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Proton pump inhibitors inhibit methotrexate transport by renal basolateral organic anion transporter hOAT3.

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Running title page: Drug interaction between MTX with PPIs and renal transporters.

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Methotrexate (MTX), Proton Pump Inhibitors (PPIs), Human Organic Anion Transporters (hOATs), P-Aminohippuric Acid (PAH), Estrone Sulfate (ES), human Embryonic Kidney (HEK).

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Abstract:

The co-administration of methotrexate (MTX) and proton pump inhibitors (PPIs) can result in a pharmacokinetic interaction that delays MTX elimination and subsequently increases the MTX blood concentrations. Human organic anion transporters (hOATs) are responsible for the renal tubular secretion of MTX and are thought to be involved in this drug interaction. The aim of this study was to evaluate the inhibitory potencies of PPIs on hOAT1 and hOAT3, which are the two isoforms of OATs predominantly expressed in kidney proximal tubules. Using stably transfected cell systems that express the uptake transporters HEK-hOAT1 and HEK-hOAT3, we analyzed the inhibitory potencies of omeprazole, lansoprazole and pantoprazole on OAT-mediated [^{3}H]ES, [^{3}H]PAH and [^{3}H]MTX uptake *in vitro*. hOAT3 is a high affinity transporter for MTX (Km = 21.17 ± 5.65 μM). Omeprazole, lansoprazole and pantoprazole inhibited [^{3}H]MTX uptake in HEK-hOAT3 cells with an IC_{50} of 6.8 ± 1.16 μM, 1.14 ± 0.26 μM and 4.45 ± 1.62 μM, respectively, and inhibited the [^{3}H]ES uptake in HEK-hOAT3 cells with an IC_{50} of 20.59 ± 4.07 μM, 3.96 ± 0.96 μM and 7.89 ± 2.31 μM, respectively. Furthermore, omeprazole, lansoprazole and pantoprazole exhibited inhibited PAH uptake on hOAT1 in a concentration-dependent manner (IC_{50} = 4.32 ± 1.26 μM, 7.58 ± 1.06 μM and 63.21 ± 4.74 μM, respectively). These *in vitro* results suggest that PPIs inhibit [^{3}H]MTX transport via hOAT3 inhibition, which most likely explains the drug-drug interactions between MTX and PPIs and should be considered for other OATs substrates.

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Introduction:

Methotrexate (MTX), an antifolate drug, is used in a wide range of doses for the treatment of certain neoplastic diseases, severe psoriasis, and rheumatoid arthritis (Jolivet et al., 1983; Tugwell et al., 1987). High-dose MTX is widely accepted as the first line treatment for lymphoid malignancy, osteogenic sarcoma and acute leukemia, with intravenous doses ranging from 300 mg/m^2 to 12 g/m^2. MTX is a highly toxic drug with a low therapeutic index. The therapeutic drug monitoring of MTX is essential to prevent toxicity from high plasma MTX concentrations, because delayed elimination can result in serious and potentially life-threatening toxicities.

Renal excretion is the primary route of MTX elimination. In humans 80% to 90% of the IV administered dose is excreted unchanged in the urine within 24 hours (Shen and Azarnoff, 1978). Renal excretion occurs via glomerular filtration and active tubular secretion mediated in proximal tubular cells uptake, followed by active efflux in tubular lumen. Organic anion transporters (OATs) are responsible for the passage from the blood to proximal tubules (uptake). Many transporters of organic anionic drugs have been identified on the apical side of the human kidney epithelium, including multidrug-resistance-related protein (MRP2/ABCC2, MRP4/ ABCC4) and breast cancer resistance protein (BCRP/ ABCG2), which are responsible for the secretion into the urine (Burckhardt and Burckhardt, 2003; Launay-Vacher et al., 2006; Nozaki et al., 2007; Takeda et al., 2002a; VanWert and Sweet, 2008).

Among OATs, hOAT1 and hOAT3 localize to the basolateral membrane of proximal tubular epithelial cells and have been shown to transport MTX (Nozaki et al., 2007; Rizwan and Burckhardt, 2007; Uwai et al., 1998). Members of the OAT family transport a variety of endogenous substances and drugs, including antineoplastic agents, antiviral agents, ß-lactam-antibiotics, diuretics and ACE inhibitors (Takeda et al., 2002b; Uwai et al., 2007; Vallon et al., 2008; Vanwert et al., 2008).

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Several drugs, including non-steroidal anti-inflammatory drugs (NSAIDs) (Maeda et al., 2008; Nozaki et al., 2004; Uwai et al., 2004; Yoshitane et al., 2007), penicillin G (Takeda et al., 2002b) and probenecid (Aherne et al., 1978), are known to inhibit the elimination of MTX. The molecular mechanism underlying these interactions partially relies on the blockade of the renal secretion of antifolate via the basal uptake transporters hOAT3 and hOAT1 (Giacomini et al., 2010).

Over the past few years, several case reports in oncology (Bauters et al., 2008; Beorlegui et al., 2000; Reid et al., 1993; Troger et al., 2002) and two retrospective cohort studies (Santucci et al., 2010; Suzuki et al., 2009) have suggested that the co-administration of PPIs, including omeprazole, pantoprazole, lansoprazole and rabeprazole, decreased the renal clearance of MTX. The elimination of MTX was significantly delayed during cycles with one PPI but normalized during subsequent cycles after PPI discontinuation or substitution with ranitidine.

Because proton pump inhibitors (PPIs) are frequently used among patients treated with MTX for cancer or autoimmune diseases, we aimed to investigate the drug-drug interaction of MTX with PPIs.

To elucidate the PPI-MTX drug interaction, we used cell systems that stably express the human uptake transporters OAT1 and OAT3 and investigated the effect of the three more commonly prescribed PPIs (omeprazole, lansoprazole and pantoprazole) on the uptake of OAT substrates.

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Materials and Methods:

Radiolabeled: [^{3}H]estrone sulfate ([^{3}H] ES) (250 µCi; 9.25 MBq); (54.26 Ci/mmol, 2.00762 TBq/mmol); 99.5 % purity; [^{3}H] p-Aminohippurate acid ([^{3}H]PAH) (1 mCi/ml; 37 mBq) (4.53 Ci/mmol, 167.61 GBq/mmol); 99 % purity, were purchased from Perkin Elmer® **(**Massachusetts, USA). [^{3}H] Méthotrexate ([^{3}H] MTX) (250 µCi; 9.25 MBq); (32.3 Ci/mmol); > 99 % purity, was purchased from Moravek Biochemicals® (California, USA).

Unlabeled: P-Aminohippuric Acid (PAH) and Estrone Sulfate (ES) uptake, which are both well-established substrates of hOAT1 and hOAT3 respectively (Burckhardt, 2012), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methotrexate ((2S)-2-[(4-[[(2,4-diaminopteridin-6-yl)methyl](methyl)amino]phenyl)formamido]pentanedioic acid) was purchased from Interchim® (Montluçon, France).

Inhibitory chemicals: probenicid (4-(dipropylsulfamoyl)benzoic acid), ibuprofen (2-[4-(2-methylpropyl)phenyl]propanoic acid), omeprazole (6-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methane]sulfinyl]-1H-1,3-benzodiazole), lansoprazole (2-([[3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl]methane]sulfinyl)-1H-1,3-benzodiazole) and pantoprazole (6-(difluoromethoxy)-2-[[(3,4-dimethoxypyridin-2-yl)methane]sulfinyl]-1H-1,3-benzodiazole) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All unlabeled solid compounds were dissolved in Dimethylsulfoxyde Organic solvent (DMSO). The concentration of DMSO in the final study medium was limited to 1% in presence or absence of inhibitors.

Scintillation fluid, Ultima-Gold® from Perkin Elmer Life Science (Boston, MA, USA).

Triton X-100, DMSO and Bicinchoninic acid (BCA) assay kits were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagles Medium (DMEM), phosphate-buffered saline (PBS), penicillin, streptomycin, zeocin, hygromycin B, were purchased from

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Gibco Invitrogen (Cergy-Pontoise, France). Fetal bovine serum (SVF) was purchased from PAA Laboratories (Vélizy Villacoublay, France). FuGENE 6 Transfection Reagent was from Roche Applied Science (Meylan, France).

Cell culture and transfection:

Stably transfected HEK cell lines were established by using the Flp-In expression system, Invitrogen® Carlsbad, USA, according to the manufacturer's protocol. HEK-293 cells were routinely grown in Dulbecco's modified Eagle's medium containing (D-MEM) containing 10% fetal calf serum and 1% Streptomycin/Ampicillin in a humidified incubator at 37°C and 5% CO_2. Briefly, in separate reactions, the cDNAs, including the open reading frames *hOAT1* or *hOAT3*, were subcloned into the Flp-In expression vector pcDNA5/FRT, which contained a FRT site linked to a hygromycin resistance gene. The constructs pcDNA5/FRT-hOAT1 and pcDNA5/FRT-hOAT3 constructs were then cotransfected with the Flp recombinase expression vector pOG44 into Flp-In HEK-293 cells. Cells stably expressing the transporters were selected in hygromycin (100 µg/ml) according to the manufacturer's protocol. The cells were grown in flasks cultured in D-MEM supplemented with 10% fetal bovine serum and hygromycin (100 µg/ml). Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were split in a 1:5 ratio every 3 to 4 days.

The function of hOAT1 and hOAT3 was evaluated using HEK293 cells stably transfected with pcDNA5/FRT vector containing hOAT1 and hOAT3 cDNA or empty vector, named HEK-hOAT1, HEK-hOAT3 and HEK-mock respectively.

Transport uptake experiments:

Cells were seeded on Poly-D-lysine-coated 12-well plates BD Biocoat from Becton, Dickinson Company (New Jersey, USA) at a density of 4×10^5 cells/well and grown for 2 days

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(37°C/5% CO2) in the absence of antibiotics. Prior to the initiation of transport experiments, the culture medium was removed, and the cells were washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min. The Krebs-Henseleit buffer (KHB) consisted of 118 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 26 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 5 mM glucose and 12.5 mM HEPES adjusted to pH 7.4.

The equilibration medium was removed before a final application of 400 µl of incubation solution per sample (buffer containing the radiolabeled compounds) in the presence or absence of prototypic OATs inhibitors used as positive control probenicid and ibuprofen. [^{3}H]ES or [^{3}H]PAH uptake was measured for 10 min in HEK cells expressing hOAT3 or 2 min in HEK-hOAT1 within the linear uptake phase. We validated the cells systems using [^{3}H]ES (10 nM) uptake in hOAT3 cells in the absence (no inhibitor) or presence of probenicid and ibuprofen and [^{3}H]PAH (50 nM) uptake in hOAT1 cells in the absence or presence of probenicid and ibuprofen. For concentration-dependant inhibition studies, PPIs were used in the following concentrations: 1; 2.5; 5; 10; 25; 50 and 100µM. After incubation at 37°C for the specified times, the uptake solutions were removed, and the cells were rapidly rinsed three times with 750µl of ice-cold PBS. The cells were dissolved in 500 µl of 1 M NaOH and neutralized after one hour with 500 µl of 1 M HCl and the radioactivity of the aliquots was determined in 4 mL of Ultima-Gold a scintillation fluid using a scintillation counter (Liquid Scintillation Counter, Tri-carb 2900TR, Perkin Elmer®, Inc. Massachusetts, USA). The cellular protein content was determined using the BCA-protein quantification system. Uptake was then normalized to the protein content in the lysates.

Transformations for kinetic calculations were performed using GraphPad Prism® software version 4 (GraphPad® Software, San Diego, CA), and the *K*m and *V*max values were calculated from the x and y intercepts of the Lineweaver-Burk plot, respectively. The *K*i values were calculated assuming competitive inhibition.

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Western blotting analysis:

Total proteins were extracted from the pellets containing HEK293 cells by homogenizing the pellets in TENTS (10 mM Tris-HCl at pH 7.4, 5 mM EDTA at pH 8, 126 mM NaCl, 1% (v/v) Triton X-100, and 0.1% (v/v) SDS) supplemented with leupeptin, aprotinin, pepstatin, and phenyl methane sulfonyl fluoride (Sigma-Aldrich®, Inc. St. Louis, USA). The suspensions were gently agitated for 1 h at 4°C and then centrifuged at 12000x g at 4°C for 20 min. The protein content of the supernatant was determined using the BCA assay. Next, 25µg of protein was separated by electrophoresis using the NuPage Novex Bis Tris MiniGels (Invitrogen®. Carlsbad, USA) according to the manufacturer's protocol and transferred electrophoretically onto nitrocellulose membranes. Free binding sites on the membranes were blocked by incubation with Tris-buffered saline containing 0.1% of Tween-20 (TTBS) and 10% nonfat dried milk for 1 h at 20–25°C. The membranes were washed with TTBS and incubated with primary antibodies (Anti-hOAT3 rabbit OAT3 (P-13): sc-107836 were purchased from Santa Cruz Biotechnology®, Inc. California, U.S.A.) and (Anti-hOAT1 rabbit were purchased from Sigma-Aldrich, Inc. St. Louis, USA) for overnight at 4°C. The primary antibodies were diluted 1: 500. The membranes were then washed with TTBS (5 times for 10 min each) and then incubated with secondary antibodies diluted at 1: 1000 for 1 h at 20–25°C. The secondary antibodies were purchased from Dako® (Glostrup, Denmark).

The membranes were washed again (5 times for 10 min) with TTBS and probed with the Western Lightning Chemiluminescence Reagent (Perkin Elmer®, Massachusetts, USA).

Quantitative real-time PCR:

Quantitative real-time PCR, for hOAT1 and hOAT3. RNA prepared from HEK Mock, HEK-hOAT1 and HEK-hOAT3 was purified on RNeasy columns (Qiagen®, Valencia, CA) and

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then reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche® Applied science) using oligo-dT as a primer. Each cDNA sample was subjected to duplicate real-time PCR reactions using a CFX96 (Bio-Rad®) thermal cycler with the following conditions: initial denaturation (95°C for 30s) followed by 44 cycles of denaturation (95°C for 2s), hybridization- extension (60°C for 5 s).

Gene expression values were normalized to that of GAPDH in the corresponding cDNA samples.

Kinetic Analyses:

Transformations for kinetic calculations were performed using GraphPad Prism® software version 4 (GraphPad® Software, San Diego, CA), and the Km and Vmax values were calculated from the x and y intercepts of the Lineweaver-Burk plot, respectively. The IC_{50} values were calculated assuming competitive inhibition. The kinetic parameters were obtained using the following Michaelis-Menten equation: one saturable component,

$$\mathrm{V} = \frac{\mathrm{Vmax}\,[\mathrm{S}]}{\mathrm{Km} + [\mathrm{S}]}$$

Where *v* is the uptake velocity of the substrate (pmoles per milligram of protein per minute). *S* is the substrate concentration of the medium (micromolar). *K*m is the Michaelis constant (micromolar). *V*max is the maximal uptake velocity (picomoles per milligram of protein per minute).

Statistics:

The uptake experiments were performed in triplicate, where the values are expressed as the mean of these replicates with error bars represent the standard error (SE). All experiments

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were performed at least three times, over 3 independent experiments in triplicate. Statistical significance was calculated by using unpaired Student's test. Differences were considered statistically significant if p values < 0.05.

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Results:

Characterization of hOAT1 and hOAT3-expressing HEK Cells:

To test the inhibitory potencies of PPIs in vitro, we stably transfected HEK cells with cDNAs encoding human OAT1, the SLC22A6 gene, or human OAT3, the SLC22A8 gene. We validated these models by examining the presence of the proteins and the function of HEK-hOAT1 or HEK-hOAT3 transfected cells. The gene and protein expression levels of hOAT1 and hOAT3 were evaluated with quantitative real-time PCR and western blot analysis. The respective recombinant OAT proteins were detected in the membrane fractions from OAT-expressing HEK cells, but not in the HEK-Mock control cells, at molecular masses of 60 kDa in the membrane fractions from hOAT1-expressing HEK cells and 62 kDa in the membrane fractions obtained from hOAT3-expressing HEK cells (Supplementary file). The quantitative real-time PCR analysis demonstrated *SLC22A6* and *SLC22A8* mRNA expression in HEK hOAT1 and HEK hOAT3 clones, respectively, which were not detected in the vector-transfected HEK Mock cells.

Both transfected HEK cell lines expressed functionally active organic anion transporters, as demonstrated by the time-dependent PAH and ES uptake, which are both well-established substrates of hOAT1 and hOAT3, respectively (Supplementary file). The stably transfected hOAT1 and hOAT3 expressing cell lines also accumulated significantly more standard substrates ([^{3}H] p-Aminohippurate acid ([^{3}H]PAH) for HEK-hOAT1 and [^{3}H]estrone sulfate ([^{3}H]ES) for HEK-hOAT3) than the control cells. The estimated Km values of PAH uptake by hOAT1 and uptake of ES by hOAT3 were 15.18 ± 1.93 µM and 28.32 ± 7.11 µM, respectively [Fig.1 (A)]. Similar to previous *in vitro* studies, probenecid and ibuprofen inhibited all mediated transport. Probenicid significantly inhibited the uptake of [^{3}H]PAH by HEK-hOAT1 and uptake of [^{3}H]ES by HEK-hOAT3, with IC_{50} values of 9.02 ± 2.28 µM and 0.76 ± 0.28 µM, respectively [Fig.1 (B)]. In addition, ibuprofen inhibited these uptakes

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with IC_{50} values of 1.45 ± 1.54 µM for hOAT3 and IC_{50} = 15.74 ± 4.35 µM for hOAT1 (Supplementary file).

MTX uptake:

To evaluate the uptake of MTX in HEK-hOAT1 and HEK-hOAT3, the cells were incubated in a solution containing 0.5 µM, MTX (for HEK-hOAT1) [Fig. 2 (A)] or 25 nM MTX (for HEK-hOAT3) [Fig. 2 (B)]. The affinity of MTX for hOAT3 was higher than that for hOAT1 [Fig. 2 (A)]. The Km values of MTX uptake by hOAT3 was 21.17 ± 5.65 µM [Fig. 2 (C)]. The higher concentration tested on HEK-hOAT1 was 0.5 µM, with an accumulation of MTX in HEK-hOAT1, which was approximately 2 fold higher than that in the control cells. The Km was not determined for HEK-hOAT1, because the difference in the accumulation of MTX was too low (data not shown). The transporter-mediated uptake of [^{3}H]MTX over time in HEK-hOAT3 is presented in [Fig. 2 (B)] and was linear up to 10 min.

Inhibition of hOAT1 and hOAT3 mediated transport by PPIs:

The inhibition of hOAT1 and hOAT3 uptake of their specific substrate by PPIs was measured within the linear uptake phase.

Regarding the inhibition of [^{3}H]PAH uptake by hOAT1, omeprazole, lansoprazole and pantoprazole inhibited the transport of PAH in HEK-hOAT1 in a concentration-dependent manner, with IC_{50} values of IC_{50} = 4.32 ± 1.26 µM, 7.58 ± 1.06 µM and 63.21 ± 4.74 µM, respectively (Fig. 3).

Each tested PPI significantly inhibited hOAT3-mediated [^{3}H]ES transport in a concentration-dependent manner (Fig. 4). The calculated half-maximal inhibitory concentration values were in the micromolar range. We obtained an IC_{50} of 20.59 ± 4.07 µM for omeprazole, an IC_{50} of 3.96 ± 0.96 µM for lansoprazole and an IC_{50} of 7.89 ± 2.31 µM for pantoprazole. Likewise,

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omeprazole, lansoprazole and pantoprazole inhibited the transport of [^{3}H]MTX in HEK-hOAT3 cells, with IC_{50} values of 6.8 ± 1.16 μM, 1.14 ± 0.26 μM and 4.45 ± 1.62 μM, respectively (Fig. 4).

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Discussion:

MTX is currently used in wide range of doses, and high dose MTX schedules are associated with an incidence of nephrotoxicity of 1.8% and a fatality rate of almost 0.1 %, despite therapeutic drug monitoring and supportive therapy (Widemann and Adamson, 2006) Although drug interactions between MTX and PPIs have been described in the clinic, the specific mechanism for this drug-drug interaction remains unknown.

Our major finding indicates that hOAT3, an uptake transporter expressed at the basolateral side of renal proximal tubular cells, selectively mediates the uptake of MTX, and this transporter is dramatically inhibited in the presence of PPIs. Different studies have suggested the involvement of multiple drug transporters in the elimination of MTX (Breedveld et al., 2004; Suzuki et al., 2009), but the uptake transporters have been well established to be the first limiting step of MTX elimination (VanWert and Sweet, 2008). Among the OATs, OAT1 and OAT3, localize to the basolateral membrane of proximal tubular cells and have been shown to play a central role in the renal uptake of anionic drugs, namely MTX.

Our study confirmed that hOAT3 is a high affinity type transporter of MTX. In our study, the estimated Km value for hOAT3 was 21.17 ± 5.65 µM, which was consistent with the Km values of MTX uptake (10.9 µM and 21.1 µM) previously described by *Cha et al*. and *Takeda et al.,* respectively (Cha et al., 2001; Takeda et al., 2002a). Because this Km value determined in human kidney sections was similar to that observed for hOAT3 in this study rather than that observed for hOAT1 (553.8 ± 43.2 µM) by *Takeda et al*. in transfected S2 cells, OAT3 likely more significantly contributes to the net uptake process involved in MTX elimination (Takeda et al., 2002a). We failed to detect MTX transport in HEK-hOAT1 below a concentration of 50 nM; because the uptake experiment required the use of 0.5 µM of MTX according to a study described by El Sheik et al., we could observe an uptake transport by incubating HEK-OAT1 with only 0.5 µM MTX (El-Sheikh et al., 2013). Unfortunately the

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difference from the mock cells was not sufficient to evaluate the drug-drug interaction. Moreover, we believe that a MTX concentration above 100 µM is not clinically relevant for therapeutic drug monitoring, because a slow elimination of MTX was defined as plasma concentrations exceeding 15 µM at 24 hours (Santucci et al., 2010). These concentrations are much higher than the human plasma concentrations of MTX and seem unlikely clinical practice. Our current results were also consistent with the findings of *Lu et al*., who cloned hPAHT (p-aminohippurate transporter, the first name of hOAT1), which exhibited no significant MTX uptake activity (Lu et al., 1999). *Uwai et al.* determined the Km value for hOAT1 mediated MTX uptake using a Xenopus laevis oocytes expression system, to be 724 µM. In fact, this higher value of Km for hOAT1 supported our result, i.e., this concentration was not clinically relevant (Uwai et al., 2004). More recently *Kurata et al.* confirmed the same result with HEK-hOAT1 (Kurata et al., 2014). *Nozaki et al.* also examined MTX uptake using human tissue sections and estimated Km values within the same range (48.9 ± 17.3 µM) we observed for hOAT3 (Nozaki et al., 2007). As mentioned previously by various authors, the discrepancy may be due to species differences in the transport activity between rat and human OAT1 or differences in the expression system (Takeda et al., 2002a; Uwai and Iwamoto, 2010; Uwai et al., 2004).

The most striking result of our study was the potent inhibition of MTX uptake transport by all 3 PPIs in HEK-hOAT3 cells. The observed PPI IC_{50} values for MTX uptake were in the micromolar range (6.80 µM, 1.14 µM and 4.45 µM for omeprazole, lansoprazole and pantoprazole, respectively). Interestingly, the IC_{50} values for the three PPIs of the MTX uptake transport of by hOAT3 were higher. The observed PPI IC_{50} values were higher for MTX than ES but were within the same concentration range as the plasma circulating concentrations. Moreover, the IC_{50} values observed for each PPI were compared to the plasma concentrations of PPIs according to the CYP2C19 genotype (Ishizaki and Horai, 1999) see

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Table 1. Indeed, PPIs are mainly metabolized by CYP2C19, and because the impact of CYP2C19 polymorphism on drug concentrations has been well established, different concentrations should be considered (Goldstein, 2001; Simon et al., 2011). A previous group described the maximum concentration of carriers of a loss of function allele in the plasma, for omeprazole (3.1 µM), lansoprazole (4.9 µM), and pantoprazole (11.5 µM) according to the CYP2C19 “poor metabolizer” (PM) phenotype (Freston et al., 2003; Ieiri et al., 2001; Pue et al., 1993; Regardh et al., 1990; Yasuda et al., 1995). The plasma concentrations were lower in carriers of the normal allele with an “extensive metabolizer” (EM) phenotype, 1.6 µM, 2.4 µM and 5.4 µM for omeprazole, lansoprazole and pantoprazole, respectively.

Until recently, most studies investigated the effects of PPIs on different *in vivo* or *in vitro* models and suggested some effect of PPIs on efflux transporters. The effect of PPIs on the uptake transporter was poorly understood. The present finding also confirms that PPIs potently interact with different uptake transporters (hOAT1 and hOAT3) and their well-established substrates. Among the 3 PPIs tested for the PAH uptake by HEK-hOAT1, two elicited a strong inhibitory effect (omeprazole IC_{50} = 4.32 ± 1.26 µM and lansoprazole 7.58 ± 1.06 µM). In agreement with our results, *Nies et al*. recently published that PPIs inhibited hOCTs mediated metformin uptake in vitro. All five tested PPIs (omepazole, pantoprazole, lansoprazole, rabeprazole, tenatoprazole) significantly inhibited metformin uptake by HEK-hOCT1, hOCT2, and hOCT3 in a concentration dependent manner. Consistent with our result, the IC_{50} values of these PPIs were in the low micromolar range (3-36µM) (Nies et al., 2011). In addition, the IC_{50} values of potent OAT drug inhibitors, such as ibuprofen, ketoprofen, piroxicam, indomethacin and probenicid, described for adefovir uptake transport by hOAT1 were 8.0, 1.3, 20.5, 3.0 and 7.4 µM, respectively (Takeda et al., 2002a). which agrees with our results.

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Although the clinical consequences are not easily predicted based on *in vitro* data, Detailed advantages and limitations of various in vitro systems for evaluation of drugs as substrates, as inhibitors, or for their potential for drug–drug interactions have been delineated (Brouwer et al., 2013; Giacomini and Huang, 2013; Hillgren et al., 2013; Zamek-Gliszczynski et al., 2013). According on the decision trees of the recommendations of the International Transporter Consortium (Giacomini et al., 2010), the values of the ration [I]/IC50 of the three PPIs tested on the uptake of MTX on HEK hOAT3 are lower than 0.1 except for lansoprazole in PM, for which the ration is higher than 0.1 giving thought to a clinical interaction between lansoprazole and MTX. It would be very interesting to confirm if this in vitro drug-drug interaction would be relevant in humans in a prospective study. Some factors support such an assumption, because plasma MTX is predictive of the risk of toxicity, therapeutic drug monitoring is often used for patients to evaluate the delayed elimination. Recently, the delayed elimination of MTX associated with serious side effects weas described in three retrospective clinical studies of patients treated with high doses of MTX and PPIs (Joerger et al., 2006; Leveque et al., 2011; Santucci et al., 2010; Suzuki et al., 2009) and one prospective study of low dose MTX (Vakily et al., 2005). Although conflicting data were reported in some case reports for either omeprazole or pantoprazole (Bauters et al., 2008; Beorlegui et al., 2000; Troger et al., 2002; Whelan et al., 1999), recent clinical studies are in line with our results suggesting that PPIs might decrease MTX renal clearance via OAT3 mediated inhibition. In the first study, *Joerger et al*. described in 76 patients who received high-dose MTX, 13 of whom received omeprazole or lansoprazole. Patients that received MTX and PPIs were associated with a 27% decrease in the clearance of MTX, which resulted in a significantly higher plasma concentration of MTX (Joerger et al., 2006). The second study is a retrospective non-interventional cohort study that included 79 French cancer patients treated with high-doses of MTX. The co-prescription of PPIs (pantoprazole, lansoprazole,

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omeprazole or esomeprazole) was found in half of the cycles with delayed elimination and only in 15% of the cycles without delayed elimination (Santucci et al., 2010). The third study examined 74 Japanese cancer patients. MTX was administered intravenously with a concomitant administration of omeprazole, lansoprazole and rabeprazole. The MTX residual concentrations (311 measurements of plasma MTX) were analyzed in 171 cycles of high dose MTX. They found that PPI co-administration was still a significant risk factor for delayed elimination after adjustment for six variables (Suzuki et al., 2009). Interestingly, the delayed elimination of plasma MTX previously mentioned in these studies was not observed in all patients who received PPIs; based on our results, this finding may be due to higher concentrations of PPIs in carriers of CYP2C19 loss-of function variant alleles (Ieiri et al., 2001; Simon et al., 2011).

Renal tubular secretion involves different uptake transporters. A recent study showed that the basolateral localization of mouse reduced folate carrier (RFC-1) in the kidney is responsible for the uptake of MTX (Nozaki et al., 2004). Others uptake transporters that are mainly expressed in the liver (OATP1B) or intestine (OATP1A2) were found to transport MTX *in vitro*. These transporters were very recently found *in vivo* in transgenic mice that expressed liver-specific human OATP1B1, OATP1B3 and OATP1A2. Further studies are necessary to confirm the influence of this transporter on the pharmacokinetic of MTX in humans.

Conversely, some ATP binding cassette transporters, such as breast cancer resistance protein (BCRP, ABCG2 (Suzuki et al., 2009)), multidrug resistance-associated protein (MRP) 2 and MRP4, which are expressed on the apical membranes of kidneys, are reportedly also involved in the excretion of MTX (Chen et al., 2002; Ito et al., 2001). *Suzuki et al.* tested the effect of PPIs on the uptake of MTX into BCRP-expressing membrane vesicles. The observed IC_{50} for each PPI was considerably higher than the plasma concentrations of the PPIs. They also concluded that the inhibitory effects of PPIs on BCRP-mediated MTX transport alone could

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not explain this drug-drug interaction (Suzuki et al., 2009). Reports on the drug-drug interaction between PPIs-MTX and MRPs transporters are lacking, and the role of these transporters should be clarified in subsequent studies.

In conclusion, we identified PPIs as an important class of drugs that inhibit OATs transporters and confirmed that MTX has a greater affinity for OAT3 than OAT1. Taken together our results indicate that hOAT1 is likely not involved in the interaction between MTX and PPIs. The growing use of PPIs to treat peptic ulcers and the widespread use of MTX for a variety of diseases, namely cancers, suggest that a number of patients may be at risk for MTX toxicity, and more intensive therapeutic drug monitoring advised. Thus, further studies are required to evaluate the clinical consequences of the pharmacological interaction between PPIs and other OAT3 substrates, such as antiviral drugs.

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Authorship Contributions:

Participated in research design: Chioukh, Noel-Hudson, and Verstuyft

Conducted experiments: Chioukh, Ribes, Noel-Hudson, and Verstuyft

Contributed new reagents or analytic tools: Fournier,

Performed data analysis: Chioukh, Noel-Hudson, Becquemont and Verstuyft

Wrote contributed to the writing of the manuscript: Chioukh, Noel-Hudson, Becquemont and Verstuyft.

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Legends for Figures:

Fig.1. Characterization of HEK cells stably transfected with cDNAs encoding human OAT1 or OAT3. **(A):** Intracellular uptake of PAH in HEK-hOAT1 and ES in HEK-hOAT3: Net intracellular PAH accumulation in HEK-hOAT1 cells after 2-min incubation with increasing PAH concentrations. Intracellular ES uptake in HEK-hOAT3 cells after 10-min incubation with increasing ES concentrations. The uptake was obtained by subtracting the uptake in vector-transfected cells (HEK Mock) from that in HEK-hOAT1 or HEK-hOAT3 expressing cells. Km and Vmax values were calculated by fitting the data to a one-site binding curve. Data are means ± SEM of 3 determinations.
(B): Probenicid inhibition in OAT expressing cells. [^{3}H]PAH (50 nM) or [^{3}H]ES (10 nM) uptake was measured for 2 min in HEK-hOAT1 or 10 min in HEK-hOAT3 respectively, in the absence or in presence of increasing inhibitor concentration. The uptake amounts of [^{3}H]PAH or [^{3}H]ES in HEK-hOAT1 or in HEK-hOAT3 respectively were determined and shown as a percentage. IC_{50} values were calculated by fitting the data to a sigmoidal dose-response regression curve. Data points are the means ± SEM of three independent experiments.

Fig.2. Intracellular [^{3}H]MTX uptake. (A): Intracellular [^{3}H]MTX uptake in HEK-hOAT1 and HEK-Mock cells: after 5 min incubation with 0.5 µM [^{3}H]MTX., Data are means ± SEM of three independent experiments. Error bars in control cells are within the borders of the bars. $^*P < 0.05$ significantly different from the control values. **(B):** [^{3}H]MTX uptake in HEK-hOAT3 and HEK-Mock cells, after 10 min incubation with 25 nM [^{3}H]MTX. Data are means ± SEM of three independent experiments. Error bars in control cells are within the borders of the bars. $^*P < 0.05$ significantly different from the control values. **(C):** Intracellular MTX uptake in HEK-hOAT3 cells: after 10 min incubation with increasing MTX concentration. The uptake was obtained by subtracting the uptake in HEK-Mock from that in HEK-hOAT3. Km and Vmax values were calculated by fitting the data to a one-site binding curve. Data are means ± SEM of 3 determinations. **(D):** Net transporter-mediated [^{3}H]MTX uptake by HEH-hOAT3 cells over time, incubation with 25 nM [^{3}H]MTX. Data are means ± SEM of three independent experiments.

Fig.3. Inhibition of hOAT1-mediated [^{3}H]PAH uptake by PPIs. Inhibitory effect of omeprazole, lansoprazole, and pantoprazole on hOAT1 mediated [^{3}H]PAH uptake after 2-min incubation. IC_{50} values were calculated by fitting the data to a sigmoidal dose-response regression curve. Data points are the means ± SEM of three independent experiments.

Fig.4. Inhibition of hOAT3-mediated [^{3}H]ES and [^{3}H] MTX uptake by PPIs. Inhibitory effect of omeprazole, lansoprazole, and pantoprazole on hOAT3 mediated [^{3}H]ES or [^{3}H]MTX uptake after 10 min incubation. IC_{50} values were calculated by fitting the data to a sigmoidal dose-response regression curve. Data points are the means ± SEM of three independent experiments.

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Table 1: Half-maximal inhibitory concentration (IC_{50}) values for hOAT3 in MTX uptake and plasmatic concentrations of proton pump inhibitors according to CYP2C19 genetic polymorphism and plasma unbound fraction.

Compounds	***Uptake MTX IC_{50} hOAT3 (µM)***	**Dose (mg)**	**Genotype CYP2C19**	**Cmax Observed in humans (µM)**	**[I], Unbound inhibitor concentration (µM)**	**[I]/IC_{50}**	**References**
Omeprazole	6.80 ± 1.16	**20**	**EM**	1.6 ± 1.0	0.05 ± 0.03	0,007	(Regardh et al., 1990; Yasuda et al., 1995)
			PM	3.1 ± 0.9	0.10 ± 0.03	0,015	
Lansoprazole	1.14 ± 0.26	**30**	**EM**	2.44 ± 0.7	0.07 ± 0.02	0,061	(Freston et al., 2003; Ieiri et al., 2001)
			PM	4.9 ± 0.08	0.15 ± 0.02	0,132	
Pantoprazole	4.45 ± 1.62	**40**	**EM**	5.4 ± 1.4	0.11 ± 0.03	0,025	(Pue et al., 1993; Regardh et al., 1990)
			PM	11.5 ± 7.80	0.23 ± 0.16	0,052	

PM: poor metabolizer phenotype. EM: extensive metabolizer phenotype. PPI dosage and maximal total PPI concentration in the systemic circulation (C_{max}) were obtained from the indicated references. [I] Unbound inhibitor concentration. [[I] = (Cmax total) * (% plasma unbound fraction) / 100].

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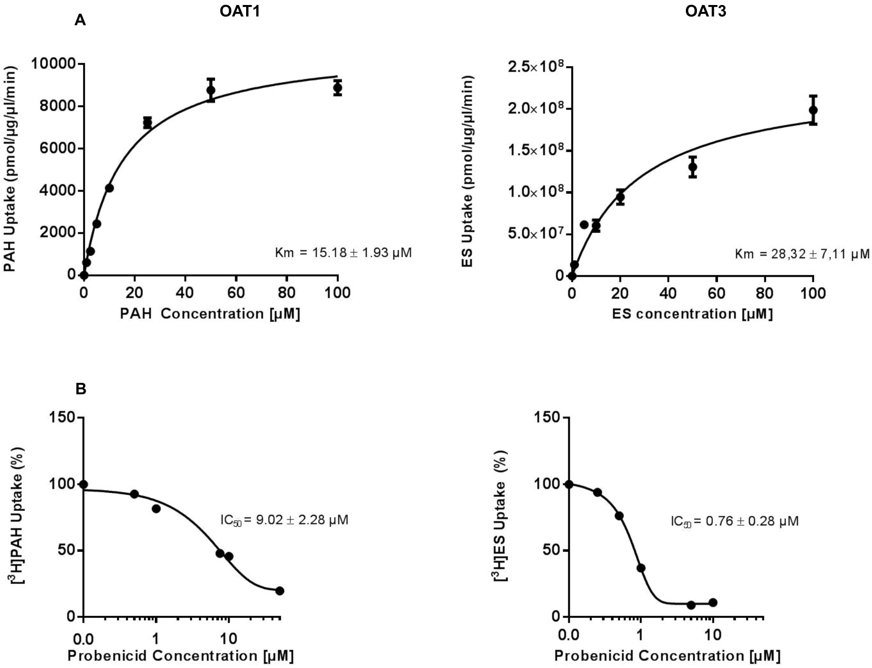
A
OAT1
OAT3
PAH Uptake (pmol/µg/µl/min)
10000
8000
6000
4000
2000
0
0
20
40
60
80
100
PAH Concentration [µM]
Km = 15.18 ± 1.93 µM
ES Uptake (pmol/µg/µl/min)
2.5×10^8
2.0×10^8
1.5×10^8
1.0×10^8
5.0×10^7
0
ES concentration [µM]
Km = 28,32 ± 7,11 µM
B
[3H]PAH Uptake (%)
150
100
50
0
0.0
1
10
Probenicid Concentration [µM]
IC50 = 9.02 ± 2.28 µM
[3H]ES Uptake (%)
IC50 = 0.76 ± 0.28 µM

Figure 1

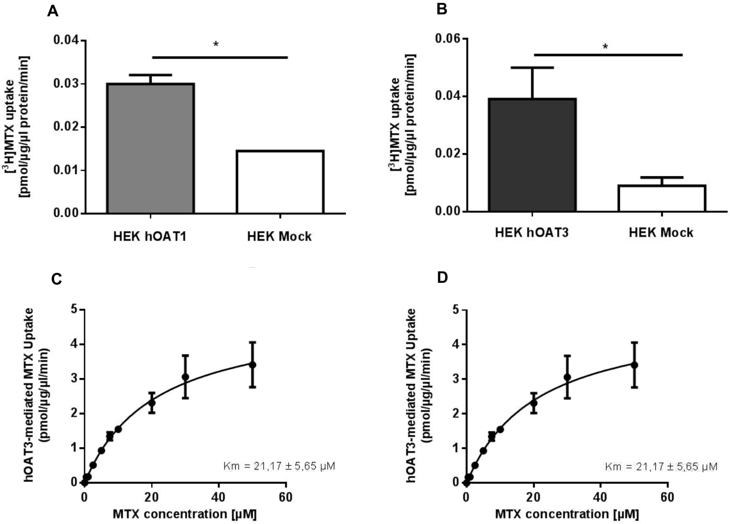
A
[^{3}H]MTX uptake [pmol/µg/µl protein/min]
0.04
0.03
0.02
0.01
0.00
*
HEK hOAT1
HEK Mock
B
[^{3}H]MTX uptake [pmol/µg/µl protein/min]
0.06
0.04
0.02
0.00
*
HEK hOAT3
HEK Mock
C
hOAT3-mediated MTX Uptake (pmol/µg/µl/min)
5
4
3
2
1
0
Km = 21,17 ± 5,65 µM
0
20
40
60
MTX concentration [µM]
D
hOAT3-mediated MTX Uptake (pmol/µg/µl/min)
5
4
3
2
1
0
Km = 21,17 ± 5,65 µM
0
20
40
60
MTX concentration [µM]

Figure 2

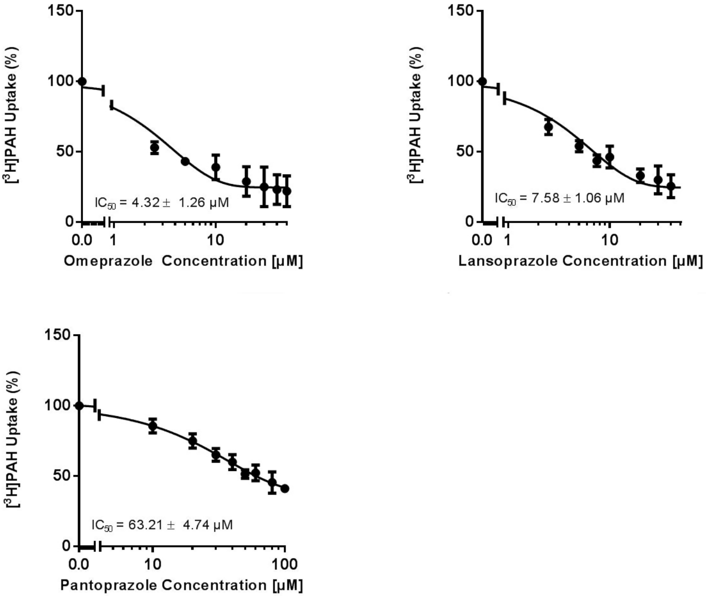
150
100
50
0
[^{3}H]PAH Uptake (%)
IC$_{50}$ = 4.32 ± 1.26 µM
0.0
1
10
Omeprazole Concentration [µM]
150
100
50
0
[^{3}H]PAH Uptake (%)
IC$_{50}$ = 7.58 ± 1.06 µM
0.0
1
10
Lansoprazole Concentration [µM]
150
100
50
0
[^{3}H]PAH Uptake (%)
IC$_{50}$ = 63.21 ± 4.74 µM
0.0
10
100
Pantoprazole Concentration [µM]

Figure 3

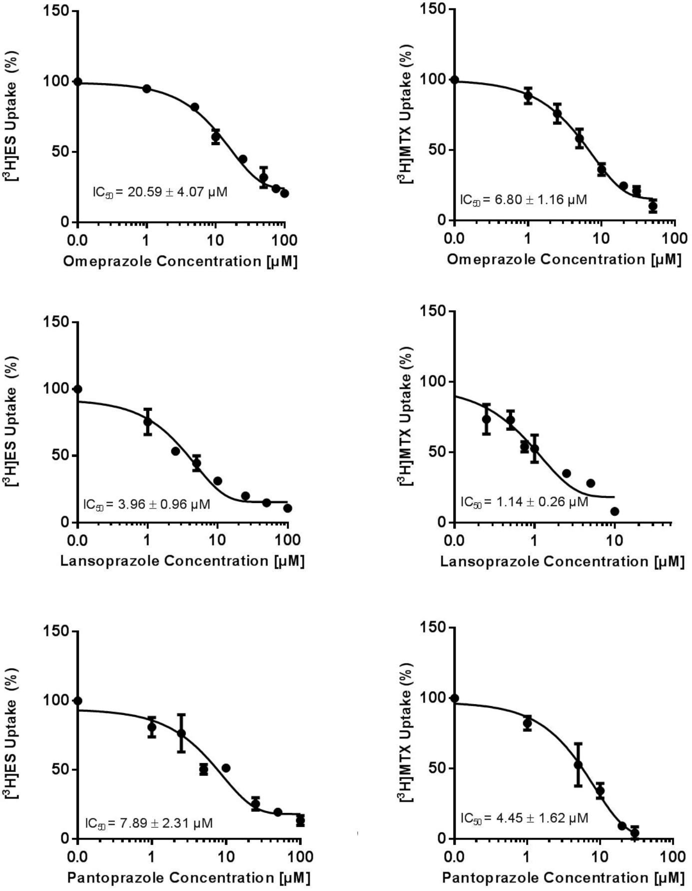
[^{3}H]ES Uptake (%)
150
100
50
0
IC$_{50}$ = 20.59 ± 4.07 µM
0.0
1
10
100
Omeprazole Concentration [µM]
[^{3}H]MTX Uptake (%)
150
100
50
0
IC$_{50}$ = 6.80 ± 1.16 µM
0.0
1
10
100
Omeprazole Concentration [µM]
[^{3}H]ES Uptake (%)
150
100
50
0
IC$_{50}$ = 3.96 ± 0.96 µM
0.0
1
10
100
Lansoprazole Concentration [µM]
[^{3}H]MTX Uptake (%)
150
100
50
0
IC$_{50}$ = 1.14 ± 0.26 µM
0.0
1
10
Lansoprazole Concentration [µM]
[^{3}H]ES Uptake (%)
150
100
50
0
IC$_{50}$ = 7.89 ± 2.31 µM
0.0
1
10
100
Pantoprazole Concentration [µM]
[^{3}H]MTX Uptake (%)
150
100
50
0
IC$_{50}$ = 4.45 ± 1.62 µM
0.0
1
10
100
Pantoprazole Concentration [µM]

Figure 4