mM NaH_2PO_4, and 15 mM glucose, pH 7.4), and cut into 5 cm for each sac after the mesenterium was removed. Following immersion in a preoxygenated buffer (pH 7.4) at room temperature for 5 min, one side of the sac was looped into a securely closed knot with a suture. Using a syringe with a blunt needle, 300 μL of warm buffer (pH 7.4 or 6.4) containing 0.75 mg/mL of MTF with or without selected compound (excipients = 10 mg/mL;[39] pyrilamine = 0.28 mg/mL) was injected into the sac. After the other side was tied and the surface was dried, each sac was placed into a tube containing 20 mL of Tyrode buffer (pH 7.4)[40,41] at 37 °C, shaken at 100 strokes/min, and constantly gassed with oxygen. Then, 0.5 mL of buffer outside of the sac was taken every 30 min for up to 120 min and replaced with 0.5 mLof fresh warm and preoxygenated buffer. The buffer samples were stored at −80 °C until the HPLC analysis. After the final sample collection, the sac was cut open and laid flat with the mucosal surface exposed to air. The length and width of each sac were measured with a ruler. The apparent permeability coefficient (P_{app}, cm/s) was calculated by eq 2.

$$P_{app} = dQ/dt/(A*60*C_0*1000) \quad (2)$$

where Q is the cumulative amount of MTF (μg), t is the sample collection time (min), A is the area of each sac (cm^2), and C_0 is the concentration of MTF in the incubation buffer (mg/mL).

Sample Preparation. For MTF cell homogenate analysis, 140 μL of the cell homogenate was mixed with 20 μL of phenformin solution (10 μM) as the internal standard and 40 μL of acetonitrile. For MTF *ex vivo* buffer sample analysis, 160 μL of *ex vivo* buffer sample was mixed with 20 μL of phenformin solution (50 μg/mL) and 20 μL of acetonitrile. The mixtures were vortexed for 3 min, followed by centrifugation at 14 000 rpm for 5 min. Then, 40 μL of the supernatant was subjected to the HPLC analysis.

For MPP^+ analysis, 140 μL of the cell homogenate was mixed with 20 μL of pyrilamine solution (180 μM) as the internal standard and 40 μL of acetonitrile. The mixture was

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vortexed for 3 min, followed by centrifugation at 14 000 rpm for 5 min. Then, 20 μL of the supernatant was subjected to the HPLC analysis.

For pyrilamine analysis, 140 μL of the cell homogenate was mixed with 20 μL of diphenhydramine solution (10 μM) as the internal standard, 20 μL of water, 500 μL of the carbonate buffer (pH 10.8), and 1 mL of ethyl acetate. The mixture was vortexed for 3 min, followed by the centrifugation at 14 000 rpm for 5 min. Aliquots of the supernatant (900 μL) were evaporated to dryness. The residue was reconstituted with 60 μL of the mobile phase, and 20 μL of the resulting solution was subjected to the HPLC analysis.

HPLC Analysis. The HPLC analysis was performed on a LC-20AT liquid chromatograph coupled with an SPD-20A detector (Shimadzu, Japan). The chromatographic conditions for MTF, pyrilamine, and MPP^+ are summarized in Table 2.

Statistical Analysis. Each data point was performed in triplicate in all experiments. Data are shown as mean ± SD. The data were analyzed by GraphPad Prism. Statistical significance was determined by Student's *t* test for a single comparison or one-way ANOVA followed by Dunnett's test for multiple comparisons. A *P*-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Effect of Excipients on MTF Uptake in Caco-2 Cells. During the long-term treatment with MTF, the patients are highly likely to receive multiple therapies, thus increasing the risk of MTF−excipient interactions. The interaction of excipients with influx transporters involved in the MTF absorption was investigated in the human intestinal Caco-2 cell line, which has been extensively used for the intestinal barrier.[42] In total, 13 excipients frequently used in many oral drug dosage forms were selected,[43−47] including ionic and nonionic excipients. The results of the *cis*-inhibitory effects of these excipients on the cellular uptake of MTF in Caco-2 cells are shown in Figure 1. When compared with meglumine and PVPP, Solutol HS 15, polysorbate 20, polysorbate 40, polysorbate 60, and polysorbate 80 showed more potent inhibitory effects on the cellular accumulation of MTF. By contrast, PEG 400, PEG 2000, PEG 4000, PEG 6000, PEG 20 000, and chitosan lactate did not decrease the MTF uptake.

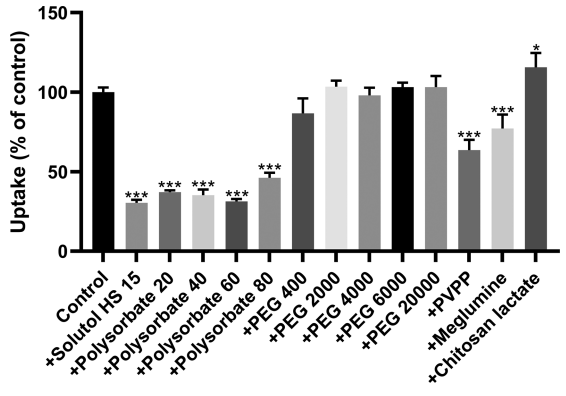

Figure 1. Effect of excipients on MTF uptake in Caco-2 cells. The uptake of MTF (300 μM) was measured at 37 °C after 90 min of incubation with or without the selected excipients. Values are represented as means ± SD (n = 3). *, P < 0.05 and ***, P < 0.001 versus control.

Solutol HS 15 is a nonionic surfactant and solubilizer with low toxicity,[48] which has been verified to inhibit the function of OCTs[34] and organic anion transporting polypeptides (OATPs).[49] Polysorbates are the widely used surfactants owing to the ability to improve the bioavailability, solubility, and permeability of certain drugs.[43] Increasing evidence demonstrated that polysorbates could modulate the function of BCRP,[14] OCTs,[34,35] and peptide transporters (PEPTs).[34] PVPP is a widely used disintegrating agent for tablets[50] and was reported to increase the absorptive transport of rhodamine 123 through the inhibition of P-gp.[51] Meglumine, a derivative of glucose, is usually used as a solubilizer and pH-adjusting agent.[52,53]

Transport Profile of MTF Uptake in Caco-2 Cells. To elucidate the underlying mechanisms of the inhibitory effects of the above excipients, it is important to understand the intestinal transport process of MTF. As shown in Figure 2A, MTF uptake in Caco-2 cells is time- and temperature-dependent. The uptake at 4 °C was significantly lower than that at 37 °C, illustrating that the transporter-mediated transport process plays a critical role in MTF uptake in Caco-2 cells.

As shown in Figure 2B, MTF uptake was concentration-dependent and composed of two saturable processes that were revealed by the two linear fittings in the Eadie−Hofstee plot. One showed high affinity (K_{m1} = 206.3 μM) and low capacity (V_{max1} = 0.0340 nmol/min/mg of protein), while the other showed low affinity (K_{m2} = 4.3 mM) and high capacity (V_{max2} = 0.571 nmol/min/mg of protein). The estimated PS_{dif} was negligible. Many studies ascribed the dose-dependent absorption of MTF to the involvement of promiscuous traditional cation-selective transporters. However, the reported affinities (K_m values) of hOCT1, hOCT2, hOCT3, hMATE1, hMATE2K, PMAT, SERT, and THTR-2 transporting MTF ranged from 0.9−3.1, 0.3−1.0, 2.3−3.6, 0.78 ± 0.1, 1.98 ± 0.48, 1.32, 4.0, and 1.15 ± 0.2 mM.[27,54] To our knowledge, this result unveiled for the first time that a transporter with high affinity also mediated the MTF uptake in Caco-2 cells.

Recently, studies of cationic drugs such as pyrilamine,[55,56] diphenhydramine,[57] verapamil,[58] oxycodone,[55] matrine,[28] and aconitum[29] led to the discovery of a novel pyrilamine-sensitive H^+/OC^+ antiporter with high affinity (K_m ranged from 7.2 to 249 μM). Hence, MTF is likely to be the substrate of the pyrilamine-sensitive H^+/OC^+ antiporter. To confirm this hypothesis, the driving force of MTF uptake in Caco-2 cells was investigated. MTF uptake was significantly decreased by a metabolic inhibitor NaN_3 (Figure 3A) and a protonophore FCCP (Figure 3B), indicating that the uptake of MTF needs energy and is driven by a proton electrochemical difference.

We further investigated the MTF uptake in Caco-2 cells at different extracellular pH values. As shown in Figure 3C, the uptake of MTF was significantly decreased at pH 6.4 but increased at 8.4. This result is consistent with the findings in the rat osteoblasts[59] but different from a report with Caco-2 cells,[60] in which pH 8.5 showed no significant effect on the uptake of either MTF or TEA, despite a slight tendency of increasing. Interestingly, the transport of TEA by OCT1 and OCT2 has been reported to be pH-dependent.[61] The possible reasons for this discrepancy can be explained by the different experimental conditions, such as the buffer composition, preincubation and incubation time, concentration, drug type (MTF versus [^{14}C]MTF), and detection method. Also, the comparison of Caco-2 cells from 10 different laboratories

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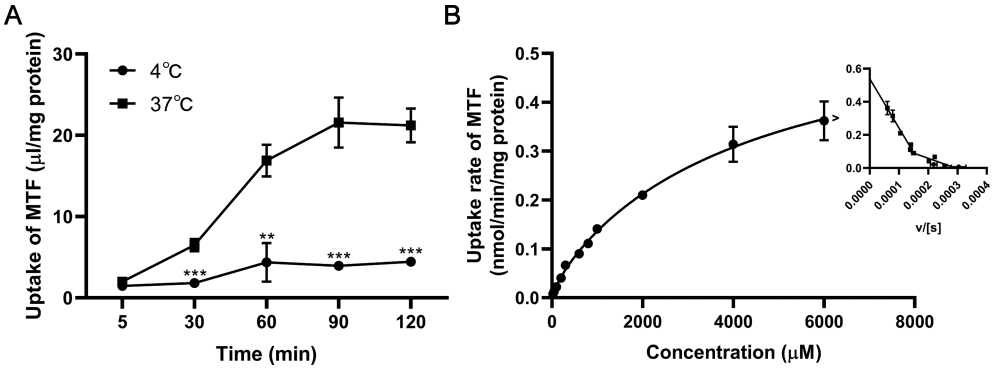

Figure 2. Time- and temperature-dependent uptake (A) and kinetic analysis (B) of MTF in Caco-2 cells. (A) The uptake of MTF (300 μM) was measured at 4 and 37 °C. (B) Concentration-dependent uptake (25−6000 μM) was measured at 37 °C for 90 min. The inset is the Eadie−Hofstee transformation. *V* and *S* represent the uptake rate (nmol/min/mg of protein) and concentration (μM). Values are represented as mean ± SD (n = 3). **, $P < 0.01$ and ***, $P < 0.001$ versus 37 °C.

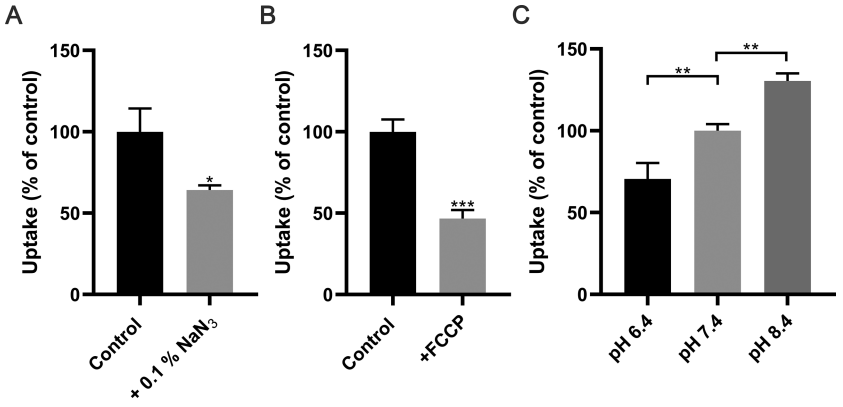

Figure 3. Effect of ATP depletion (A), protonophore (B), and extracellular pH (C) on MTF uptake in Caco-2 cells. The uptake of MTF (300 μM) was measured at 37 °C for 90 min with or without 0.1% NaN_3 (A) and 25 μM FCCP (B) or with a pH of 6.4, 7.4, and 8.4 (C). Values are represented as mean ± SD (n = 3). *, $P < 0.05$ and ***, $P < 0.001$ versus control; **, $P < 0.01$ versus pH 7.4.

revealed that even small differences in culture conditions had significant impacts on the gene expression of some transporters.[62] We thus profiled the expression of the traditional organic-cation-selective transporters, and the result is shown in Figure 4. Compared with the expressions of OCTN1, MATE1−2, and CHT, transporters including OCT1−3, OCTN2, PMAT, SERT, and THTR-2 were abundantly expressed in the Caco-2 cells.

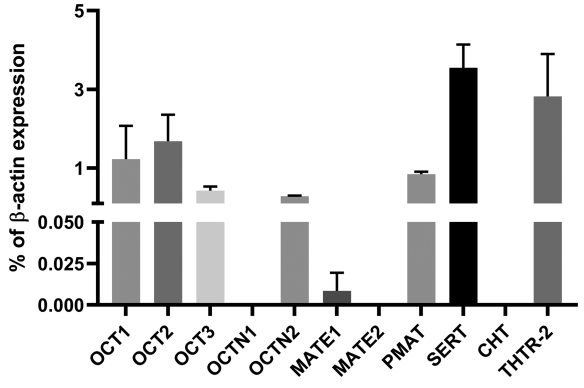

Figure 4. mRNA expression levels of traditional cation-selective transporters in Caco-2 cells. Values are represented as means ± SD (n = 4).

A *trans*-stimulation study was further used to verify the bidirectional transport of MTF in Caco-2 cells. The preloaded intracellular MTF underwent trans-stimulated efflux into the extracellular buffer of pH 6.4 as compared to 8.4 (Figure 5A). Meanwhile, preloaded intracellular diphenhydramine significantly trans-stimulated MTF uptake from the extracellular compartment (Figure 5B), confirming that MTF shares the same antiporter with diphenhydramine, at least in part.

Identification of the Involved Uptake Transporters for MTF in Caco-2 Cells. To unveil the intestinal uptake mechanism of MTF, a series of prototypical inhibitors of cation-selective transporters[28,29] was employed (Figure 6). The uptake was considerably decreased by all chemical inhibitors except L-carnitine and ergothioneine, which are the substrates of OCTN2 and OCTN1, respectively.[63,64] This result is consistent with the undetectable expression of OCTN1 in Caco-2 cells (Figure 4) and the previous report that MTF transport was not mediated by OCTN2.[26] Thus, OCTN1−2 are not involved in the cellular uptake of MTF.

Among the inhibitors of traditional cation-selective transporters, i.e., amantadine (inhibitor of OCT1 and OCT2),[65] cimetidine (inhibitor of OCT2 and MATEs),[66] TEA (inhibitor of OCTs, OCTNs, MATEs, and PMAT),[67,68] pyrimethamine (inhibitor of MATEs),[69] and MPP^+ (inhibitor of OCTs, MATEs, PMAT, CHT, SERT, and THTR-2),[26,27,70] MPP^+ was the most potent inhibitor, indicating that the total contribution of traditional cation-selective transporters ac-

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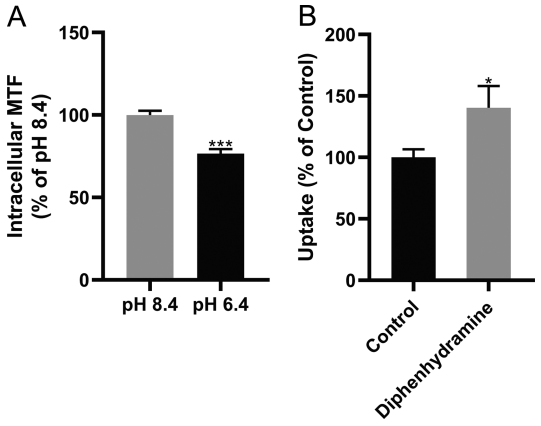

Figure 5. *Trans*-stimulation of MTF uptake in Caco-2 cells by pH (A) and diphenhydramine (B). As for pH, the Caco-2 cells were preincubated with MTF (300 μM, 60 min) and then exposed to blank buffer (pH 6.4 or 8.4) for 40 min. As for diphenhydramine, the Caco-2 cells were preincubated with diphenhydramine (0.5 mM) for 30 min before being exposed to MTF (300 μM) for 60 min. Values are represented as mean ± SD (n = 3). ***, P < 0.001 versus pH 8.4; *, P < 0.05 versus control.

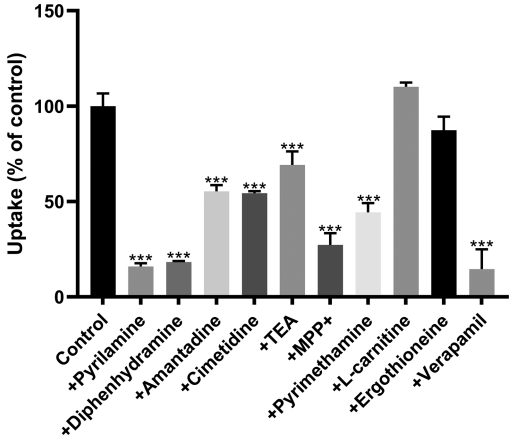

Figure 6. Effects of prototypical inhibitors on MTF uptake in Caco-2 cells. The uptake of MTF (300 μM) was measured at 37 °C for 90 min with or without each inhibitor. Values are represented as mean ± SD (n = 3). ***, P < 0.001 versus control.

counts for approximate 70% of MTF uptake. The inhibitors of pyrilamine-sensitive H^+/OC^+ antiporter like verapamil, diphenhydramine, and pyrilamine showed more potent inhibitory effects with an 82−85% reduction in MTF uptake. Verapamil is an inhibitor of OCTs, OCTNs, MATEs, THTR-2, and pyrilamine-sensitive H^+/OC^+ antiporter.[27,70−72] Diphenhydramine and pyrilamine could not only inhibit the pyrilamine-sensitive H^+/OC^+ antiporter[73] but also significantly inhibit the cellular uptake of MPP^+ (Figure 7A). To our knowledge, the inhibitory effect of pyrilamine on the uptake of MPP^+ has not been reported before. Hence, among the multiple cation-selective influx transporters involved in the gut absorption of MTF, the contribution of the pyrilamine-sensitive H^+/OC^+ antiporter exceeds 10%.

Effect of Nonionic Excipients on Pyrilamine and MPP^+ Uptake in Caco-2 Cells. To confirm whether the inhibitory effect of the above excipients was achieved by inhibiting the function of influx transporters involved, we further evaluated the effects of nonionic excipients on the cellular uptake of probe substrates of transporters (Figure 7). The uptake of MPP^+, a common substrate of OCTs, PMAT, MATEs, SERT, and THTR-2, was significantly inhibited by Solutol HS 15, polysorbate 40, polysorbate 60, and polysorbate 80. This result is consistent with previous reports that Solutol HS 15,[34] polysorbate 60,[35] and polysorbate 80[34,35] could inhibit the transport function of OCTs. Meanwhile, Solutol HS 15, polysorbate 20, polysorbate 40, and polysorbate 60 showed significant inhibitory activity on the uptake of pyrilamine, a probe substrate of the pyrilamine-sensitive H^+/OC^+ antiporter. Taken together, the suppressive effects of Solutol HS 15, polysorbate 40, and polysorbate 60 on MTF uptake were due to the synergistic inhibition toward multiple involved transporters. As emerging evidence showed that the pyrilamine-sensitive H^+/OC^+ antiporter played critical roles in the influx transport of many cationic therapeutic drugs, its potential interactions with excipients need to be further studied to ensure clinical efficacy and safety.

Cytotoxicity in Caco-2 Cells. The cytotoxicity was investigated to exclude the possibility that the inhibitory effects of the excipients and inhibitors were caused by their toxicity toward the cells. As shown in Figure 8, the viability of cells treated with each selected compound had no significant

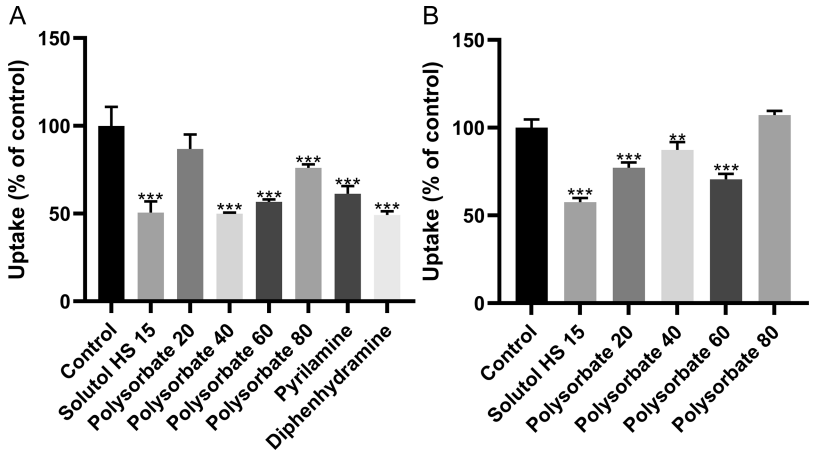

Figure 7. Effects of selected nonionic excipients or transporter inhibitors on MPP^+ (A) and pyrilamine (B) uptake in Caco-2 cells. The uptake of pyrilamine or MPP^+ (150 μM) was measured at 37 °C for 90 min with or without the test compound. Values are represented as mean ± SD (n = 3). **, P < 0.01 and ***, P < 0.001 versus control.

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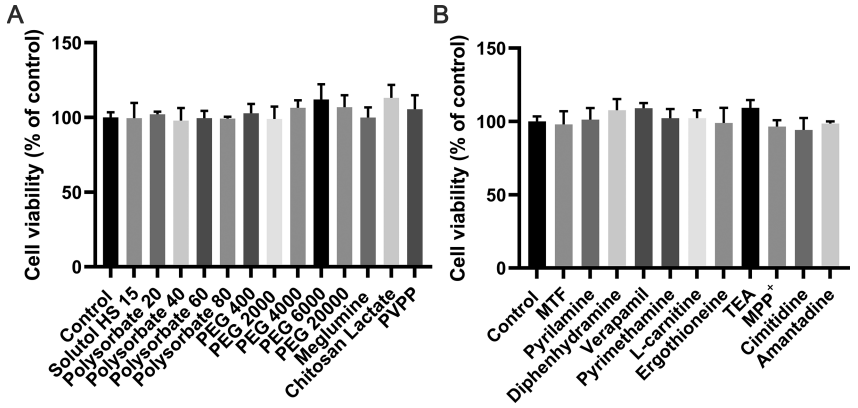

Figure 8. Effects of excipients (A) and transporter inhibitors (B) on the viability of Caco-2 cells. Cells were incubated with buffer containing excipients or inhibitors at 37 °C for 2 h. Values are represented as mean ± SD (n = 3).

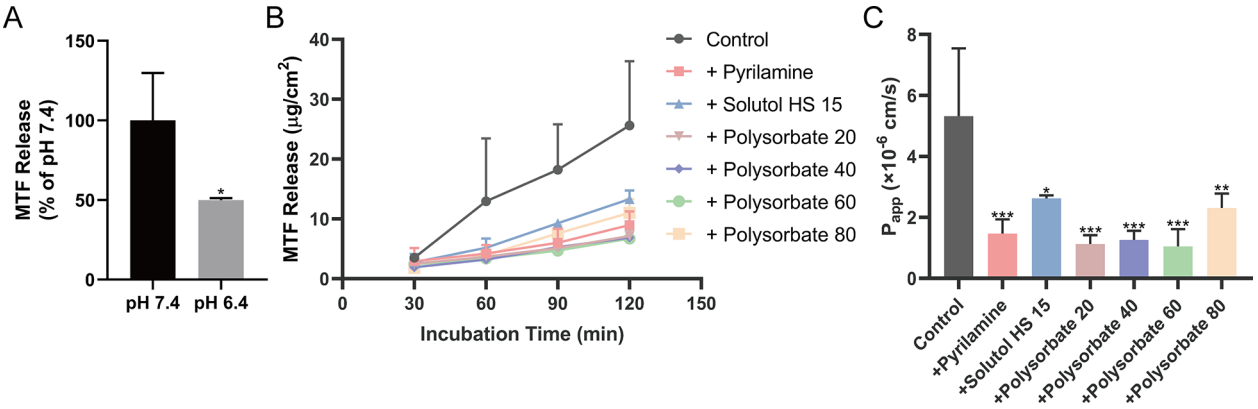

Figure 9. Effects of luminal buffer pH (A) and selected compounds on MTF release (B) and P_{app} (C). The uptake of MTF (0.75 mg/mL) was measured at 37 °C with or without selected compounds. Values are represented as mean ± SD (n = 3). *, P < 0.05 versus pH 7.4; *, P < 0.05, **, P < 0.01, and ***, P < 0.001 versus control.

difference when compared with that of the control group, suggesting that MTF, excipients, and inhibitors had no cytotoxic effect at each investigated concentration on Caco-2 cells.

Effect of Selected Nonionic Excipients on *Ex Vivo* Intestinal Absorption of MTF. In healthy subjects, the gastrointestinal pH changes from highly acidic conditions in the stomach to about pH 6 in the duodenum, pH 7.4 in the terminal ileum, pH 5.7 in the cecum, and pH 6.7 in the rectum.[74] Thus, the effect of the pH was studied with the *ex vivo* mouse non-everted gut sac model. The acidic luminal buffer (pH 6.4) significantly decreased the mouse intestinal absorption of MTF (Figure 9A), which is consistent with the Caco-2 result. Therefore, the intestinal absorption of MTF may vary in different gastrointestinal sections, which should be taken into consideration during the formulation design and clinical application.

Then, the potential effects of the nonionic excipients on the intestinal absorption of MTF were characterized. The time course of absorptive transport of MTF across the duodenum segments and the apparent permeability coefficients are shown in Figure 9B,C. Consistent with the Caco-2 results, Solutol HS 15, polysorbate 20, polysorbate 40, polysorbate 60, and polysorbate 80 significantly decreased the gut absorption of MTF in mice. As the inhibition potencies of polysorbates were similar to that of pyrilamine, the effects of these excipients on other cationic drugs warranted further clarification.

Besides the inhibition of the transcellular transport mediated by the cation-selective transporters, another possible reason is the decreased paracellular transport. It was postulated that the contributions of the paracellular of MTF were much larger than those of the transcellular transport in a kinetic model.[75] The saturable paracellular transport of MTF caused by the electrostatic interactions between MTF and the tight junction proteins, claudins, in particular claudin-2,[75] may be altered by the excipients.

Interestingly, it was previously reported that polysorbate 20 markedly increased MTF transport across Caco-2 monolayers in a concentration-dependent manner, accompanied by increased cell damages.[76] This contradictory result may be caused by the difference between the toxic sensitivity of the two-dimensional cultured Caco-2 monolayer and the *ex vivo* mouse intestine as well as the species difference between human and mouse.

■ CONCLUSION

The present study demonstrates that a novel transport process mediated by the pyrilamine-sensitive H^+/OC^+ antiporter contributes to the intestinal absorption of MTF in conjunction with the traditional OCTs, PMAT, MATEs, SERT, CHT, and THTR-2. The intestinal transport of MTF was markedly reduced by the nonionic excipients, among which the inhibitory effects of Solutol HS 15, polysorbate 40, and

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polysorbate 60 are due to the synergistic dysfunction of the multiple transporters.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

API, active pharmaceutical ingredient; BCRP, breast cancer resistance protein; CHT, choline high-affinity transporter; DDI, drug−drug interaction; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; HPLC, high-performance liquid chromatography; MATE, multidrug and toxin extrusion transporter; MPP^+, 1-methyl-4-phenylpyridinium; MRP2, multidrug resistance-associated protein 2; MTF, metformin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; OCTN, organic cation/carnitine transporter; PBS, phosphate buffer saline; PEG, poly(ethylene glycol); PEPT, peptide transporter; P-gp, P-glycoprotein; PMAT, plasma membrane monoamine transporter; PVPP, crospovidone; RT-qPCR, real-time quantitative polymerase chain reaction; SERT, serotonin reuptake transporter; TEA, tetraethylammonium; THTR-2, thiamine transporter-2

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