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Enhanced and Persistent Inhibition of Organic Cation Transporter 1 (OCT1) Activity by Preincubation of Cyclosporine A

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Running title: Preincubation effects on inhibition of OCT1 by CsA

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Nonstandard Abbreviations: *AUC*, area under the plasma concentration-time curve; BCRP, breast cancer resistance protein; Cyclosporine A, CsA; CYP, Cytochrome P450; DDI, drug-drug interaction; FDA, Food and Drug Administration; f_T, fraction transported; f_u, unbound fraction in plasma; HEK293, human embryonic kidney 293 cells; IC_{50}, concentration required to inhibit transport by 50%; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MATE, multidrug and toxin extrusion protein; NME, new molecular entity; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; P-gp, P-glycoprotein; SD, standard deviation.

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ABSTRACT

Recent pharmacogenetic evidence indicates that hepatic organic cation transporter 1 (OCT1) can serve as the locus of drug-drug interactions (DDIs) with significant pharmacokinetic and pharmacodynamic consequences. We examined the impact of preincubation on the extent of OCT1 inhibition in transfected human embryonic kidney 293 (HEK293) cells. Following a 30 minute preincubation with an inhibitor, approximately 50-fold higher inhibition potency was observed for Cyclosporine (CsA) against OCT1-mediated uptake of metformin as compared to coincubation, with IC_{50} values of 0.43 ± 0.12 and 21.6 ± 4.5 μM, respectively. By comparison, only small shifts ($\leq$ 2-fold) in preincubation IC_{50} versus coincubation were observed for quinidine, pyrimethamine, ritonavir, and trimethoprim. The shift in CsA OCT1 IC_{50} is substrate-dependent as it ranged from > 1.2 fold to 50.2-fold using different experimental substrates. The inhibition potential of CsA towards OCT1 is confirmed by fenoterol hepatocyte uptake experiment. Furthermore, no shift in CsA IC_{50} was observed with HEK293 cells transfected with OCT2 and organic anion transporter (OAT) 1 and OAT3. A short (30 min) exposure to 10 μM CsA produced long-lasting (at least 120 min) inhibition of the OCT1-mediated uptake of metformin in OCT1-HEK293 cells, which was likely attributable to the retention of CsA in the cells, as shown by the fact that inhibitory cellular concentrations of CsA are maintained long after the removal of the compound from incubation buffer. The potent and persistent inhibitory effect after exposure to CsA warrants careful consideration in the design and interpretation of clinical OCT1 DDI studies.

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SINIFICANCE STATEMENT

Preincubation of OATP1B1 and OATP1B3 with their inhibitor may result in the enhancement of the inhibitory potency in a cell-based assay. However, limited data are available on potentiation of OCT1 inhibition by preincubation, which is a clinically relevant drug transporter. For the first time, we observed a 50-fold increase in CsA inhibitory potency against OCT1-mediated transport of metformin following a preincubation step. The CsA preincubation effect on OCT1 inhibition is substrate-dependent. Moreover, the inhibition potential of CsA towards OCT1 is confirmed by hepatocyte uptake experiment. This study delivers clear evidences about the potent and persistent inhibitory effect on OCT1 after exposure to CsA. Further studies are needed to assess the effect of CsA on OCT1 drug substrates in vivo.

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INTRODUCTION

The organic cation transporter 1 (OCT1, SLC22A1) is an emerging transporter of clinical importance due to its involvement in the hepatic uptake and clearance of many currently prescribed drugs (Zamek-Gliszczynski et al., 2018a, Zamek-Gliszczynski et al., 2018b). Being a solute carrier protein expressed at the blood-facing membrane of hepatocytes, OCT1 is involved in the transport of more than 120 cationic compounds from the blood into hepatocytes, which include platinum drugs (cisplatin and oxaliplatin), H-2 receptor antagonists (cimetidine, famotidine, ranitidine, and nizatidine), antibiotics (pentamidine, sulpiride, and prothionamide), kinase inhibitor agents (imatinib, pazopanib, and nilotinib), and biguanide agents (metformin and phenformin) (The University of Washington Metabolism and Transport Drug Interaction Database). The perturbation of OCT1 activity by concomitant medications, along with genetic and disease alterations, could lead to inter-individual variability in treatment response and even adverse reactions. For example, the genetic deficiency of OCT1 resulted in a 1.9-fold increase in the systemic exposure of fenoterol (Tzvetkov et al., 2018). As a result, more severe cardiovascular and metabolic adverse reactions, including 1.5- and 3.4-fold increases in heart rates and blood glucose levels, respectively, were observed in the individuals carrying inactive OCT1 genotypes (i.e., OCT1*3, OCT1*4, OCT1*5, and OCT1*6) (Tzvetkov et al., 2018). Because of the rising importance of OCT1 in drug hepatic uptake and clearance, the assessment of the inhibition potential of a new chemical entity towards OCT1 was recently recommended as a part of the drug discovery and development process by the International Transporter Consortium (ITC) (Zamek-Gliszczynski et al., 2018a, Zamek-Gliszczynski et al., 2018b).

CsA, a large lipophilic cyclic polypeptide with unexpectedly high membrane permeability, is used for preventing graft rejection following solid organ transplantation and bone marrow transplantation (Faulds et al., 1993). It is also used for the treatment of patients with severe psoriasis and rheumatoid arthritis. CsA exhibits solvent-dependent conformation and hydrogen-bonding capacity, from an apolar solvent where CsA is internally hydrogen-bonded to polar solvent where it exposes its hydrogen-bonding groups to the solvent (Kessler et al., 1990, el Tayar et al., 1993, O'Donohue et al., 1995). The interconversion process is slow on the molecular dynamics time scale (el Tayar et al., 1993), Furthermore, CsA is well known for a relatively high incidence of pharmacokinetic DDIs as a perpetrator of transporters and drug metabolizing enzymes (Armstrong et al., 2002, Gertz et al., 2013). CsA decreases the clearance of statins (atorvastatin, simvastatin, rosuvastatin, and pitavastatin) and antivirals (daclatasvir, danoprevir, letermovir, and velpatasvir) by reducing OATP1B1 and OATP1B3 activity. In addition, CsA increases the oral

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absorption systemic exposures of several taxanes (docetaxel and paclitaxel) and anthracyclines (doxorubicin and idarubicin) by inhibiting P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). Large inter-laboratory variability in transporter IC_{50} values of CsA is observed even for data obtained from the same transfected cell model (Gertz et al., 2013, Vaidyanathan et al., 2016).

CsA is a preincubation time-dependent inhibitor of OATP1B1 and OATP1B3 because the IC_{50} values of CsA are approximately 5- to 22-fold lower after a 20- to 60-minute preincubation compared to coincubation (Amundsen et al., 2010, Shitara et al., 2012, Gertz et al., 2013, Shitara et al., 2013, Izumi et al., 2015). The IC_{50} values after preincubation are closer to the *in vivo* transporter inhibition rate constant estimated by fitting clinical OATP1B DDI data to a physiologically-based pharmacokinetic (PBPK) model (Gertz et al., 2013, Shitara and Sugiyama, 2017). Furthermore, the inhibitory effect of CsA against OATP1B1 and OATP1B3 persisted for at least 90 min after CsA was removed from the incubation buffer (Oh et al., 2018). In agreement, CsA exhibited a lasting OATP inhibitory effect *in vivo*. When 15 mg/kg CsA was administered subcutaneously to rats 21 hours before intravenous administration of sulfobromophthalein, a substrate for Oatps, it increased the area under the plasma concentration-time curve (*AUC*) of sulfobromophthalein 2.7-fold (Shitara et al., 2009). Taken together, these results indicate the time-dependent inhibition of hepatic OATP1B1 and OATP1B3, although the underlying mechanism(s) is unknown. In the current drug-drug interaction draft guideline (https://www.fda.gov/downloads/Drugs/Guidances/UCM581965.pdf), the FDA recommended assessing *in vitro* OATP1B1 and OATP1B3 inhibition potency following a preincubation step to reduce false negative prediction rates using the mechanistic static model approach (Vaidyanathan et al., 2016). However, the preincubation-dependent inhibitory effect towards other transporters including OCT1 remains uncharacterized.

The current study aimed to investigate the time-dependence of the inhibition potential of CsA and other inhibitors towards OCT1-mediated transport of metformin. The preincubation effect of CsA on OCT1 inhibition was further investigated by using other experimental substrates including 1-methyl-4-phenylpyridinium (MPP^+), tetraethylammonium (TEA), ranitidine, sumatriptan, and cycloguanil. In addition, *in vitro* inhibitory potency and time-dependence of CsA against other key drug uptake transporters, including OCT2, OAT1, and OAT3, were investigated. The time course of cellular accumulation and efflux of CsA in stably transfected human embryonic kidney 293 (HEK293) cells expressing OCT1- and Mock-HEK293 were determined in order to elucidate the mechanism of significantly increased inhibition potency of CsA after preincubation observed for OCT1.

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MATERIALS AND METHODS

Chemicals. CsA, CsA-13C2, d4, quinidine, pyrimethamine, ritonavir, trimethoprim, probenecid, ranitidine, ranitidine-d6, fenoterol, fenoterol-d6, sumatriptan, sumatriptan-d6, and cycloguanil, and cycloguanil-d4 were obtained from Toronto Research Chemicals Inc. (North York, ON). [^{14}C]Metformin was obtained from Moravek Biochemicals and Radiochemicals (Brea, CA). [^{3}H]1-Methyl-4-phenylpyridinium ([^{3}H]MPP^{+}; 107 Ci/mmol), [^{14}C]tetraethylammonium ([^{14}C]TEA; 3.2 mCi/mmol), [^{3}H] *p*-aminohippuric acid, and [^{3}H]estrone-3-sulfate were purchased from PerkinElmer Life (Downers Grove, IL). Radiochemical purity of all compounds was >97% as determined by high-performance liquid chromatography (HPLC). EcoLite (+) liquid scintillation fluid was obtained from MP Biomedicals (Solon, OH). The protein quantitation assay kit was purchased from Pierce (Rockford, IL). Dulbecco's modified Eagle medium (DMEM), hygromycin, Hank's balanced salt solution, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and fetal bovine serum were obtained from Corning (Manassas, VA). Methanol, acetonitrile, and water were of analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ). Cryopreserved human primary hepatocytes were purchased from Celsis *In Vitro* Technologies (Baltimore, MD). All other chemicals were obtained from standard sources.

Preparation and Cell Culture of Human OCT1-, OCT2-, OAT1-, and OAT3-Expressing Cells. The stably transfected OCT1-HEK293, OCT2-HEK293, OAT1-HEK293, OAT3-HEK293, and Mock-HEK293 cell lines were previously developed (Han et al., 2010b, Han et al., 2010a). HEK293 cells were routinely grown in 75-cm^2 culture flasks (BD Biosciences, Franklin Lakes, NJ) in a humidified incubator at 37°C and 5% CO_2 in DMEM containing 10% (vol/vol) fetal calf serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 µg/mL hygromycin B, and 100 U/ml penicillin and streptomycin. To prepare cell monolayers for transporter experiments, after wash and detachment steps, the cells were seeded on 24-well poly-D-lysine-coated Biocoat plates (BD Biosciences, Franklin Lakes, NJ) at a density of 500,000 cells/well. Cells were grown at 37°C with 5% CO_2 in the DMEM cell culture medium and were ready to use 2 or 3 days after seeding.

Inhibition of Uptake into Human OCT1-, OCT2-, OAT1, and OAT3-Expressing Cells. To assess inhibition potential of CsA and other compounds towards OCT1, OCT2, OAT1, and OAT3, individual radiolabeled probe substrates plus testing compound at various concentrations were freshly prepared in Hanks' balanced salt

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solution (HBSS) buffer supplemented with 10 mM HEPES (HBSS-HEPES) (pH 7.4) solution and kept at 37°C. The cell monolayers were washed twice with 2 mL of pre-warmed HBSS and preincubated with the HBSS-HEPES buffer containing testing compound at various concentrations for 30 min. A 30 min preincubation period is the standard time used in the protocol for assessment of inhibition of OATP1B1 and OATP1B3. Two-hundred (200) µL of the incubation solutions containing probe substrate plus testing compound were added to cell monolayers to initiate the incubation. The probe substrates include 2 µM [^{14}C]metformin (OCT1 and OCT2), 1 µM [^{3}H]MPP^{+} (OCT1), 2 µM [^{14}C]TEA (OCT1), 1 µM ranitidine (OCT1), 1 µM fenoterol (OCT1), 1 µM sumatriptan (OCT1), 1 µM cycloguanil (OCT1), 1 µM [^{3}H] *p*-aminohippuric acid (OAT1), and 1 µM [^{3}H]estrone-3-sulfate (OAT3). Incubation proceeded for 2 min at 37°C. After 2 min, uptake buffer was then removed and the cell monolayers were immediately washed three times with 1.5 mL ice-cold HBSS buffer to terminate the uptake process. To analyze the concentrations of radiolabeled compounds, cells in the dried plate were lysed with 300 µL 0.1% Triton-X 100. Aliquots of 200 and 20 µL cell lysate were used for radioactivity counting and protein concentration analysis, respectively. After the addition of 5 mL of scintillation cocktail, radioactivity was measured on a dual-channel liquid scintillation counter (Tri-Carb 3100TR liquid scintillation counter; PerkinElmer Life Sciences, Boston, MA). Cellular protein content was determined using a protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. Cellular uptake was normalized based on the protein amount in each well. To analyze the concentrations of nonradiolabeled compounds, cells in the dried plate were lysed in 300 µL methanol and water (3:1, volume/volume) with fenoterol-d6, ranitidine-d6, sumatriptan-d6, and cycloguanil-d4 [internal standard (IS)]. The concentration of ranitidine, fenoterol, sumatriptan, and cycloguani in the cell lysates was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Inhibition of Fenoterol Uptake Clearance in the Presence of CsA in Human Hepatocytes. Cryopreserved human hepatocytes from 3 male donors were thawed according to the manufacturer's protocol, and the cells were pooled in Krebs-Henseleit buffer to give a cell density of 2,000,000 viable cells per milliliter, which was determined by trypan blue staining. After pre-warmed in a water bath at 37°C for 3 min, hepatocytes were preincubated with Krebs-Henseleit buffer with or without an inhibitor for 60 min. Uptake experiment was initiated by adding an equal amount of fenoteroln solution into the cell suspension at 37°C, resulting in a final cell density of 1,000,000 viable cells per milliliter and 1 µM fenoterol with or without inhibitor. A 100 µL aliquot of cell suspension was then transferred

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into centrifuge tubes containing a top layer of 100 µL oil mixture (silicone oil and mineral oil at density of 1.015 g/mL) and a bottom layer of 50 µL 2 M ammonium acetate (pH 4.8) at time points of 0.25, 1, and 1.5 min. The tubes were immediately centrifuged at 10,000 x g for 10 sec at room temperature in a tabletop centrifuge to spin down the hepatocytes through the oil layer into the ammonium acetate solution after transferring. The centrifuge tubes were placed on dry ice and the frozen bottom layer containing the cell pellet was then cut. The cell pellets were lysed and extracted with acetonitrile and water (2:1, volume/volume) containing fenoterol-d6 at room temperature for one hour. The lysates were filtered through a 96-well filter plate, and the filtrate was then dried under nitrogen gas. Finally, the dried contents were reconstituted in 100 µL of water for LC-MS/MS analysis.

Uptake and Efflux of CsA in Human OCT1-HEK293 and Mock-HEK293 Cells. CsA uptake and efflux studies were performed in human OCT1-expressing cells and control cells. The uptake of CsA was studied in a manner similar to that described above for the transporter inhibition studies. Cell monolayers were washed twice with 2 mL pre-warmed HBSS and were then incubated with HBSS-HEPES buffer containing 1 and 10 µM CsA for 0, 2, 5, 15, 30 and 60 min. At the end of the incubation period, cells were washed three time with 1.5 mL ice-cold HBSS buffer.

After drying the plate at room temperature overnight, cells were solubilized and lysed with 300 µL of a solution of methanol/water (75%:25% vol/vol) containing CsA-13C2,d4 (50 nM) as the internal standard. Cells were lysed by shaking the plate for 60 min. A 200 µL aliquot of each cell lysate sample was added into an individual well of a 96 well filter plate. A 96-deep- well receiving plate was placed under the filter plate. Both plates were centrifuged at 3,700 rpm for 15 min. The contents in each well of the receiving plate were dried under nitrogen, and were then reconstituted in 100 µL of a solution of water/acetonitrile (75%:25% vol/vol). Working stock solutions for the CsA standard curves in acetonitrile/water (75%:25% vol/vol) at 0, 2.74, 8.23, 24.7, 55.6, 74.1, 222, 667, and 2000 nM were utilized. Thirty microliters of each standard 10X solution was spiked into individual wells of a washed cell plate. The contents of the plate were dried and then 300 µL of methanol/water (75%:25% vol/vol) with CsA-13C2,d4 was added to each well. The plate was then processed following the same procedure described for study samples. An aliquot of each sample and standard (20 µL) was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). A cell volume of 1.4 µL/million cells was applied to calculate the intracellular concentration of CsA in the uptake and efflux study (Poirier et al., 2008),

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In the second set of experiments, OCT1-HEK293 and Mock-HEK293 cells were incubated with HBSS-HEPES containing 10 μM CsA for 30 min. The cell monolayers were washed three times with 1.5 mL warm HBSS buffer and then placed into 200 μL of fresh HBSS-HEPES buffer. At designated times, 150-μL aliquots of buffer were taken and CsA was measured in the buffer by LC-MS/MS. At the collection time, cell monolayers were washed and then measured for remaining drug using LC-MS/MS.

LC-MS/MS Analysis of CsA, Ranitidine, Fenoterol, Sumatriptan, and Cycloguanil in Cell Lysates and Buffer.

A high-performance liquid chromatography (HPLC)-mass spectrometer system, which consisted of two Nexera X2 LC-30AD pumps (Shimadzu scientific Instruments, Inc., Kyoto, Japan), a Nexera X2 SIL LC-30AC autosampler (Shimadzu scientific Instruments, Inc., Kyoto, Japan), and a triple quadrupole 6500 mass spectrometer (AB SCIEX, Concord, Ontario, Canada), was used for LC/MS/MS analysis. The analytical column was Waters UPLC Protein BEH C4 (2.1 ×150mm, 1.7 μm) (CsA) or Waters UPLC HSS T3 (2.1 ×50 mm, 1.8 μm, Waters, Milford, MA, USA) (ranitidine, fenoterol, cycloguanil, and sumatriptan). The mobile phase consisted of 0.1% of formic acid in water (A) and 0.1% formic acid in methanol (CsA) or acetonitrile (ranitidine, fenoterol, cycloguanil, and sumatriptan) (B). For CsA, the initial condition was 45 % B, ramped up to 98% B in 4.00 minutes, further ramped to 99% in 0.5 minutes before going back to initial condition for a total run time of 6.5 minutes at a flow rate of 0.5 mL/min. For fenoterol and cycloguanil, the LC gradient started at 5% B, ramped up to 50% B in 1.5 minutes, further ramped up to 95% to flush the column then going back to initial condition for a total run time of 2.5 minutes at a flow rate of 0.6 mL/min. For ranitidine and sumatriptan, the LC conditions were essentially the same as those for cycloguanil, except that the initial ramp to 50% B took only 1.4 minutes.

The LC eluent was introduced into the mass spectrometer in positive electrospray ionization mode with source temperature at 450°C and ion spray voltage at 4500 volts. The multiple reaction monitoring transitions used were: m/z 1202.5 → 1184.6 for CsA, m/z 314.7 → 101.9 for ranitidine, m/z 304.0 → 106.9 for fenoterol, m/z 296.0 → 58.0 for sumatriptan, and m/z 251.9 → 58.0 for cycloguanil, respectively. All analytes were quantified within the Analyst software with calibration curves for the mass spec peak area ratio to their respective stable isotope labeled internal standards.

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Data Analysis. Experiments were performed at least three times in triplicate. The net transport of probe substrate by a transporter was calculated after subtracting the uptake in mock-transfected cells from the total uptake in the transporter-expressing cells. IC_{50} values, the concentration of CsA or other compounds required for 50% inhibition of transport of probe substrate, were calculated with WinNonlin (Pharsight Inc.; Mountain View, CA) using the following equation:

$$V = V_0 - \left(\frac{I_{\max} \bullet C^{\gamma}}{IC_{50}{}^{\gamma} + C^{\lambda}} \right)$$

where V is defined as the rate of probe transport measured at a given CsA concentration, γ is the slope factor, C is the CsA concentration, V_0 refers to the rate of probe transport measured in the absence of CsA, and I_{max} refers to the maximum inhibitory effect.

To test for statistically significant difference in persistent inhibitory effect of CsA on OCT1-mediated uptake of metformin among multiple time points, two-way analysis of variance was performed. When the F ratio showed that there were significant differences among days, the Tukey's method of multiple comparisons was used to determine which treatments differ. The two-sided paired Student's *t* test was used to assess the statistical significance of differences in time course of CsA uptake between OCT1- and Mock-HEK 293 cells. The same statistics test was used to evaluate the statistical significance of differences in time course of CsA efflux between OCT1- and Mock-HEK 293 cells. All statistical analyses were performed using Prism version 7.03 (GraphPad Software, Inc.; San Diego, CA). Results are presented as the mean ± standard deviation of the mean (SD). Differences were considered statistically significant when $p < 0.05$.

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RESULTS

Inhibitory Effect of CsA on OCT1. To determine whether the inhibitory effect of CsA was potentiated by preincubation, we investigated cellular uptake of metformin into OCT1-HEK293 cells under conditions of coincubation and preincubation, where preincubation was performed with HBSS-HEPES buffer containing CsA at various concentrations for 30 min. As shown in Figure 1A, the inhibition potency of CsA towards OCT1 was increased 50.2-fold with preincubation compared to coincubation (IC_{50} values of 0.43 ± 0.12 and 21.6 ± 4.5 μM, respectively) (Table 1), a finding consistent with CsA demonstrating 10.4- to 22-fold higher inhibitory capacity against OATP1B1 under preincubation conditions (Amundsen et al., 2010, Gertz et al., 2013). Specificity of the preincubation-dependent inhibition of CsA was examined using OCT2-, OAT1-, and OAT3 overexpressing HEK293 cells. As shown in Figures 1B, 1C, and 1D, the inhibition potency of CsA towards OCT2, OAT1, and OAT3 was slightly affected or unaffected by preincubation compared to coincubation.

In addition, we assessed the effect of CsA preincubation on the OCT1-mdiated transport of other substrates into HEK293 cells. Toward this end, 5 known OCT1 substrates were chosen that would represent a wide range of chemical structures and physiochemical properties (Figure 2 and Table 2). The CsA preincubation markedly potentiated the inhibition of OCT1-mediated transport of MPP^+, TEA, ranitidine, and fenoterol,compared to coincubation (Figure 2). Although the preincubation had no apparent potentiation of inhibition on OCT1-mediated transport of sumatriptan and cycloguanil (recommended OCT1 clinical probes by ITC), their IC_{50}s were in good agreement with that of metformin under preincubation condition (0.77 ± 0.18 and 0.15 ± 0.04 μM versus 0.43 ± 0.12 μM) (Table 2).These results suggest that CsA is a potent OCT1 inhibitor when the full inhibitory potency is attained. Moreover, the inhibitory effect of CsA on OCT1 was confirmed with human hepatocyte uptake experiment. The 60-min preincubation of 2 and 10 μM CsA inhibited the uptake of fenoterol, a OCT1 substrate drug, in human hepatocytes by 48 and 54%, respectively (Figure 3 and Table 3). The inhibition of fenoterol hepatic uptake by 2 and 10 μM CsA are slightly smaller than 100 μM pyrimethamine which is a potent inhibitor of OCT1 (48 and 54% versus 80%).

To further compare specificity of the preincubation-dependent inhibition against OCT1, we examined the inhibition of other inhibitors including quinidine, pyrimethamine, ritonavir, and trimethoprim under conditions of coincubation and preincubation. The IC_{50} values are summarized in Table 1. In contrast to a marked shift in the IC_{50} of CsA, the IC_{50} values of quinidine, pyrimethamine, ritonavir, and trimethoprim against OCT1 under conditions of

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preincubation were similar to that of coincubation, with IC_{50} shifts within 2-fold (1.7-, 1.4-, 2.0-, and 1.1-fold, respectively) (Table 1).

Figure 4 shows OCT1 activity over 120 min after OCT1- and Mock-HEK293 cells are preincubated with HBSS-HEPES buffer containing 10 μM CsA and control buffer. The OCT1-mediated uptake was evaluated by subtracting the uptake of metformin in mock-transfected cells from total uptake in the OCT1-expressing cells. Preincubation with 10 μM CsA for 30 min resulted in the significant reduction in OCT1-mediated uptake of metformin compared to the buffer control (approximately 19 versus 3 pmol/min/mg protein) ($p < 0.001$). The inhibitory effect on OCT1 is observed up to 120 min (approximately 80% inhibition of control) (Figure 4). The extent of reduction in OCT1 activity by the 30-min preincubation is similar to the reduction observed in the cells constantly under CsA exposure in the media up until the metformin transport is performed.

Time-Dependent Cellular Accumulation and Release of CsA in OCT1- and Mock-HEK293 Cells. Having established that preincubation with CsA resulted in a potent and lasting inhibition of OCT1, the cellular accumulation and release kinetics of CsA were examined. As shown in Figure 5, the cellular uptake of 1 and 10 μM CsA into the HEK293 cells was rapid as the intracellular concentrations were approximately 50 and 500 pmol/mg protein after 2-min incubation and reached plateau values of approximately 120 and 1200 pmol/mg protein at 15 min at 1 and 10 μM concentrations of drug, respectively. The cellular uptake pattern of CsA was similar in OCT1- and Mock-HEK cells (Figure 5) (p values are 0.06 and 0.25 at 1 and 10 μM CsA, respectively). Given its medium concentrations of 1 and 10 μM and assuming a water content of 9.3 μL/mg cells (Poirier et al., 2008), the cellular water-to-extracellular concentration ratio of CsA was approximately 13 (i.e., 13 and 130 μM cellular CsA concentrations, respectively).

As shown in Figure 6A, the efflux rate of CsA in OCT1-HEK293 cells was similar to that of Mock-HEK293 cells at all time-points up to 120 min. CsA was slowly released from the cells after preincubation with HBSS-HEPES buffer containing 10 μM CsA for 30 min, and the cellular concentrations of CsA remained at approximately 130 μM even after 120 min in buffer with no significant difference between OCT1- and Mock-HEK293 cells ($p > 0.05$), indicating lasting cellular retention of cyclosporin A in HEK293 cells In addition, on average, lower OCT1 activity was observed at higher concentration of intracellular CsA in OCT1-HEK293 cells, indicated by a decrease in metformin uptake rate (Figure 6B).

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DISCUSSION

In the present study, several new findings were revealed with respect to the inhibition of CsA towards OCT1. First, a 50-fold increase in CsA inhibitory potency against OCT1-mediated transport of metformin was observed following a preincubation step. Second, the CsA preincubation effect on OCT1 inhibition is substrate-dependent. Third, the inhibition potential of CsA towards OCT1 is confirmed by fenoterol hepatocyte uptake experiment. Fourth, CsA had a preferential preincubation-dependent inhibition effect on OCT1 over other drug transporters (OCT2, OAT1, and OAT3) and compounds (quinidine, pyrimethamine, ritonavir, and trimethoprim). Fifth, the accumulation of CsA in OCT1-HEK293 cells was a nonsaturable and rapid process, whereas the release of CsA from cells was extremely slow. Given the emerging clinical importance of OCT1, the results demonstrate that CsA is a clinical inhibitor that can be used to assess the worst-case scenario of transporter-based DDI mediated by OCT1, OATP1B1, OATP1B3, P-gp, and BCRP.

A major challenge in the *in vitro*-to-*in vivo* extrapolation of drug-drug interactions mediated by transporters, including OCT1, is to accurately determine IC_{50} and minimize IC_{50} variability (Tweedie et al., 2013, Lee et al., 2017). Even though *in vitro* inhibition assays are generally good predictors of clinical transporter-mediated DDIs, the highly variable inhibition potency among laboratories, which differ on models, probe substrates, incubation conditions, and data analysis methods, result in markedly wide DDI predictions (Vaidyanathan et al., 2016). In particular, it has been demonstrated in OATP1B1-HEK293 cells that a preincubation step decreased the IC_{50} of CsA 12- to 22-fold, with a less pronounced decrease for OATP1B3 (5.2-fold) (Amundsen et al., 2010, Gertz et al., 2013). The present studies show a 50-fold increase in CsA inhibitory potency against OCT1-mediated transport of metformin after a preincubation step, with IC_{50} values of 21.6 ± 4.5 and 0.43 ± 0.12 μM under conditions of coincubation and preincubation, respectively (Figure 1A and Table 1), which suggests that CsA is a potent inhibitor of OCT1. In addition, preincubation of OCT1-HEK293 cells with CsA resulted in the apparent enhancement of the inhibitory potency of CsA against the transport of MPP^+, TEA, ranitidine, and fenoterol mediated by OCT1 (Figure 2). CsA is a potent inhibitor of OCT1-mediated uptake of sumatriptan and cycloguanil, however, the potentiation of transporter inhibition by CsA preincubation was not observed with sumatriptan and cycloguanil. Moreover, 2 and 10 μM CsA reduced the uptake of fenoterol, a OCT1 probe, in human hepatocytes by 48 and 54%, respectively (Figure 3 and Table 3). In contrast, OCT2, OAT1, and OAT3 have been shown not to be affected by a 30-min preincubation of CsA compared to coincubation (Figures 1B, 1C, and 1D), which suggests that the preincubation with CsA does not

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potentiate its inhibitory effect on OCT2, OAT1, and OAT3. While this paper was in preparation, Tátrai et al. (2019) systematically assessed the in vitro inhibitor preincubation effect on multiple clinically relevant transporters using transfected cell lines (Tatrai et al., 2019). The inhibitor preincubation effect was prevalent among OCT1, OCT2, OATP1B1, and OATP1B3 but not among OAT1, OAT3, multidrug and toxin extrusion protein 1 (MATE1), and MATE2-K, and most instances of the effect persisted after controlling for toxicity and nonspecific binding. Although the effect of CsA preincubation on OCT1 inhibition has not been examined in that study, several inhibitors such as ledipasvir, irinotecan, saquinavir, and daclatasvir exhibited 3.4- to > 594-fold enhancement of OCT1 inhibition by preincubation (Tatrai et al., 2019). Unfortunately, only very limited data are presently available describing a clinical drug interaction between CsA and OCT1 substrates, in particular whether discrepancy exists in OCT1 inhibition potency between *in vivo* and *in vitro* assessments. One possible reason is that CsA is an inhibitor of multiple drug metabolizing enzymes and transporters including CYP3A4, P-gp, BCRP, and OATP1B1, and OATP1B3, and its inhibition on the other enzymes and transporters might mask the OCT1 inhibitory effect.

Because OCT1 activity is strongly inhibited by CsA following a preincubation step (IC_{50} of 0.43 ± 0.12 μM) (Table 1) and the free maximum concentration of CsA at the inlet to the liver is pronounced ($I_{in,max,u}$) [approximately 0.5 to 2.5 μM estimated by using CsA pharmacokinetics parameters including dose and maximum plasma concentration (C_{max}) (Stein et al., 2001), and fraction of unbound drug (f_u) (Legg and Rowland, 1987), the predicted systemic exposure ratio of a victim drug in the presence and absence of CsA (*AUC*R) is 6.7 using IC_{50} of 0.43 μM determined under preincubation condition ($AUCR = 1 + I_{in,max,u}/IC_{50}$), suggesting a drug interaction potential of CsA with OCT1. In contrast, the *AUCR* is 1.1 using IC_{50} of 21.6 μM determined under coincubation condition. The importance of OCT1 in the disposition of metformin (Shu et al., 2008), tropisetron (Tzvetkov et al., 2012), sumatriptan (Matthaei et al., 2016), O-desmethyltramadol (Stamer et al., 2016), fenoterol (Tzvetkov et al., 2018), and cycloguanil (Matthaei et al., 2018) has been demonstrated in OCT1 pharmacogenomics studies in which significantly higher systemic exposures were observed in the individuals carrying loss-of-function OCT1 polymorphisms compared to individuals carrying wild-type OCT1. However, clinical DDI studies on OCT1 substrates cannot be conducted in the absence of pharmocogenomic enrichment because no selective clinical inhibitors of OCT1 have been reported (Zamek-Gliszczynski et al., 2018a). Quinidine, ritonavir, trimethoprim, and pyrimethamine are inhibitors of OCT1 (Table 1), however significant overlap exists in inhibitor selectivity of these compounds for OCT1, OCT2, MATE1, and MATE2-K. CsA did not significantly affect OCT2, OAT1, or OAT3 activities up to 20 μM (Figures 1B, 1C, and

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1D), and CsA is not an inhibitor of MATE1 or MATE2-K (unpublished data). However, CsA is an inhibitor of cytochrome P450 (CYP) 3A4, OATP1B1, OATP1B3, P-gp and BCRP (Gertz et al., 2013). Pharmacogenomic analysis has been proposed for studying the role and involvement of OCT1 in drug disposition (Zamek-Gliszczynski et al., 2018a). One must use caution in analyzing pharmacogenomics data with OCT1.

Several common single nucleotide polymorphisms (SNPs) in the human OCT1 gene (*SLC22A1*) have been demonstrated to cause significant reduction or complete loss of OCT1 activity and the substitutions constitute four major OCT1 alleles OCT1*3, OCT1*4, OCT1*5, and OCT1*6: arginine61-to-cysteine, cysteine88-to-arginine, glycine401-to-serine, and glycine465-to-arginine (Kerb et al., 2002, Shu et al., 2003, Seitz et al., 2015). However, the total frequencies of these homozygous and heterogonous OCT1activity-altering SNPs are 9.7, 5.5, and 3.5% in European, American, and worldwide populations, respectively (Shu et al., 2003, Gertz et al., 2013). As a result, less than 1% of Europeans and Caucasians Americans are homozygous carriers of the OCT1 variant alleles and have heritable OCT1 deficiency. In this regard, among 217 to 249 Caucasians, only 3, 0, 0, and 0 subjects were found to carry the homozygous genotypes of arginine61-to-cysteine, cysteine88-to-arginine, glycine401-to-serine, or glycine465-to-arginine, respectively. Taken together, CsA is an appropriate inhibitor that can be used in a clinical DDI study to assess the worst-case scenario of transporter-based DDI mediated by OCT1, OATP1B, P-gp, and BCRP.

Although there is an increasing number of studies examining the mechanisms, there is much less known about how preincubation with CsA enhances transporter inhibition potency and why its inhibitory effect lasts so long. The current study indicates that, in cultured OCT1- and Mock-HEK293 cells, the uptake of CsA occurs via a two-step, nonsaturable process (e.g., possibly diffusion): a rapid increase in uptake during the first 2 min, followed by a significant slow-down, with the uptake ultimately reaching a plateau at 15 min (Figure 4). In contrast, CsA demonstrated long intracellular retention in OCT1- and Mock-HEK293 cells, with extremely slow movement out of the cells, resulting in no significant change of intracellular concentration of CsA during the first 120 min incubation (Figure 5). The long intracellular retention may explain how CsA exerted a lasting inhibitory effect of OCT1 up to 120 min even after it was removed from incubation buffer (Figure 3). A model consisting of *cis*- and *trans*-inhibition, occurring outside and inside cells, respectively, was proposed recently as a possible mechanism of preincubation-dependent and long-lasting inhibition of OATP1B1 and OATP1B3 by CsA (Shitara and Sugiyama, 2017). The authors propose that CsA slowly permeates cell membranes and then exerts *trans*-inhibition to enhance inhibition of OATP1B1 and OATP1B3. Recently, Tátrai et al. also tried to elucidate the mechanism of the inhibitor preincubation

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effect on drug transporters by measuring time-course intracellular drug concentration and transporter inhibition (Tatrai et al., 2019). The intracellular concentration and inhibitory potency of ledipasvir, venetoclax, and CsA followed greatly similar time course profiles. In addition, the enhancement of transporter inhibition by preincubation correlated significantly with the time required to reach steady-state cellular concentration which is governed by inhibitor passive permeation rate combined with its cellular binding extent. As a result, they concluded that intracellular unbound inhibitor concentration is a major determinant of transporter inhibitory potency, and thus the magnitude of potentiation of transporter inhibition by preincubation depends on the equilibration time. However, as shown in Figure 4, our data indicate that some amount of CsA permeated membranes rapidly into HEK293 cells during the first 2-min incubation and reached cellular uptake of approximately 50 and 500 pmol/mg or intracellular concentration of 5.4 and 54 μM after 2-min incubation with 1 and 10 μM CsA, respectively. It has been reported that changes of phosphorylation and subcellular localization of OATP1B3 and OATP2B1 via protein kinase activation result in altered transport activity (Kock et al., 2010, Powell et al., 2014). It is unknown whether the same mechanism is responsible for the potentiation of OCT1 inhibition by CsA incubation. There are no reports that describe the clinical drug-drug interactions involving protein kinase activation (Shitara and Sugiyama, 2017). Shitara et al. examined the expression of OATP1B1 on cell surface membrane after CsA treatment in vitro by using immunostaining (Shitara et al., 2012). The expression of OATP1B1 protein was not apparently affected by incubation of MDCK-OATP1B1 cells with CsA and no obvious alteration in OATP1B1 subcellular localization was observed. But it is controversial if immunohistochemistry and microscope are able to produce the resolution to quantify subcellular expression of transporter protein. Taken together, the mechanism(s) behind potentiation of transporter inhibition by inhibitor preincubation remains unknown. Our findings suggest that the enhancement of the OCT1 inhibitory potency unlikely shares common cellular mechanism(s) and the physicochemical property of a particular drug may not play a solely decisive role in its inhibition effects.

In conclusion, our results are unique in demonstrating the effect of preincubation on OCT1 by CsA. These findings provide the mechanistic basis for the use of CsA as a clinical inhibitor to assess the worst-case scenario of DDI mediated by drug transporters. Future studies will be focused on studying the *in vivo* clinical pharmacokinetics of a selective drug substrate of OCT1 – if applicable, in the presence and absence of CsA.

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Authorship Contributions

Participated in research design: Panfen, Chen, Zhang, Gan, and Shen

Conducted experiments: Panfen, Zhang, and Chen

Contributed new reagents or analytic tools: Panfen, Zhang, and Shen

Performed data analysis: Panfen, Chen, Zhang, Gan, and Shen

Wrote or contributed to the writing of the manuscript: Panfen, Chen, Sinz, Marathe, Gan, and Shen

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Footnotes

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Figure Legends

Figure 1. Representative inhibition plots of OCT1 (A), OCT2 (B), OAT1 (C), and OAT3 (D) by CsA coincubation (•) and preincubation (Δ). Data points are mean ± S.D. values of triplicate uptake rates in transporter-expressing HEK293 cells. The extent of inhibition of transport is expressed as a percentage of that in the absence of CsA. Nonlinear regression analysis of transporter-mediated uptake was used to determine apparent IC_{50} values.

Figure 2. Representative inhibition plots of OCT1-mediated uptake of MPP^+ (A), TEA (B), ranitidine (C), fenoterol (D), sumatriptan (E), and cycloguanil (F) by CsA coincubation (•) and preincubation (Δ). Data points are mean ± S.D. values of triplicate OCT1-mediated uptake rates. The extent of inhibition of transport is expressed as a percentage of that in the absence of CsA. Nonlinear regression analysis of transporter-mediated uptake was used to determine apparent IC_{50} values (Table 2).

Figure 3. Effect of CsA (2 and 10 μM) and pyrimethamine (100 μM) on the uptake of fenoterol into human hepatocytes. Open circles, squares, triangles, and diamonds represent the uptake of 1 μM fenoterol alone, with 2 μM CsA, 10 μM CsA, and 100 μM pyrimethamine, respectively. Close circles represent the uptake of 1 μM fenoterol at 4°C. The uptake of fenoterol was performed after hepatoxytes were preincubated with solutions containing inhibitor or vehicle control for 60 min. Each value shows the mean ± S.D. of three separate determinations (n = 3).

Figure 4. Persistent inhibitory effect of CsA on the OCT1-mediated transport of metformin. Data points are mean ± S.D. values of triplicate OCT1-mediated uptake. After a preincubation with HBSS-HEPES buffer only (closed circles) and 10 μM CsA (open circles and triangles) for 30 min, OCT1-HEK293 cells were then incubated for 0-120 min with fresh HBSS-HEPES buffer, and the OCT1 activity was further measured by incubating the cells with the HBSS-HEPES buffer containing 2 μM [^{14}C]metformin (closed and open circles). The transport activities were also examined after OCT1-HEK293 cells constantly under CsA exposure in the media up until the metformin transport was performed (open triangles). ***$p < 0.001$ represents statistically significant difference compared to the preincubation with HBSS-HEPES.

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Figure 5. Time course of CsA uptake into OCT1- and Mock-HEK293 cells. Data points are mean ± S.D. values of triplicate CsA uptake into cells. HEK293 cells stably transfected with recombinant OCT1 (black bars) or mock plasmid vector (gray bars) were incubated for the indicated times at 37°C with 1 and 10 μM CsA. *$p < 0.05$, statistically significant difference compared with Mock-HEK293 cells.

Figure 6. Time course of CsA efflux from OCT1- (black bars) and Mock-HEK293 cells (gray bars) (A), where cells were preloaded for 30 min with 10 μM CsA. Correlation of OCT1 transport activity with CsA cellular concentration is analyzed (B). OCT1-mediated transport rate of metformin correlated negatively with CsA remaining in the cells. Data points are mean ± S.D. values of triplicate concentration of CsA remaining in cells and effluxed into buffer as a function of time. *$p < 0.05$, statistically significant difference compared with Mock-HEK293 cells.

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Table 1. Inhibition of OCT1 by CsA and other Drugs with and without 30-Min Preincubation in Stably Transfected HEK Cells

Drugs	IC_{50} (μM)		IC_{50} fold shift
	Without 30-min preincubation	With 30-min preincubation	
Cyclosporine A	21.6 ± 4.5	0.43 ± 0.12	50.2
Quinidine	7.7 ± 0.9	4.5 ± 0.7	1.7
Pyrimethamine	1.8 ± 0.2	1.3 ± 0.1	1.4
Ritonavir	2.2 ± 0.3	1.1 ± 0.1	2.0
Trimethoprim	9.6 ± 1.2	9.2 ± 0.7	1.1

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Data are represent the mean ± S.D. The IC_{50} values were estimated by Phoenix as indicated under *Materials and Methods*. [^{14}C]Metformin (2 μM) was used as a probe substrate. Therefore, the IC_{50} values are considered as K_i (assuming competitive inhibition) because the ratio of metformin concentration to K_m is small (< 0.2).

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Table 2. Inhibition of OCT1-Mediated Uptake of Metformin, MPP+, TEA, and Clinical OCT1 Substrate Drugs with and without 30-Min Preincubation in Stably Transfected HEK Cells

Substrate Drugs	CsA IC_{50} (μM)		IC_{50} fold shift
	Without 30-min preincubation	With 30-min preincubation	
Metformin	21.6 ± 4.5	0.43 ± 0.12	50.2
MPP^+	> 20	16.1 ± 6.0	> 1.2
TEA	> 20	12.1 ± 1.7	> 1.7
Ranitidine	> 20	4.6 ± 0.6	> 4.3
Fenoterol	> 20	2.4 ± 0.9	> 8.3
Sumatriptan	2.5 ± 1.2	0.77 ± 0.18	3.2
Cycloguanil	0.32 ± 0.07	0.15 ± 0.04	2.1

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Data are represent the mean ± S.D. The IC_{50} values were estimated by Phoenix as indicated under *Materials and Methods*.

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Table 3. Inhibition of Uptake of Fenoterol in Human Hepatocytes by CsA (2 and 10 μM) and Pyrimethamine (100 μM)

Parameter	Control	With 2 μM CsA	With 10 μM CsA	With 100 μM Pyrimethamine	4°C
Uptake Clearance (μL/min/10^6 cells)	12.2	6.4	5.7	2.5	1.2
Uptake Clearance Inhibition (%)	NA	48	54	80	90

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The hepatic uptake clearance was determined using data in Figure 3.

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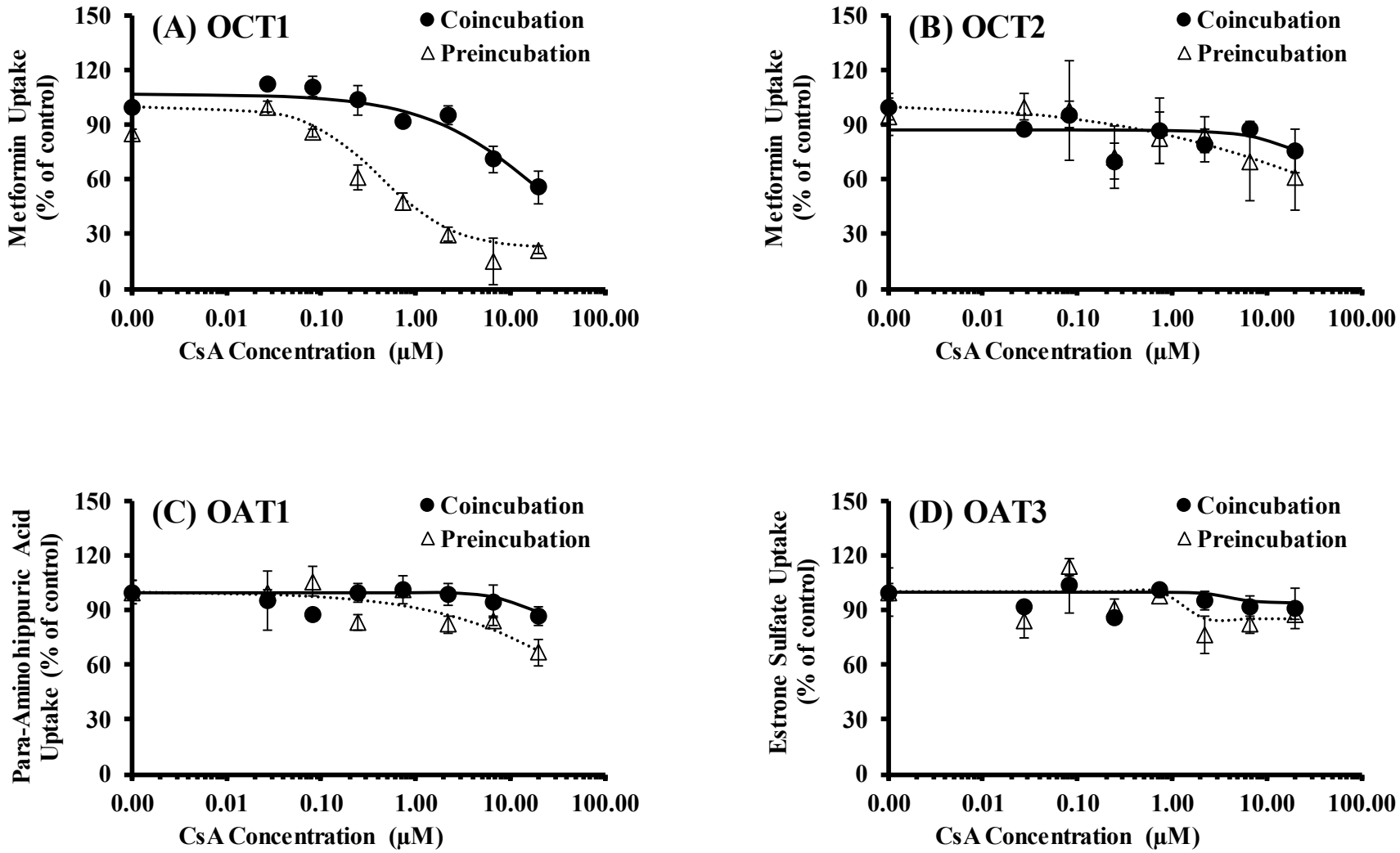

Figure 1

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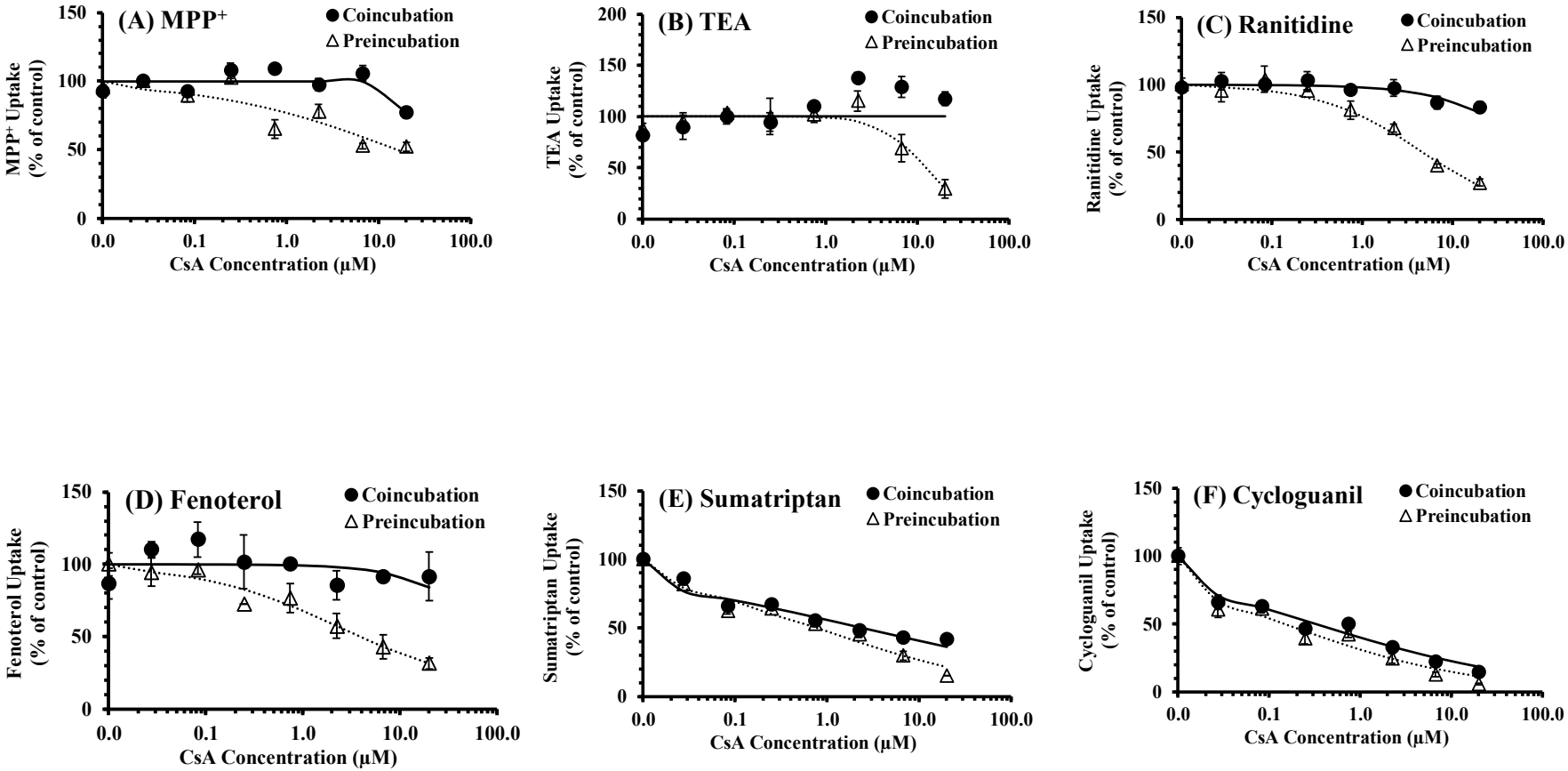

Figure 2

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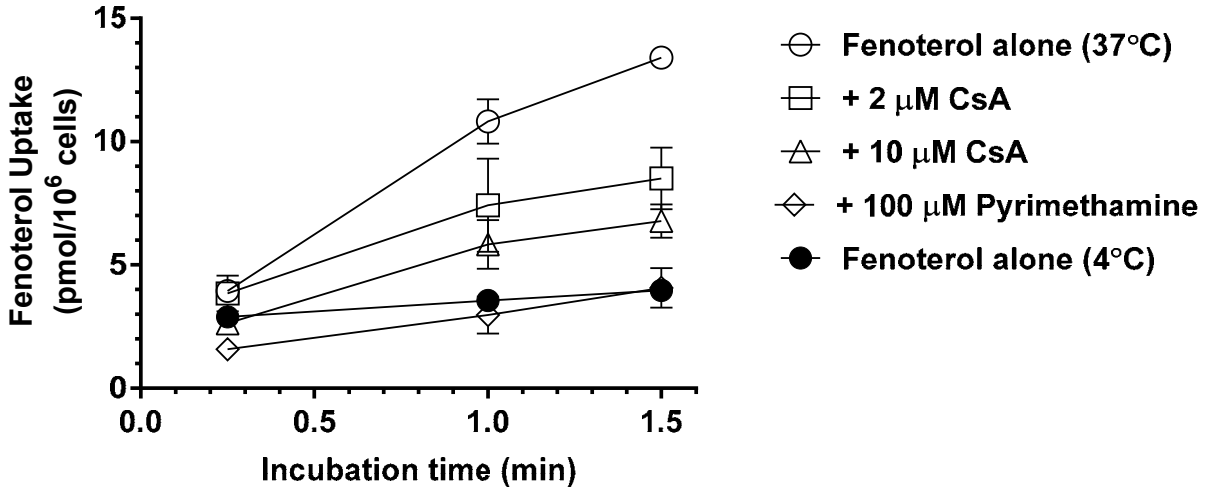

Figure 3

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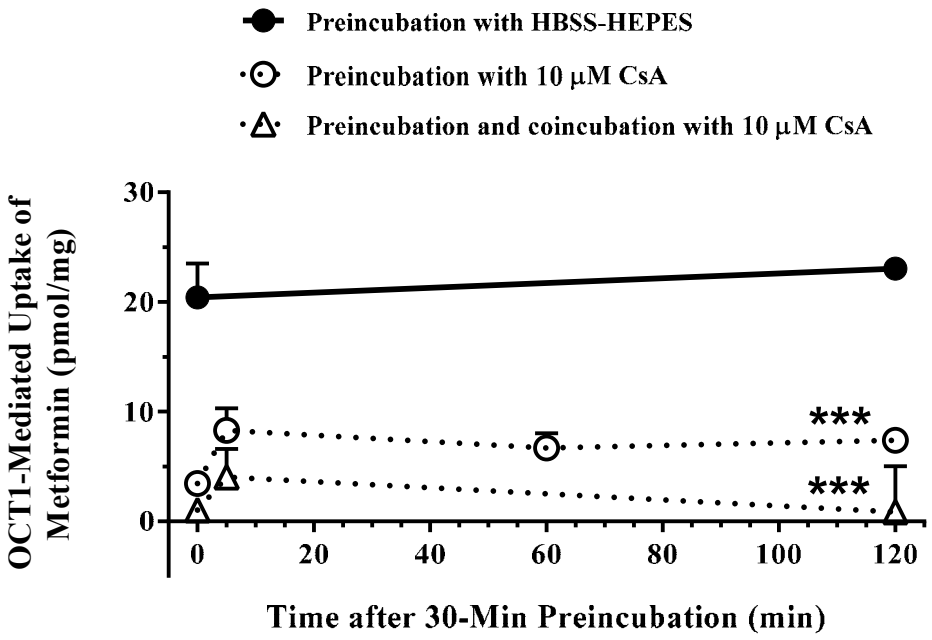

Figure 4

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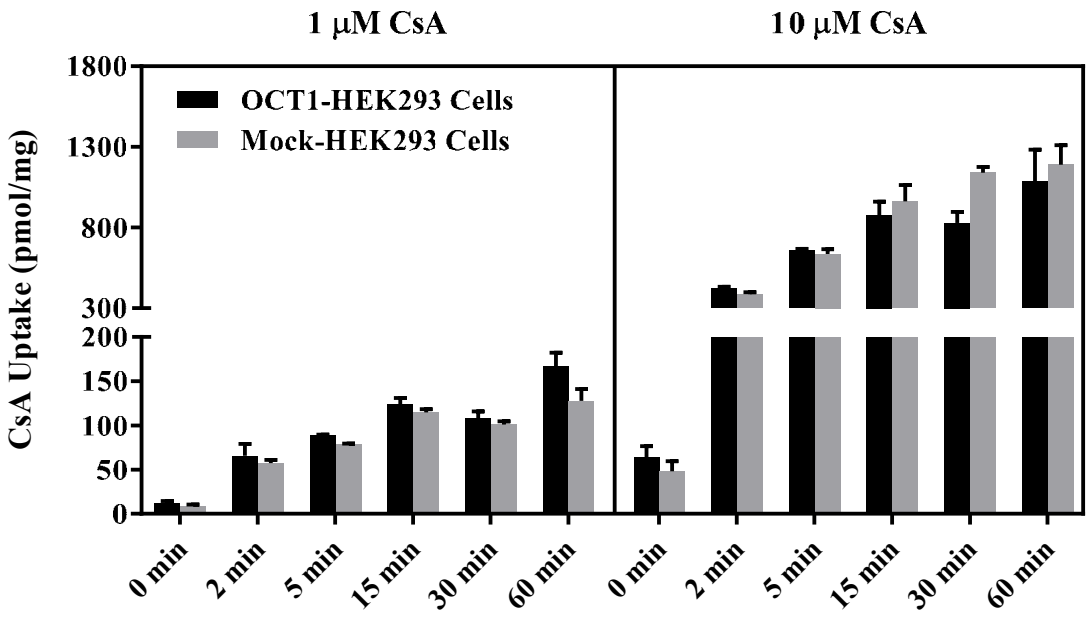

Figure 5

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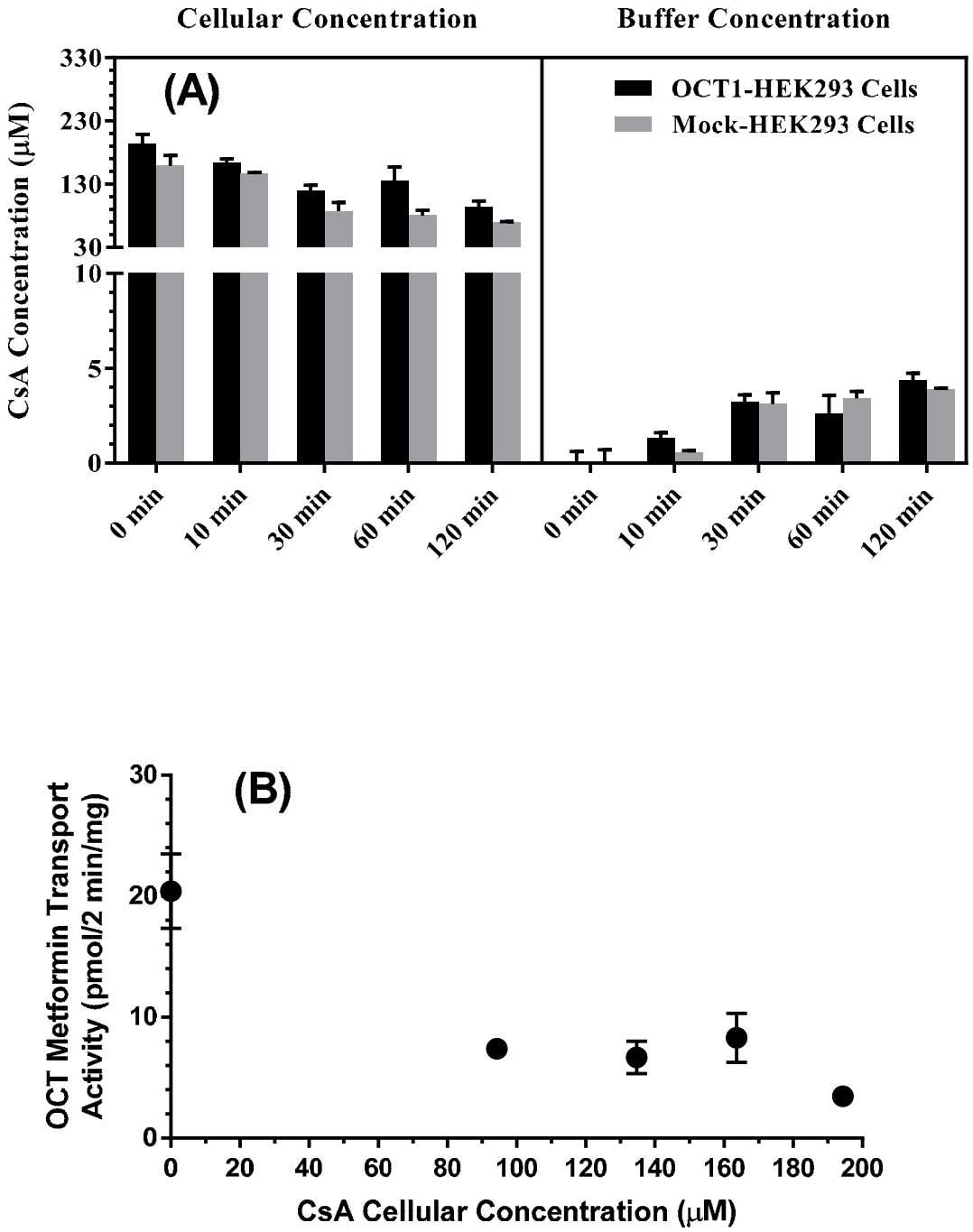

Figure 6

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