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Assessment of Vandetanib as an Inhibitor of Various Human Renal Transporters: Inhibition of Multidrug and Toxin Extrusion (MATE1 and MATE2K) as a Possible Mechanism Leading to Decreased Cisplatin and Creatinine Clearance

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Running title: Inhibition of MATE1 and MATE2K by vandetanib

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Abbreviations: AUC, area under the concentration-time curve; Cisplatin, *cis*-diamminedichloroplatinum II; DDI, drug-drug interaction; E3S, estrone-3-sulfate; FRT, Flp recombination target; HBSS, Hank's balanced salt solution; HEK-293: human embryonic kidney 293 cells; MATE, multidrug and toxin extrusion; MMP^+, 1-methyl-4-phenylpyridinium; NSCLC, non–small cell lung cancer; OCT, organic cation transporter; OAT, organic anion transporter; PAH, *p*-aminohippuric acid; PYR, pyrimethamine.

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Abstract

Vandetanib was evaluated as an inhibitor of human OAT1, OAT3 (organic anion transporter 1 and 3), OCT2 (organic cation transporter 2), and multidrug and toxin extrusion (MATE1 and MATE2K) transfected (individually) into human embryonic kidney (HEK) 293 cells. Although no inhibition of OAT1 and OAT3 was observed, inhibition of OCT2-mediated uptake of 1-methyl-4-phenylpyridinium (MPP^+) and metformin was evident (IC_{50} of 73.4 ± 14.8 µM and 8.8 ± 1.9 µM, respectively). However, vandetanib was an even more potent inhibitor of MATE1- and MATE2K-mediated uptake of MPP^+ (IC_{50} of 1.23 ± 0.05 µM and 1.26 ± 0.06 µM, respectively) and metformin (IC_{50} of 0.16 ± 0.05 µM and 0.30 ± 0.09 µM, respectively). Subsequent cytotoxicity studies demonstrated that transport inhibition by vandetanib (2.5 µM) significantly decreased the sensitivity (right shift in concentration of cisplatin giving rise to 50% cell death; $IC_{50(CN)}$) of MATE1-HEK and MATE2K-HEK cells to cisplatin ($IC_{50(CN)}$ of 1.12 ± 0.13 versus 2.39 ± 0.44 µM; 0.85 ± 0.09 versus 1.99 ± 0.16 µM; $P < 0.05$), but not OCT2-HEK cells (1.36 ± 0.19 versus 1.47 ± 0.24 µM), versus vandetanib untreated cells and Mock-HEK cells ($IC_{50(CN)}$ of 2.34 ± 0.31 µM). In summary, the results show that vandetanib is a potent inhibitor of MATE1 and MATE2K (versus OCT2). Inhibition of the two transporters may explain why there are reports of decreased creatinine clearance, and increased cisplatin nephrotoxicity (reduced cisplatin clearance), in some subjects receiving vandetanib therapy.

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Introduction

Vandetanib [N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine], an oral tyrosine kinase inhibitor of vascular endothelial growth factor receptor-, epidermal growth factor receptor-, and rearranged during transfection-dependent signaling, has been approved for the treatment of symptomatic or progressive medullary thyroid cancer (Langmuir and Yver, 2012; Frampton, 2012). Vandetanib is also being developed for the treatment of non–small cell lung cancer (NSCLC) (Natale et al., 2009; Stinchcombe and Socinski, 2009; McKeage 1995). Recently, Blackhall et al. (2010) investigated the safety of vandetanib in combination with either gemcitabine plus cisplatin (*cis*-diamminedichloroplatinum II) or vinorelbine plus cisplatin. While vandetanib in combination with the cisplatin-based doublets had no effect on the pharmacokinetics of vinorelbine and gemcitabine, a 30% increase in the exposure of cisplatin was observed compared to cisplatin alone. In addition, an increase in plasma creatinine was more common in patients (16% vs. 1%) who received 300 mg vandetanib (versus placebo) (US Department of Health and Human Services; Caprelsa (vandetanib) safety label, 2012). Although the mechanisms underlying the apparent interaction with cisplatin and creatinine are unknown, inhibition of renal transporters is suspected.

In the case of cisplatin, it is known that renal elimination accounts for more than 90% of the dose in human subjects and that its renal clearance exceeds creatinine clearance and glomerular filtration rate. Therefore, it is likely that cisplatin and other platinum-containing molecules are actively secreted by the kidneys (Go and Adjei, 1999; Jacobs et al., 1980; US Department of Health and Human Services; cisplatin label, 2011). Human OCT2 (organic cation transporter 2), MATE1 (multidrug and toxin extrusion 1) and MATE2K (multidrug and toxin extrusion 2K) are highly expressed in the brush-border membrane of kidneys (proximal tubule

epithelial cells) and are thought to play a role in the clearance of cisplatin (Ciarimboli et al., 2010 and 2011; Liu et al., 2012; Sprowl et al., 2012). In agreement, the recovery of a cisplatin dose in urine is markedly reduced in mice deficient in both Oct1 and Oct2 (Filipski, et al., 2008). Similarly, inhibition by cimetidine and gene deletion decreases OCT2-mediated renal uptake of cisplatin and ameliorates nephrotoxicity (Franke et al., 2010). Furthermore, OCT2 genotype is associated with cisplatin-induced nephrotoxicity (Filipski et al., 2009).

From the standpoint of MATE1- and MATE2K-mediated cisplatin renal clearance, it has been shown that cisplatin plasma concentrations are increased, and renal accumulation is higher, in Mate1 (-/-) mice (versus wild-type animals) (Nakamura et al., 2010). Unlike mice, however, genotype-phenotype associations in humans are more complex because of the expression of two forms of MATE (MATE1, SLC47A1; and MATE2K, SLC47A2) (Yonezawa and Inui, 2011). Therefore, although a number of non-synonymous single-nucleotide polymorphisms have been identified for MATE1, Iwata et al (2012) determined that a single point mutation (rs2289669 G>A) had no effect on either cisplatin plasma concentrations or toxicity. Unfortunately, the authors did not consider MATE2K and that it might compensate for the loss of function of MATE1. Unfortunately, no clinical information is available describing the effect of a dual (MATE1 and MATE2K) inhibitor like pyrimethamine (PYR) on cisplatin systemic exposure (Ito et al., 2012; Kusuhara et al., 2011).

Given the clinical observations described above, and to garner additional mechanistic information regarding the interaction with cisplatin, an attempt was made to study vandetanib as an inhibitor of 5 renal transporters in vitro (OCT2, MATE1, MATE2K, and organic anion transporter 1 and 3; OAT1 and 3). Like cisplatin, creatinine serves as an OCT2, MATE1 and MATE2K substrate (Urakami et al., 2004; Tanihara et al., 2007; Imamura et al., 2011;

Ciarimboli et al., 2012), therefore, the possibility that vandetanib impacts creatinine clearance by inhibiting one or more of these transporters was considered also. Inhibition of transporters by vandetanib may influence the renal elimination and systemic exposure of certain transporter substrates, leading to drug-drug interactions (DDIs) and increased toxicity. It should be noted that the vandetinib (Caprelsa®) product label does contain information regarding the inhibition of OCT2-mediated uptake of creatinine into HEK293 cells (IC_{50} = 4.4 µM; 2.1 µg/mL) (AstraZeneca study KMX083; US Department of Health and Human Services; Caprelsa (vandetanib) safety label, 2012). However, inhibition of MATE1 and MATE2K has not been reported to date.

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Material and Methods

Materials. HEK293 cells containing the Flp Recombination Target (FRT) recombination site, Lipofectamine 2000, hygromycin B, Zeocin, Flp-in system, and water-soluble probenecid were purchased from Invitrogen Corporation (Carlsbad, CA). [^{3}H]*para*-Aminohippuric acid (PAH; 4.3 Ci/mmol), [^{3}H]Estrone-3-sulfate (E3S; 45.6 Ci/mmol), and [^{3}H]1-methyl-4-phenylpyridinium (MPP^+; 83.9 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{14}C]Metformin hydrochloride (92.7 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Vandetanib, cisplatin and PYR were supplied by Toronto Research Chemicals Inc. (North York, Ontario). Cell culture reagents including Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), trypsin, Hanks' balanced salt solution (HBSS), nonessential amino acids, and L-glutamine were purchased from Mediatech (Herndon, VA). Biocoat poly-D-lysine-coated 24-well plates were purchased from Becton Dickenson (Bedford, MA). BCA protein assay kit was purchased from (Pierce Chemical, Rockfold, IL). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Transfection. HEK293 cells were cultured in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum, nonessential amino acids (0.1 mM), and L-glutamate (2 mM), in an atmosphere of CO_2 (5%) and air (95%) at 37°C, and used as host cells for transfection. The stable transfected HEK293 cell lines expressing human OAT3, OCT2, and MATE1 were established as previously described (Han et al., 2010).

For generation of a stable human OAT1 and MATE2K-transfected cell lines, the cDNAs including the open reading frame of OAT1 (SLC22A6) and MATE2K (SLC47A2, transcript variant 2), were subcloned into the Gateway entry vector pDONR221 (Invitrogen, Carlsbad, CA)

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using standard methods and the sequence was confirmed in both cases. The Gateway entry clones were recombined into a Gateway adapted version of the expression vector pcDNA5/FRT/TO (Invitrogen, Carlsbad, CA) using LR clonase II (LifeTechnologies, Carlsbad, CA). The sequences of the expression constructs were confirmed by DNA sequencing. HEK293 cells stably expressing OAT1 (or MATE2K) were established by using the Flp-In™ expression system (Invitrogen, Carlsbad, CA) as described previously (Shen et al., 2011). In brief, the recombinant pcDNA5/Flp recombination target construct containing OAT1 (or MATE2K) was co-transfected with pOG44, a Flp recombinase expression plasmid, into an HEK293 cell line using lipofectamine 2000. The cells stably expressing the transporter were then selected with HEK293 cell medium (Dulbeccos's modified eagle's medium supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 2 mM L-glutamate) supplemented with the antibiotic hygromycin B (100 μg/ml). Single hygromycin-resistant colony cells were sorted into 24-well plates containing HEK293-conditioned medium. After expansion, clones were screened for expression of functional OAT1 and MATE2K activity on the basis of increased accumulation of probe substrates (i.e., [^{3}H]PAH for OAT1; [^{3}H]MPP^{+} and [^{14}C]metformin for MATE2K). The expression of OAT1 and MATE2K was verified by PCR analysis. Real time PCR results showed significant over-expression of OAT1 and MATE2K mRNA in the cells, compared to mock cells (data not shown).

To support the transport inhibition experiments, cells were seeded in 24-well poly-D-lysine coated plates (500,000 cells per well) 2 to 3 days prior to performing the transport experiments. For the assessment of cytotoxicity, cells were seeded (5,000 cells per well) on 96-well culture plates and incubated overnight before assaying.

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Assessment of Transporter Inhibition. All transport experiments were performed manually in 24-well plate on a thermostatic VWR symphony incubating microplate shaker (Thorofare, NJ) calibrated at 37 °C following the protocol described previously with minor modifications (Shen et al., 2013). In brief, cells grown in 24-well plates were rinsed twice with 1.5 ml of pre-warmed Hanks' balanced salt solution (HBSS). The transport experiment was initiated by adding pre-warmed HBSS containing the radiolabeled probe substrate (buffered with 10 mM HEPES to pH 7.4 for OAT1, OAT3 and OCT2, and to pH 8.4 for MATE1 and MATE2K) to enable inhibition curves to be derived for vandetanib. A low concentration (< K_m) of radiolabeled PAH and E3S (1 μM) was used for the measurement of OAT1 and OAT3 activity, respectively (Takeda et al., 2001). Both MPP^+ and metformin (1 μM) were used as probe substrates for OCT2, MATE1, and MATE2K. In each case, the substrate concentration chosen was well below the K_m reported in the literature (Kimura et al., 2005; Tanihara et al., 2007). Probenecid was used as a control inhibitor of OAT1 and OAT3, and to confirm the functional activities of each transporter in the test system (Takeda et al., 2001). PYR was used as a positive control inhibitor of OCT2, MATE1 and MATE2K (Kusuhara et al., 2011). Subsequently, the transport experiments were stopped at the designated time by removing the buffer and immediately washing the cells three times with 1 ml of ice-cold HBSS. The cells were lysed with 0.3 ml of 0.1% Triton X-100. The cell lysate samples obtained in the study were analyzed by liquid scintillation counting (LS 6500; Beckman Coulter, Inc., Fullerton, CA). Accumulation was normalized to the protein content of the HEK293 cells in each well measured using the BCA protein assay kit.

Assessment of Cell Growth and Cytotoxicity. Cytotoxicity of cisplatin in OCT2, MATE1, MATE2K, and mock cells was measured using the XTT colorimetric assay (Roche

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Applied Science, Mannheim, Germany) performed in 96-well plates. Cells were harvested from culture flasks by trypsinization and seeded in 50 μL aliquots (in DMEM supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, and 2 mM L-glutamate 10% fetal calf serum, 0.1 mM nonessential amino acids, and 2 mM L-glutamate) into 96-well microculture plates at the density of 5,000 viable cells per well. Cells were allowed to settle and resume exponential growth in drug-free complete culture medium for 24 h, followed by the addition of dilutions of vandetanib (50 μL per well) of the same medium. Following the addition of vandetanib, incubations were continued for 72 h at which time XTT labeling mixture (50 μL per well) was added. After incubation for 16 h, the resulting orange formazan product formed by viable cells was spectrophotometrically quantified with a Micro Plate Reader (Molecular Devices, Sunnyvale, CA), using a reference wavelength of 690 nm to correct for nonspecific absorption.

Data Analysis Methods. *Determination of IC_{50} Values for Inhibition of Transporter-Mediated Uptake.* The transporter-mediated net uptake rate (v) of radiolabeled probe substrate was obtained by subtracting the uptake in mock cells from the uptake in transporter stably transfected cells (measured as total radioactivity). The results were reported as mean ± S.D. (n = 3 determinations). The decrease in net uptake rate of probe substrate in the presence of increasing concentrations of the putative inhibitors (probenecid, PYR, and vandetanib) was determined to assess their relative potency and to calculate IC_{50} values (concentration of inhibitor giving rise to 50% inhibition of activity). The IC_{50} values were determined using equation 1 (WinNonlin 5.0.1, Pharsight Inc.; Mountain View, CA).

$$v = v_0 x\left(1 - \frac{I^n}{I^n + IC_{50}^n}\right) \quad (1)$$

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where v_0 is net uptake without inhibitor, I is the inhibitor concentration, and n is a sigmoidicity factor that determines the slope of the relationship.

Determination of IC_{50} Values for Inhibition of Cell Growth. The quantity of viable cells was expressed as a percentage of the number of cells in the untreated controls (taken as 100%) according to equation 2. The results were expressed as mean ± S.D. (n = 4 determinations):

$$\% \, Control = \left(\frac{N_I}{N_0} \right) x100 \tag{2}$$

where N_I represents the number of viable cells measured in the presence of inhibitor and various concentrations of cisplatin, and N_0 represents the number of viable cells in the absence of inhibitor. The concentration of cisplatin that decreases the number of cells by 50% ($IC_{50(CN)}$) was determined by fitting the cytotoxicity data to equation 3:

$$\% \, Control = 100\% \, x \left(1 - \frac{I^n}{I^n + IC_{50(CN)}^n} \right) \tag{3}$$

where I was cisplatin concentration, and n was a sigmoidicity factor. The nonlinear regression analysis was performed with WinNonlin 5.0.1 software (Pharsight Inc.; Mountain View, CA).

Statistical Analysis. Data are reported as mean ± S.D. To test for statistically significant differences among different cell lines and substrates for IC_{50}, two-way analysis of variance (ANOVA) was performed. When the F ratio showed that there were significant differences among groups, the Bonferroni posttest was used to determine which groups differ. To test for statistically significant differences in cytotoxicity among different cell lines treated with cisplatin alone or among multiple treatments for a given cell line, one-way ANOVA was performed. When the F ratio showed that there were significant differences among treatments, the Dunnett

method of multiple comparisons was used to determine which treatments differ. All statistical analyses were performed using Prism version 5 (GraphPad Software, Inc., San Diego, CA).

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Results

Vandetanib Does Not Inhibit Transport Mediated by Human OAT1 and OAT3. Human OAT1- and OAT3-HEK cells in this study exhibited transport characteristics similar to those reported previously (Han et al., 2010). Intracellular uptake of [^{3}H]PAH (1 μM) and [^{3}H]E3S (1 μM), prototypical substrates for human OAT1 and OAT3, respectively (Lee and Kim, 2004; Han et al., 2010), was time-dependent and significantly greater than in mock cells (Figs. 1 A and 1D), demonstrating the functional activity of each transporter. As shown in Figs. 1B and 1E, vandetanib (0.08-100 μM) did not inhibit human OAT1-mediated [^{3}H]PAH uptake (Fig. 1B) and OAT3-mediated [^{3}H]E3S uptake (Fig. 1C), which was calculated after subtracting the uptake by the mock cells from the uptake by OAT1 or OAT3 cDNA-transfected cells. In contrast, probenecid inhibited OAT1-mediated [^{3}H]PAH uptake (Fig. 1C) and OAT3-mediated [^{3}H]E3S uptake (Fig. 1F) with IC_{50} values of 27.4 ± 5.6 μM and 18.7 ± 5.3 μM, respectively (Table 1). These results suggested that vandetanib is not an inhibitor of OAT1 and OAT3.

Vandetanib as an Inhibitor of MATE1, MATE2K and OCT2. Vandetanib was assessed as an inhibitor of OCT2, MATE1 and MATE2K employing MPP^+ and metformin as substrates (Tanihara et al., 2007; Han et al., 2010; Kusuhara et al., 2011).

The uptake of [^{3}H]MPP^+ (1 μM) and [^{14}C]metformin (1 μM) by OCT2-HEK cells was time-dependent and significantly greater than in mock vector transfected cells (Figs. 2A and 2D). As shown in Figs. 2B and 2E, vandetanib was able to reduce the intracellular accumulation of both [^{3}H]MPP^+ and [^{14}C]metformin in OCT2-HEK cells at concentrations higher than those observed in plasma (approximately 1.7 μM) following multiple (300 mg) doses. However, the inhibitory effect of vandetanib appeared to be substrate dependent (MPP^+ versus metformin, $P < 0.0001$; Table 1). Specifically, [^{3}H]MPP^+ transport was only weakly inhibited by vandetanib

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(IC_{50} value of 73.4 ± 14.8 μM) (Fig. 2 B), whereas [^{14}C]metformin transport was more sensitive to vandetanib (IC_{50} value of 8.8 ± 1.9 μM; Fig. 2 E). PYR, a known inhibitor of human OCT2, exhibited similar substrate-dependent inhibition on OCT2-mediated MPP^+ and metformin uptake (IC_{50} values of 23.6 ± 3.7 and 4.8 ± 1.0 μM, respectively; Figs. 2C and 2F, and Table 1).

In addition to OCT2-HEK cells, MATE1-HEK and MATE2K-HEK cells exhibited efficient intracellular accumulation of MPP^+ and metformin. As shown in Figs. 3A and 4A, MATE1- and MATE2K-mediated uptake of MPP^+ was significantly greater than in mock vector transfected cells, and was linear during the initial 10 min. After 10 min of incubation, [^{3}H]MPP^+ accumulated in MATE1-HEK and MATE2K-HEK cells to levels that were 10- and 11-fold, respectively, higher than that observed with Mock-HEK cells under the same conditions. Uptake of [^{14}C]metformin into MATE1-HEK and MATE2K-HEK cells at 10 min was approximately 20- to 40-fold higher than with Mock-HEK cells (Figs. 3D and 4D).

Unlike OCT2-mediated uptake, vandetanib inhibited both MATE1- and MATE2K-mediated uptake of [^{3}H]MPP^+ and [^{14}C]metformin at clinically relevant concentrations. Vandetanib was a potent inhibitor of transport of MPP^+ and metformin by MATE1-HEK cells with IC_{50} values of 1.23 ± 0.05 and 0.16 ± 0.05 μM, respectively (Figs. 3B and 3E, and Table 1). Similar IC_{50}s were obtained with MATE2K-HEK cells for both MPP^+ (1.26 ± 0.06 μM) and metformin (0.30 ± 0.09 μM) (Figs. 4B and 4E, and Table 1). Therefore, vandetanib demonstrates 29- to 60-fold higher inhibitory potency for MATE1 and MATE2K versus OCT2 ($P < 0.0001$; Table 1). Substrate-dependent inhibition by vandetanib was observed also with both MATE1 and MATE2K (MPP^+ versus metformin) ($P < 0.0001$; Table 1).

Vandetanib Significantly Decreases the Sensitivity of MATE1-HEK and MATE2K-HEK Cells to Cisplatin-Dependent Cytotoxicity. To evaluate the potential role of OCT2,

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MATE1, and MATE2K in governing the cytotoxicity of cisplatin and to determine whether vandetanib inhibition contributes to differences in activities and renal excretion of cisplatin, an attempt was made to determine the sensitivities ($IC_{50(CN)}$) of the variously transfected (OCT2, MATE1, or MATE2K) HEK cells to cisplatin in the presence and absence of vandetanib (2.5 μM) and PYR (2.5 or 50 μM). In the absence of an inhibitor, the IC_{50} values of cisplatin in OCT2-HEK, MATE1-HEK, and MATE2K-HEK cells after 3 days of drug exposure were all significantly lower than those in Mock-HEK cells (Fig. 5; Table 2). Resistance factor (RF), defined as the ratio of the $IC_{50(CN)}$ value in Mock-HEK cells to that in the corresponding transporter transfected cells, was 1.7, 2.1, and 2.8 for OCT2-HEK, MATE1-HEK, and MATE2K-HEK cells, respectively ($P < 0.05$; Table 2). Co-incubation with vandetanib (2.5 μM) significantly increased the $IC_{50(CN)}$ values of cisplatin in MATE1-HEK and MATE2K-HEK cells ($P < 0.05$), but had little effect in OCT2-HEK and Mock-HEK cells tested in parallel (Table 2 and Fig. 5). The RF values in the presence of vandetanib (defined as the ratio of the $IC_{50(CN)}$ value in the presence of vandetanib to that in Mock-HEK cells incubated under identical test conditions) were 1.6, 1.0, and 1.2 for OCT2-HEK, MATE1-HEK, and MATE2K-HEK cells respectively (Table 2). Vandetanib itself did not manifest any cytotoxicity up to a concentration of 10 μM under the same test conditions (data not shown). These results indicate that vandetanib (2.5 μM) inhibits the transport of cisplatin by MATE1 and MATE2K, but not OCT2, and thus ameliorates the cytotoxicity of cisplatin in the cells expressing either MATE1 or MATE2K. Similarly, co-incubation with PYR (2.5 μM) substantially increased the cisplatin $IC_{50(CN)}$ in MATE1-HEK and MATE2K-HEK cells (control versus vandetanib treated, 1.12 ± 0.13 vs. 2.28 ± 0.19 μM and 0.85 ± 0.09 vs. 2.23 ± 0.28 μM, respectively). In the presence of higher concentration of PYR (i.e., 50 μM), as shown in Table 2 and Fig. 5A, sensitivity of OCT2-HEK

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cells was substantially reduced, resulting in $IC_{50(CN)}$ value comparable with that in Mock-HEK cells (2.38 ± 0.31 vs. 2.39 ± 0.42 μM). PYR (50 μM) itself did not exhibit cytotoxicity under the same experiment conditions.

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Discussion

As described herein, vandetanib is able to inhibit the activity of various human renal transporters in vitro. The results indicate that vandetanib is a significantly more potent inhibitor of [^{3}H]MPP^{+} and [^{14}C]metformin uptake by MATE1- and MATE2K-HEK cells than by OCT2-HEK cells under the same conditions. Vandetanib can also modulate cisplatin-induced cytotoxicity in vitro by inhibiting MATE1 and MATE2K (Table 2). Moreover, vandetanib exhibits substrate-dependent inhibition (Table 1).

First, we examined the selectivity and inhibition potency of vandetanib in HEK293 cells stably transfected with 5 different renal transporters. Vandetanib did not affect the uptake by OAT1 and OAT3 up to a concentration of 100 μM (Fig. 1). In contrast, marked inhibition of uptake in MATE1-HEK and MATE2K-HEK cells was observed (Table 1). Given that vandetanib had no effect on both MPP^{+} and metformin uptake by OCT2-HEK cells at a clinically relevant concentration (i.e., 1.7 μM), and that vandetanib inhibited the uptake by MATE1- and MATE2K-HEK cells (IC_{50} values ranging from 0.16 to 1.26 μM), it is hypothesized that vandetanib can selectively inhibit the luminal efflux mediated by MATE1 and MATE2K without blocking the uptake process mediated by OCT2. This may be important, with respect to localized renal epithelium cytotoxicity, because of intracellular accumulation of toxic MATE1 and MATE2K substrates caused by vandetanib inhibition, even though their plasma levels may not be affected significantly.

Second, the effects of vandetanib on cisplatin-induced cytotoxicity in HEK293 cells stably transfected with human OCT2, MATE1, and MATE2K were investigated. The lower concentration of vandetinib tested (2.5 μM) did not impact the sensitivity of OCT2-overexpressing cells to cisplatin. On the other hand, PYR (50 μM), a well characterized OCT2

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inhibitor (Kusuhara et al. 2011; Ito et al. 2012), completely desensitized the OCT2-HEK cells to cisplatin compared to Mock-HEK cells (Table 2). Vandetanib itself exhibited cytotoxicity at a concentration of 50 μM under the same test conditions. As a result, the effect of this higher vandetanib concentration on the cytotoxicity could not be studied and demonstrated. Importantly, the lower concentration of vandetanib tested was well below the observed IC_{50} values describing the inhibition of OCT2-mediated uptake (i.e., 73.4 ± 14.8 μM and 8.8 ± 1.9 μM) and close to or above the IC_{50} values obtained with MATE1-HEK and MATE2K-HEK cells (0.16 to 1.26 μM). In agreement, the addition of vandetanib (2.5 μM) to MATE1-HEK and MATE2K-HEK cells after 3 days of culture almost completely decreased their sensitivity to cisplatin compared with mock-transfected cells (Figs. 5A and 5B, Table 2). Thus, for vandetanib, there was concordance between the ability to inhibit probe substrate accumulation into OCT2-HEK, MATE1-HEK, and MATE2K-HEK cells and the degree of desensitization of the cells to cisplatin. Likewise, the addition of PYR (2.5 μM), a potent MATE1 and MATE2K inhibitor, was cytoprotective when added to MATE1-HEK and MATE2K-HEK cells (Figs. 5A and 5B, Table 2).

In the present study, vandetanib was found to be a more potent inhibitor of MATE1-HEK and MATE2K-HEK cells compared to OCT2-HEK cells. The results complement those of Minematsu and Giacomini (2011), who evaluated eight kinase inhibitors as OCT2, MATE1 and MATE2K inhibitors employing metformin as substrate (HEK293 cells). Three of the kinase inhibitors (gefitinib, erlotinib, and lapatinib) are variously substituted quinazolines, similar to vandetinib (supplement Fig. 1). As reported herein, the metformin IC_{50} ratio (OCT2/MATE) for vandetinib with MATE1-HEK and MATE2K-HEK was 55 and 29, respectively (Table 1). The same ratios for gefitinib, which is most structurally similar to vandetinib, were 13 and 128, respectively (Minematsu and Giacomini, 2011). The two remaining quinazolines exhibited a

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different profile. For example, the metformin IC_{50} ratio was ~1.0 for erlotinib with both MATE1 and MATE2K, and only weak inhibition of OCT2, MATE1 and MATE2K (IC_{50} > 30 μM) was observed with lapatinib. So it is possible that these various kinase inhibitors exhibit differential effects on creatinine, metformin and cisplatin clearance. Interestingly, like vandatenib, gefitinib and sunitinib are known to be potent inhibitors of both MATE1 and MATE2K in vitro and increase plasma creatinine levels in cancer patients (Petrylak et al., 2010; Khan et al., 2010).

Although the protein expression levels of MATE1-, MATE2K-, and OCT2-HEK cells have not been measured due to lack of specific antibodies, quantitative real-time polymerase chain reaction (RT-PCR) analysis indicated that the expression of MATE1 and MATE2K was comparable to that of OCT2 in the HEK293 cells (data not shown). To some extent, this permits a comparison across transporters in vitro (inhibition of OCT2 versus MATE1 and MATE2K). Consideration of OCT2 as well as MATE1 and MATE2K is important when attempting to understand DDIs and their consequences for drugs that are cleared renally. Both OCT2 at the basolateral membrane and MATE1 and MATE2K at the apical membrane of proximal tubule cells play an important role in vectorial transport and govern intracellular and circulating concentrations of organic cation compounds such as metformin and cisplatin (Morrissey et al., 2013; Motohashi et al., 2013). In some cases, the inhibition of MATE1 and MATE2K at the apical membrane likely increases drug concentrations within the renal cells, resulting in enhanced renal toxicity. Similar to vandetanib, cimetidine is a more potent inhibitor of MATE1 and MATE2K than OCT2, and cimetidine at low concentration inhibits apical MATE1 rather than basolateral OCT2 (Tsuda et al., 2009). Likewise, in vitro inhibition potency values against cell lines indicate that PYR is a more potent inhibitor of MATE1- and MATE2K-HEK cells (P < 0.0001; Table 1), which is consistent with recent reports suggesting that they serve as the loci of

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the PYR-metformin and PYR-N-methylnicotinamide DDI (Kusuhara et al., 2012; Ito et al., 2012).

We observed that vandetanib was a more potent inhibitor of transport of metformin by OCT2-, MATE1- and MATE2K-HEK cells compared to MPP^+ transport (MPP^+/metformin IC_{50} ratio 4.2 to 8.3; $P < 0.0001$, Table 1). This significant substrate dependence was also observed with PYR ($P < 0.0001$; Table 1). For OCT2 (metformin K_m of 990 µM; MPP^+ K_m of 2.7 to 24.5 µM), MATE1 (metformin K_m of 780 µM; MPP^+ K_m of 100 µM) and MATE2K (metformin K_m of 1980 µM; MPP^+ K_m of 110 µM), the result cannot be explained solely in terms of the reported differences in K_m values; (Kimura et al., 2005; Tanihara et al., 2007). In all cases, IC_{50}s were generated at a low concentration of substrate (1 µM; < K_m). In agreement, substrate-dependent inhibition (MPP^+ versus metformin) of OCT2 has been observed. For example, Zolk et al (2009) compared the potency of seven OCT2 inhibitors by plotting IC_{50} values for the inhibition of MPP^+ uptake versus metformin uptake, and revealed a significant linear correlation (r = 0.92) with a mean MPP^+/metformin IC_{50} ratio of ~4 (ranging from 2 to 14). In order to further explore OCT2 substrate dependency, an attempt was made to interrogate the University of Washington Metabolism and Transport Drug Interaction Database. It was possible to download IC_{50} data for 16 different inhibitors with the two substrates. As shown in supplement Fig. 2, despite the IC_{50} values being generated by various laboratories under different experiment conditions, a good correlation was observed, with IC_{50}s for MPP^+ trending higher (mean MPP^+/metformin IC_{50} ratio ~3; r = 0.88; MPP^+/metformin IC_{50} ratios ranging from 1 to 35). More recently, substrate-dependent inhibition has been reported for MATE1 also (Martinez-Guerrero and Wright, 2013).

In summary, we have shown that vandetanib is a relatively potent inhibitor of substrate transport by MATE1-HEK and MATE2K-HEK cells (versus OCT2-HEK cells) in vitro.

Inhibition of the two transporters may explain why there are reports of decreased creatinine clearance, and increased cisplatin nephrotoxicity, in some subjects receiving vandetanib (Blackhall et al., 2010; US Department of Health and Human Services; Caprelsa [vandetanib] safety label, 2012). In agreement with numerous reports, the results also indicate that the potency of OCT2, MATE1 and MATE2K inhibition in vitro is substrate-dependent. Although such a finding needs to be confirmed clinically, it implies that one has to be judicious when choosing a renal transporter probe to support a clinical DDI study, when attempting in vitro-in vivo extrapolations and modeling DDIs involving renal transporters, and when interpreting renal transporter (biomarker) data.

Authorship Contributions

Participated in research design: Shen, Yang, and Rodrigues.

Conducted experiments: Shen.

Contributed new reagents or analytic tools: Shen, Zhao, and Zhang.

Performed data analysis: Shen, Yang and Rodrigues.

Wrote or contributed to the writing of the manuscript: Shen and Rodrigues.

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Footnotes

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Figure Legends

Fig. 1. Effects of vandetanib and probenecid on the uptake of PAH by OAT1-HEK cells and uptake of E3S by OAT3-HEK cells. Time-dependent uptake of 1 μM [^{3}H]PAH (A) and 1 μM [^{3}H]E3S (D) in HEK293 cells stably transfected with human OAT1 or OAT3 (closed triangle), and vector only (open triangle). The OAT1- and OAT3-mediated uptake labeled probe substrate (closed circle) was examined by subtracting uptake in mock cells from that observed with OAT1- and OAT3-transfected cells. Experiments were conducted with a labeled probe substrate alone or in the presence of 0.08 to 100 μM vandetanib (B and E) or 0.14 to 100 μM probenecid (C and F). Values shown are mean ± S.D. for experiments performed in triplicate.

Fig. 2. Effects of vandetanib and PYR on the uptake of MPP^+ and metformin by OCT2-HEK cells. Time-dependent uptake of 1 μM [^{3}H]MPP^+ (A) and 1 μM [^{14}C]metformin (D) in HEK293 cells stably transfected with human OCT2 (closed triangle), and vector only (open triangle). The OCT2-mediated uptake of probe substrate (closed circle) was examined by subtracting the uptake in mock cells from that in OCT2-transfected cells. Experiments were conducted with a labeled probe substrate alone or in the presence of 0.08 to 100 μM vandetanib (B and E) or 0.14 to 100 μM PYR (C and F). Values shown are mean ± S.D. for experiments performed in triplicate.

Fig. 3. Effects of vandetanib and PYR on the uptake of MPP^+ and metformin by MATE1-HEK cells. Time-dependent uptake of 1 μM [^{3}H]MPP^+ (A) and 1 μM [^{14}C]metformin (D) in HEK293 cells stably transfected with human MATE1 (closed triangle), and vector only (open triangle). The MATE1-mediated uptake of probe substrate (closed circle) was examined by subtracting

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uptake in mock cells from that in MATE1-transfected cells. Experiments were conducted with a labeled probe substrate alone or in the presence of 0.08 to 100 μM vandetanib (B and E) or 0.02 to 5 μM PYR (C and F). Values shown are mean ± S.D. for experiments performed in triplicate.

Fig. 4. Effects of vandetanib and PYR on the uptake of MPP^+ and metformin by MATE2K-HEK cells. Time-dependent uptake of 1 μM [^{3}H]MPP^+ (A) and 1 μM [^{14}C]metformin (D) in HEK293 cells stably transfected with human MATE2K (closed triangle), and vector only (open triangle). MATE2K-mediated uptake of probe substrate (closed circle) was examined by subtracting uptake in mock cells from that in MATE2K-transfected cells. Experiments were conducted with a labeled probe substrate alone or in the presence of 0.08 to 100 μM vandetanib (B and E) or 0.02 to 5 μM PYR (C and F). Values shown are mean ± S.D. for experiments performed in triplicate.

Fig. 5. Effects of vandetanib and PYR on cisplatin-induced cytotoxicity in OCT2- (A), MATE1- (B), and MATE2K- (C) transfected cells. The HEK293 cells stably transfected with transporter cDNA (or vector only cells) were cultured for 3 days at 37°C in the absence and presence of vandetanib (2.5 μM), or PYR (50 or 2.5 μM), with increasing amounts of cisplatin. Cell density was then evaluated by the XTT method (see Materials and Methods). Values shown are mean ± S.D. for experiments performed in quadruple.

TABLE 1

Inhibition of substrate uptake into transporter-transfected HEK293 cells

Inhibitor	IC_{50} (μM)[a]							
	OAT1	OAT3	OCT2		MATE1		MATE2K	
	PAH[b]	E3S	MPP^+	Metformin	MPP^+	Metformin	MPP^+	Metformin
Vandetanib	> 100	> 100	73.4 ± 14.8	8.8 ± 1.9£	1.23 ± 0.05†	0.16 ± 0.05‡£	1.26 ± 0.06†	0.30 ± 0.09‡£
					(60)[c]	(55)	(58)	(29)
Probenecid	27.4 ± 5.6	18.7 ± 5.3	ND	ND	ND	ND	ND	ND
PYR	ND	ND	23.6 ± 3.7	4.8 ± 1.0£	0.63 ± 0.07†	0.11 ± 0.02‡£	0.52 ± 0.17†	0.10 ± 0.02‡£
					(37)[c]	(44)	(45)	(48)

Table 1 Legend:

[a]IC_{50} value obtained at a final substrate concentration of 1 μM (see Materials and Method). Data are reported as mean ± S.D. (n = 3 determinations).

[b]Substrate used to generate IC_{50}.

[c]Number in parentheses represents the IC_{50} ratio (OCT2 versus MATE1 or MATE2K).

†Statistically significant different compared with IC_{50} of MPP^+ uptake in OCT2-HEK cells ($P < 0.0001$).

‡Statistically significant different compared with IC_{50} of metformin uptake in OCT2-HEK cells ($P < 0.0001$).

£Statistically significant different compared with IC_{50} of MPP^+ uptake in same cell line ($P < 0.0001$).

PYR: pyrimethamine.

E3S: estrone-3-sulfate.

PAH: p-aminohippuric acid.

MPP^+: 1-methyl-4-phenylpyridinium

TABLE 2

Effect of vandetanib and PYR on the cytotoxicity of cisplatin when added to variously transfected HEK293 cells[a]

Drug	Mock-HEK	OCT2-HEK		MATE1-HEK		MATE2K-HEK	
	$IC_{50(CN)}$	$IC_{50(CN)}$[c]	RF[b]	$IC_{50(CN)}$	RF[b]	$IC_{50(CN)}$	RF[b]
Cisplatin only	2.34 ± 0.31	1.36 ± 0.19	1.7	1.12 ± 0.13	2.1	0.85 ± 0.09†	2.8
Cisplatin + vandetanib (2.5 μM)	2.31 ± 0.31	1.47 ± 0.24	1.6	2.39 ± 0.44‡	1.0	1.99 ± 0.16‡	1.2
Cisplatin + PYR (2.5 μM)	ND	ND	-	2.28 ± 0.19	1.1	2.23 ± 0.28‡	1.1
Cisplatin + PYR (50 μM)	2.39 ± 0.42	2.38 ± 0.31	1.0	ND	-	ND	-

Table 2 Legend:

[a]HEK293 cells stably transfected with transporter (or vector only) were treated with various concentrations of cisplatin in the presence or absence of vandetanib or PYR. After 3 days, cells were counted and $IC_{50(CN)}$ values were generated (see Materials and Methods). Data are reported as mean ± S.D. (n = 4 determinations).

[b]Resistance factor (RF); $IC_{50(CN)}$ after addition of cisplatin to HEK293 cells stably transfected with transporter versus the $IC_{50(CN)}$ after addition of cisplatin (alone) to vector only (mock) transfected HEK293 cells.

[c]$IC_{50(CN)}$; concentration of cisplatin that decreases cell number by 50%.

†Statistically significant left shift in $IC_{50(CN)}$ versus cisplatin alone in Mock-HEK cells ($P < 0.05$).

‡For MATE1-HEK and MATE2K-HEK cells, there was a statistically significant right shift in $IC_{50(CN)}$ versus cisplatin alone in MATE1-HEK and MATE2K-HEK ($P < 0.05$).

PYR: pyrimethamine.

ND: Not determined.

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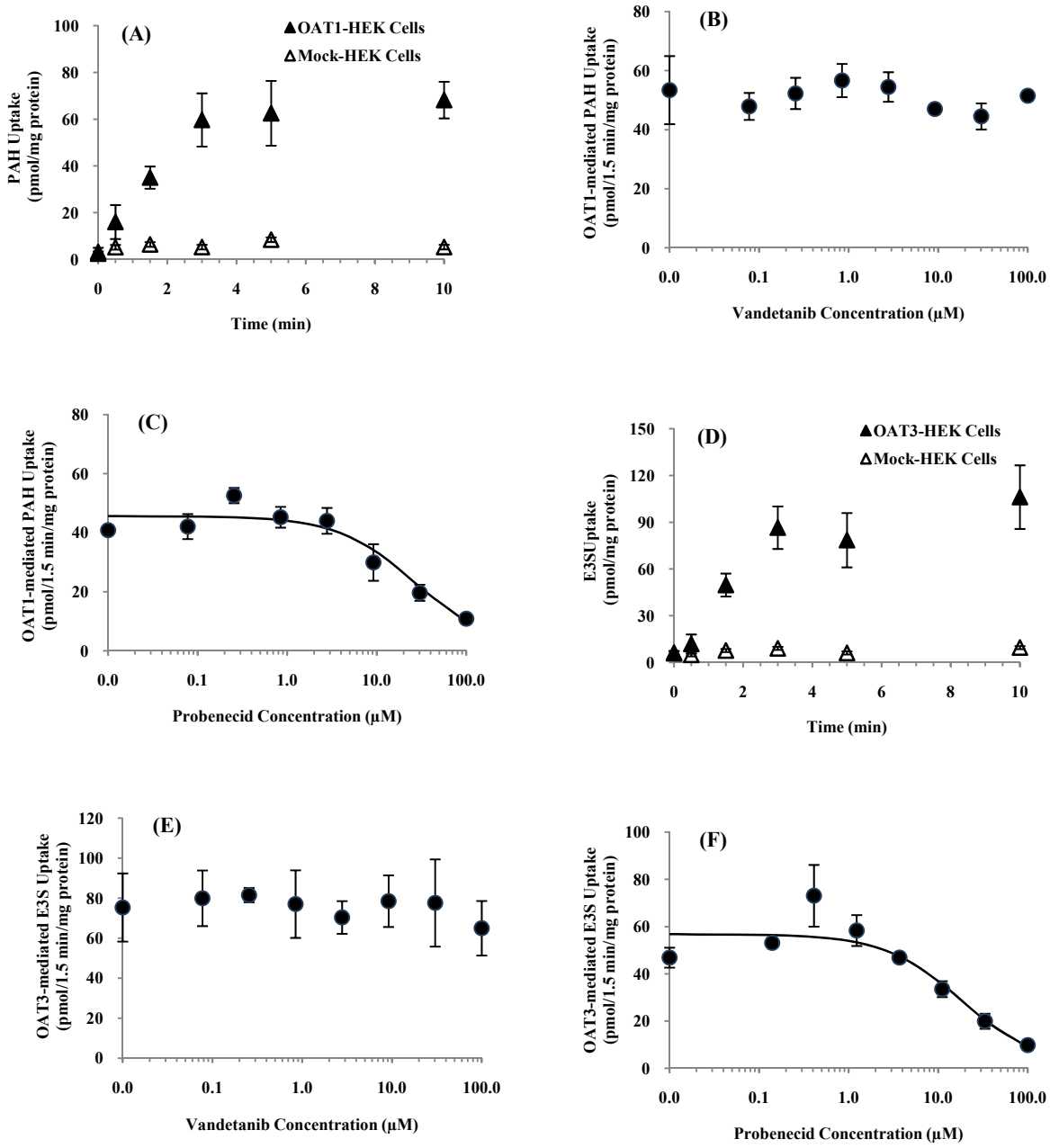

Figure 1

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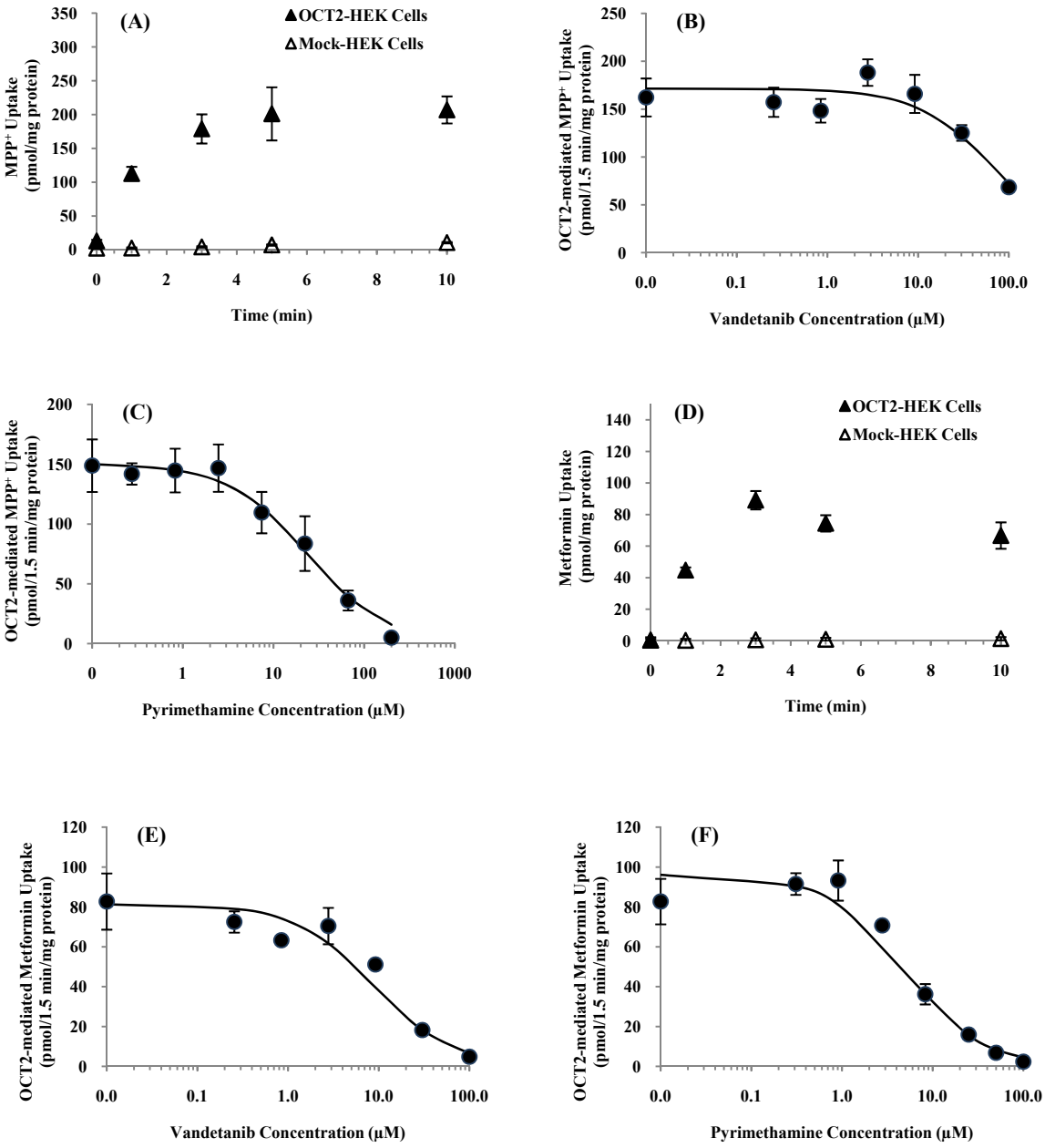

Figure 2

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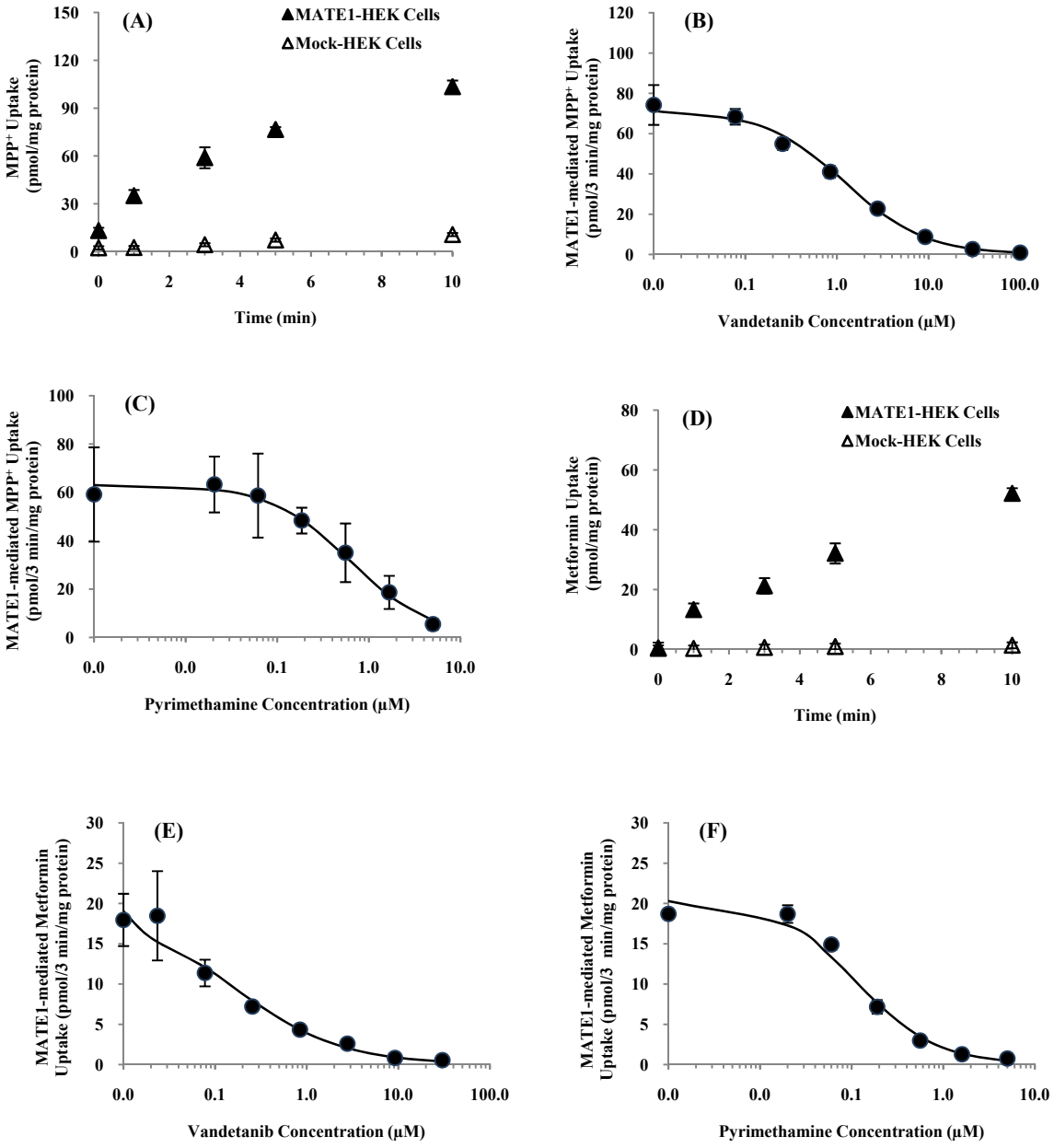

Figure 3

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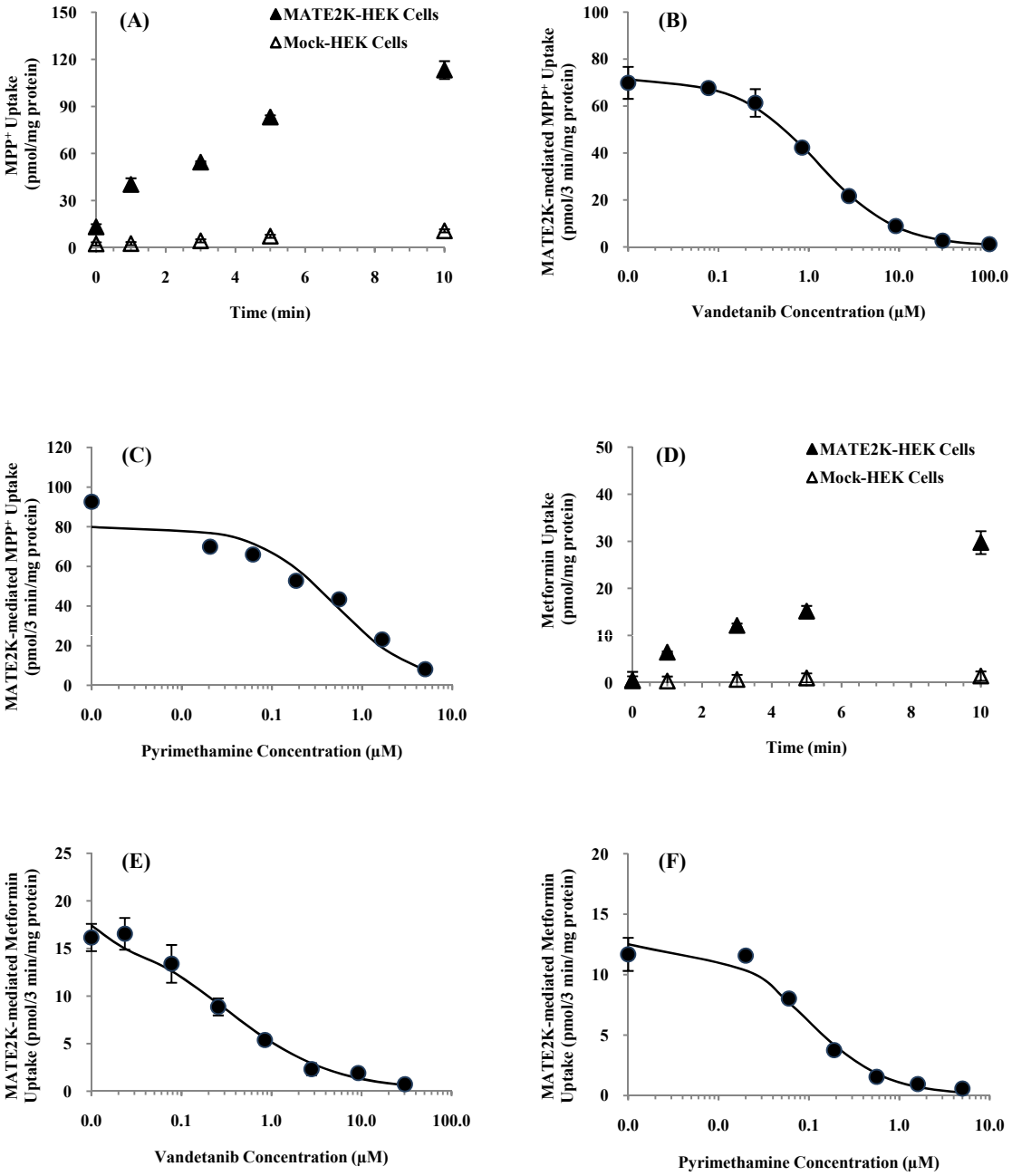

Figure 4

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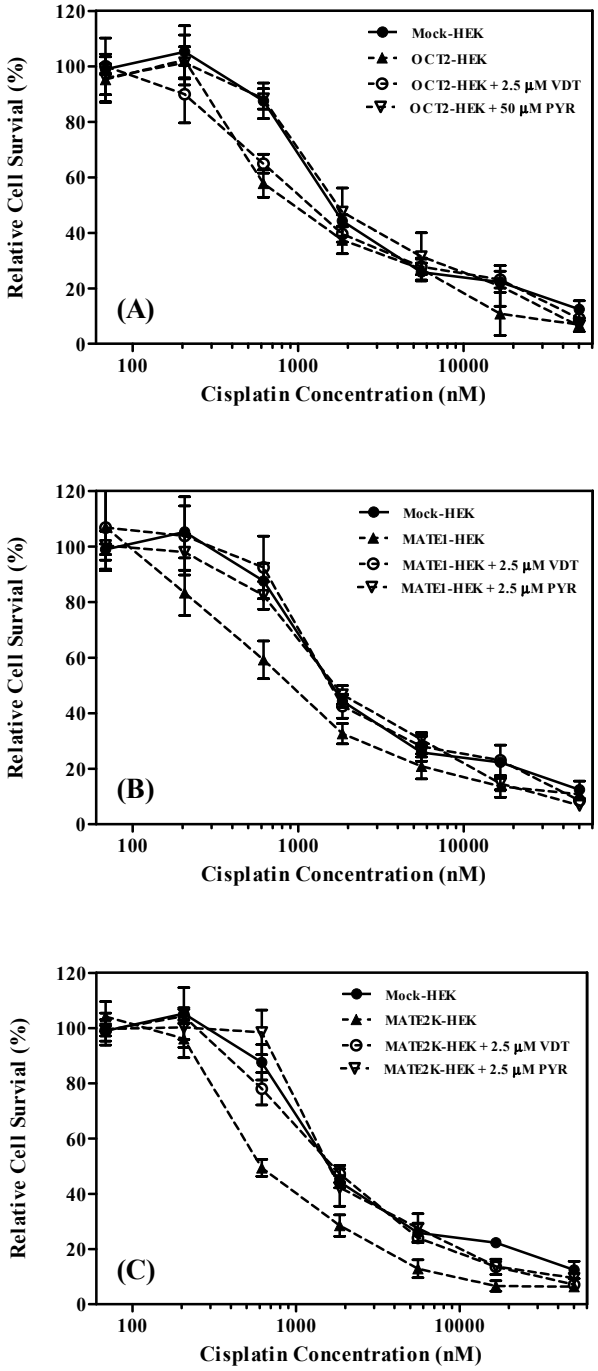

Figure 5