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Characterization of the inhibitory effects of N-butylpyridinium chloride and structurally related ionic liquids on OCT1/2 and hMATE1/2-K in vitro and in vivo

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Abbreviations:

AUC	area under the curve
Bmim-Cl	1-butyl-3-methylimidazolium chloride;
BmPy-Cl	N-butyl-N-methylpyrrolidinium chloride;
CHO	Chinese hamster ovary;
CL	systemic clearance;
EtPy-Cl	1-ethylpyridinium chloride;
HePy-Cl	1-hexylpyridinium chloride;
IC_{50}	concentration of inhibitor that results in half maximal transport;
IL, ILs	ionic liquid(s);
K_t	concentration of substrate that results in half maximal transport;
LSC	liquid scintillation counter;
MATEs	multidrug and toxic extrusion transporters
MPP	1-methyl-4-phenylpyridinium;
NBuPy-Cl	N-butylpyridinium chloride;
OCTs	organic cation transporters;
Py-Cl	pyridine hydrochloride;
SAR	structure activity relationship;
T1/2	elimination half-life;
TEA	tetraethylammonium;
Vss	volume of distribution.

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Abstract:

Ionic liquids (ILs) are a class of salts that are expected to be used as a new source of solvents and for many other applications. Our previous studies revealed that selected ILs, structurally related organic cations, are eliminated exclusively in urine as parent compound, partially mediated by renal transporters. This study investigated the inhibitory effects of N-butylpyridinium chloride (NBuPy-Cl) and structurally related ILs on organic cations transporters (OCTs) and multidrug and toxic extrusion transporters (MATEs) in vitro and in vivo. After CHO cells expressing rOCT1, rOCT2, hOCT2, hMATE1 or hMATE2-K were constructed, the ability of NBuPy-Cl, 1-methyl-3-butylimidazolium chloride (Bmim-Cl), N-butyl-N-methylpyrrolidinium chloride (BmPy-Cl) and alkyl substituted pyridinium ILs to inhibit these transporters was determined in vitro. NBuPy-Cl (0, 0.5, or 2 mg/kg/h) was also infused into rats to assess its effect on the pharmacokinetics of metformin, a substrate for OCTs and MATEs. NBuPy-Cl, Bmim-Cl and BmPy-Cl displayed strong inhibitory effects on these transporters (IC_{50}: 0.2~8.5 μM). In addition, the inhibitory effects of alkyl substituted pyridinium ILs on OCTs increased dramatically as the length of the alkyl chain increased. The IC_{50} values were 0.1, 3.8, 14 and 671 μM (hexyl-, butyl-, ethyl-pyridinium

and pyridinium chloride) for rOCT2 mediated metformin transport. Similar structurally related inhibitory kinetics were also observed for rOCT1 and hOCT2. The in vivo co-administration study revealed that NBuPy-Cl reduced the renal clearance of metformin in rats. These results demonstrate that ILs compete with other substrates of OCTs and MATEs, and could alter the in vivo pharmacokinetics of such substrates.

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Introduction:

Ionic liquids (ILs) are a growing class of industrial chemicals that are being increasingly investigated for a variety of applications (Plechkova and Seddon, 2008). They are usually composed of organic cations and variable inorganic or organic anions. They have melting points at or less than 100 °C. Because of the chemical diversity of the cations/anions, the number of available ILs is almost unlimited (Baker et al., 2005). ILs with specific melting points, viscosities, densities or ionic conductivities can be formulated for special applications (Welton, 1999). Therefore, ILs are expected to be used widely in analytical methods, engineering processes, consumer products and biomedical applications (Plechkova and Seddon, 2008). Because of their extremely low vapor pressure, the capacity to pollute the air is minimal. Thus, a primary application of these compounds is to replace the classical volatile organic solvents (Rogers and Seddon, 2003).

Although the physical/chemical characteristics of ILs may minimize the risk of atmospheric contamination, other sources of environmental exposure are of concern. For example, Couling et al. (2005) suggested that the increased use of ILs on a large scale in industry could result in water pollution. Exposures from

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occupational and consumer products are also likely to occur. Some studies have already investigated the toxic effects caused by pyridinium and imidazolium based ILs. On a molecular level, such ILs altered the function of some biological enzymes, such as oxidoreductase and mushroom tyrosinase (Pinto et al., 2008; Yang et al., 2009). Zhao et al. (2007) have summarized the toxicities of these two groups of ILs to various organisms, such as water algae, bacteria, fungi and mammalian cell lines. To some organisms, ILs are much more toxic than the conventional organic solvents (Ranke et al., 2004).

ILs based on imidazolium, pyridinium, phosphonium, and ammonium have been tested on Cyclotella meneghiniana, Selenastrum capricornutum, Daphnia magna, Pseudokirchneriella subcapitata, and zebrafish (Latala et al., 2005; Cho et al., 2007; Yu et al., 2009; Pretti et al., 2009). It was found that shorter alkyl substituted chains demonstrated lower toxicity, when compared with the cations with longer alkyl substituent. This structure activity relationship (SAR) was also observed for biological enzymes, such as acetylcholine esterase (Stock et al., 2004; Stasiewicz et al., 2008). Lactic acid production by Lactobacillus, an acid producing bacterium, was decreased as the alkyl chain length on the imidazolium cations increased (Matsumoto et al., 2004). Ranke et al. (2007) compared the cation lipophilicity and the cytotoxicity of 74 ILs on IPC-81 Leukemia cells and

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suggested that the length of alkyl chain played a dominant role on their cytotoxicity.

Few studies have investigated the disposition and toxicity of ILs in mammals. Because N-butylpyridinium chloride (NBuPy-Cl), 1-methyl-3-butylimidazolium chloride (Bmim-Cl) and N-butyl-N-methylpyrrolidinium (BmPy-Cl) are starting materials for many other ILs, they were selected by the National Toxicology Program for investigating their disposition, metabolism and toxicity. In previous studies, we reported that these three compounds are absorbed (40-70%) from the rodent gastrointestinal tract and then widely distributed in rats (Sipes et al., 2008; Cheng et al., 2009; Knudsen et al., 2009). For the three ILs, the portion of the dose that became systemically available was eliminated exclusively in the urine as parent compound. Renal elimination was facilitated, in part, by tubular secretion, presumably mediated by organic cation transporter 2 (OCT2).

In this study, we examined the in vitro inhibitory effects of NBuPy-Cl, Bmim-Cl and BmPy-Cl on rat OCT1/2 and human OCT2 and also determined if these ILs inhibited human MATE1/2-K. How the length of the alkyl chain affected the degree of inhibition was further investigated on OCTs. The ILs used were pyridine hydrochloride (Py-Cl), 1-ethylpyridinium chloride (EtPy-Cl) and 1-hexylpyridinium chloride (HePy-Cl), derivatives of NBuPy-Cl (Figure 1). In

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addition, in vivo studies were conducted to determine if NBuPy-Cl can influence the pharmacokinetics of metformin, a known substrate of OCTs and MATEs.

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Methods:

Chemicals and materials:

[^{3}H]Tetraethylammonium trifluoroacetate (TEA, 54 Ci/mmol) was synthesized by GE Healthcare (Little Chalfont, Buckinghamshire, UK). [^{14}C]Metformin (112 mCi/mmol) was received from Moravek Biochemicals (Brea, CA). [^{3}H]1-methyl-4-phenylpyridinium (MPP, 80 Ci/mmol) was synthesized by the Department of Chemistry and Biochemistry, University of Arizona (Tucson, AZ). Metformin (98% purity) was purchased from Sigma-Aldrich (St. Louis, MO). N-butylpyridinium chloride (NBuPy-Cl), 1-methyl-3-butylimidazolium chloride (Bmim-Cl), and N-butyl-N-methylpyrrolidinium chloride (BmPy-Cl) (all≥98% purity, Figure 1) were obtained from Merck KGaA (Darmstadt, Germany). Pyridine hydrochloride (Py-Cl), 1-ethylpyridinium chloride (EtPy-Cl) and 1-hexylpyridinium chloride (HePy-Cl) (all≥98% purity) were purchased from Acros Organics (Geel, Belgium). Ketamine/xylazine and pentobarbital sodium salt were purchased from Sigma-Aldrich (St. Louis, MO). Solvable® and Pico-Flour scintillation cocktail solution were obtained from Perkin Elmer (Torrance, Ca). Fetal bovine serum (FBS), penicillin/streptomycin, zeocin and hygromycin B were obtained from Invitrogen (Carlsbad, CA). Kaighn's modification (F12K) medium

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and other chemicals (the highest quality available) were purchased from Sigma-Aldrich (St. Louis, MO).

In vitro studies

Cell Culture and Transfection

Chinese Hamster Ovary cells containing a single integrated Flp Recombination Target site(CHO Flp-In) were acquired from Invitrogen and were used for stable expression of the rat ortholog of OCT1 (rOCT1) and OCT2 (rOCT2), the human ortholog of OCT2 (hOCT2), MATE1 (hMATE1) and MATE2-K (hMATE2-K). Transporter cDNAs were inserted into the pcDNA5/FRT/V5-His-TOPO® plasmid vector (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The sequences were confirmed by the DNA Sequencing Facility at the University of Arizona (Tucson, AZ). CHO cells were maintained in F12K media with 10% FBS, penicillin (100 unit/ml), streptomycin (100 µg/ml) and zeocin (100 µg/ml) at 37 ^{o}C in a humidified atmosphere with 5% CO_2. 5x10^6 cells in 400 µL of media were electroporated (BTX ECM 630) at 260 V (time constant of ~25 ms) with 10 µg of salmon sperm DNA (Invitrogen, Carlsbad, CA), 18 µg of pOG44 and 2 µg of the plasmid vector containing transporter cDNA. The cells were then transferred to T75 cell culture flasks. After 24 h incubation, media

containing hygromycin (300 μg/ml), instead of zeocin, was applied as selection pressure for 2 weeks.

Transport studies

The transport of [^{3}H]TEA (12 nM) and [^{14}C]metformin (9 μM) was first characterized over time in CHO_hOCT2, CHO_rOCT1 and CHO_rOCT2 cells, and [^{3}H]MPP (15 nM) in CHO_hMATE1 and CHO_hMATE2-K cells. Based on the time dependent transport curve, the inhibitory effects of ILs towards OCTs were determined during the linear phase of uptake (30 s for [^{3}H]TEA uptake and 60 s for [^{14}C]metformin uptake by hOCT2; 120 s for [^{3}H]TEA uptake by rOCT1, [^{14}C]metformin uptake by rOCT2, and [^{3}H]MPP uptake by hMATE1 and hMATE2-K.

To determine the inhibitory effects of ILs, the transport of [^{3}H]TEA (12 nM) by rOCT1 and hOCT2, and [^{14}C]metformin (9 μM) by rOCT2 and hOCT2 were measured in the presence of increasing concentrations of NBuPy-Cl, Bmim-Cl, BmPy-Cl, Py-Cl, EtPy-Cl or HePy-Cl. Various concentration of NBuPy-Cl were used to investigate its inhibitory effects on hMATE1 or hMATE2-K mediated [^{3}H]MPP transport. The transport assay was performed as described previously (Cheng et al., 2009). Typically, cells were cultured in 12-well plates. Once reaching confluence, they were incubated in 0.4 ml of Waymouth's buffer

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containing [^{14}C]metformin, [^{3}H]TEA or [^{3}H]MPP and one of the ILs for a predetermined time as described above. Following solublization of the cells with 0.4 ml of NaOH (0.5 M) with 1% SDS and neutralization with 0.2 ml of HCL (1 M), the cell lysate solution (0.5 ml) was counted using a liquid scintillation counter (LSC).

Animal studies

Animal surgeries:

Male Fischer-344 rats (300-350 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The rats were housed in the University of Arizona Animal Care Facility (accredited by the Association for Assessment and Accreditation for Laboratory Animal Care) with controlled temperature (25^{o}C), humidity (40-60%) and light/dark cycle (12 h). Following 7-10 days acclimation, 1 ml/kg of ketamine/xylazine solution was administered (i.p.) to induce anesthesia. Once the animals were totally anesthetized, the jugular vein and carotid artery were cannulated with the PE-50 tubing (I.D. 0.58 mm, O.D. 0.965 mm; BD, Franklin Lakes, NJ). The surgical openings were covered with water saturated gauze sponges. After surgery, pentobarbital (65 µg/kg) was administered subcutaneously to maintain anesthesia. The rats were then placed in a ventral

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position on a heating pad maintained at 37^{o}C. A syringe infusion pump (Harvard Apparatus, Holliston, MA) was used for the infusion of NBuPy-Cl. All the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arizona.

Dosing selection and sample collection

Previously, it was observed that NBuPy-Cl (5 mg/kg) did not affect the GFR in rats following a single i.v. dose (Cheng et al., 2009). However, when this dose (5 mg/kg/h, 2 ml/kg/h) was infused over 4 hr (total dose 20 mg/kg), the clearance of the metformin (assessed over 3 h) was reduced to <1% of that observed in the control (saline group). Therefore, two lower doses (2 mg/kg/h, 0.5 mg/kg/h) of NBuPy-Cl were used in the studies described here. They provided total doses of 8 and 2 mg/kg, respectively.

Following surgery, the jugular vein was infused with saline (2 ml/kg/h) or NBuPy-Cl (0.5 or 2 mg/kg/h, 2 ml/kg/h) over 4 h. A bolus dose of [^{14}C]metformin (5 mg/kg, 50 µCi/kg) was administered through the jugular vein after 1 h of perfusion. Blood samples (300 µL) were then collected at 7.5, 15, 30, 45, 60, 90, 120, 150 and 180 min from the carotid artery. An equal volume of saline was administered to replace the blood which was withdrawn. At the end of the experiment (3 h after metformin dosing), animals were euthanized by CO_2

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inhalation. Selected tissues (adipose, heart, kidney, liver, lung, muscle, spleen, and testes) were collected.

Sample analysis

Blood samples were centrifuged at 750g for 10 min. Aliquots of the plasma samples (2x50 µL) were mixed with 15 ml of the Pico-Flour scintillation cocktail solution and samples were counted by LSC. Tissues samples were analyzed as described by Sipes et al. (2008). Briefly, the samples were solubilized with Solvable®, quenched with H_2O_2 (30%), and counted by LSC.

Data analysis

For the in vitro transport studies, estimates of the IC_{50} values were determined by the modified Michaelis-Menten Equation as shown below (Malo and Bertloot, 1991; Groves et al, 1994). J is the transport rate of radio-labeled compound at the concentration of [T*]; J_{mapp} is the J_{max} for [*T] transport times the ratio of K_i/K_t; and IC_{50} is equal to $K_i(1+[T^*/K_t])$; [I] is the concentration of unlabeled inhibitor; and C is a constant representing the component of total uptake that is not saturable over the concentration range tested.

$$J = \frac{J_{mapp}[T^*]}{IC_{50} + [I]} + C$$

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The application of this equation, given its structure, carries the tacit assumption that the inhibitory interactions observed are competitive in nature and reflect binding of substrate and inhibitor at a common binding site. Although the substrates/inhibitors studied here have all been shown to be transported by OCTs, inhibitory interactions between OCT substrate have, in some cases, been shown to reflect a "mixed-type" inhibitory profile, presumably reflecting both competition for a common binding region within the transporter and longer range allosteric interactions (e.g., Koepsell et al., 2007). Consequently, we refer to the kinetic constants calculated through the above equation as 'IC_{50}' values, and make no claim as to their precise mechanistic basis.

The pharmacokinetics of metformin following i.v. administration in rats was defined by the WinNonlin software (Version 6.1, Pharsight Corp) using a non-compartment model. All individual data points are presented as mean±SEM (standard error of mean). The data were analyzed statistically using student t-test or one way ANOVA with the Newman-Keuls post test by GraphPad Prism 4 Statistics Program. A level of $p<0.05$ was considered significant.

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Results

Functional expression of OCTs and MATEs in CHO cells

The accumulation of 12 nM [^{3}H]TEA by CHO cells stably expressing OCTs was time-dependent, i.e., nearly linear for at least 60 sec, and approached steady-state by 10 minutes (Figure 2-A). In all three cell lines, the presence of 5 mM unlabeled TEA reduced the 10 min accumulation of [^{3}H]TEA by 90% or more. The uptake of [^{3}H]TEA displayed kinetic characteristics of carrier-mediated transport minimally influenced by surface binding (Suhre et al., 2005). The profiles of hOCT2 and rOCT2 mediated [^{14}C]metformin transport were similar to that of [^{3}H]TEA, but the transport rate of [^{14}C]metformin by rOCT1 was too low to conduct an inhibition study (data not shown). Therefore, uptake of [^{3}H]TEA was used to determine the inhibitory effects of ILs on rOCT1. The function of CHO cells expressing hMATE1 or hMATE2-K was also determined and the results are shown in Figure 2-B. As with the OCTs, MATE1 or MATE2-K mediated transport of radiolabeled substrate, in this case, [^{3}H]MPP, was blocked more than 90% by unradiolabeled 1 mM of MPP. These results demonstrate that OCTs and MATEs were expressed in the CHO cells and were functionally active.

Inhibitory effect of NBuPy-Cl, Bmim-Cl and BmPy-Cl on OCTs

In the presence of NBuPy-Cl, the intracellular uptake of [^{3}H]TEA by rOCT1 was inhibited in a concentration dependent manner (Figure 3-A). Similar inhibitory kinetics of NBuPy-Cl on [^{14}C]metformin transport were observed for rOCT2 and hOCT2 (Figure 3-B, C). The IC_{50} values, which are the

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concentrations of inhibitors (ILs) that result in half-maximum transport of TEA or metformin by OCTs, are presented in Table 1. The three ILs were potent inhibitors of rOCT1/2 and hOCT2, with IC_{50} values ranging from 0.15 to 7.53 μM. As shown in Table 1, IC_{50} values were significantly lower for hOCT2 when metformin was used as the probe substrate.

Inhibition of OCTs by alkyl-substituted pyridiniums

To determine the relationship between the structure of ILs and their inhibitory effects (structure activity relationship) on OCTs, pyridnium-based ILs with different alkyl chain lengths were investigated. Uptake of TEA or metformin by OCTs was measured in the presence of increasing concentrations of Py-Cl, EtPy-Cl, NBuPy-Cl and HePy-Cl. As shown in Figure 4-A, all the tested pyridinium compounds inhibited rOCT2 mediated [^{14}C]metformin transport. Py-Cl was the weakest inhibitor with an IC_{50} of 671 μM (Table 1). The inhibition curve shifted left gradually and the IC_{50} values decreased around 10 fold for every 2 carbons added to the alkyl chain. HePy-Cl showed the strongest inhibitory activity (IC_{50}: 0.1 μM). The structurally related inhibitory effects of these pyridnium ILs on rOCT1 and hOCT2 were similar to those of rOCT2 (Figure 4-B). The IC_{50} values are presented in Table 1 for both probe substrates, TEA and metformin.

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Inhibitory effects of NBuPy-Cl on hMATE1 and hMATE2-K

The inhibitory effects of NBuPy-Cl were also determined on the apical membrane transporters, hMATE1 and hMATE2-K. Figure 5 shows the inhibitory kinetic curves of NBuPy-Cl on intracellular uptake of [^{3}H]MPP by CHO cells expressing hMATE1 or hMATE2-K. The IC_{50} values were 8.5±2.6 μM and 1.6±0.2 μM, respectively.

Intravenous co-administration of NBuPy-Cl and metformin

Pharmacokinetic analysis using a non-compartment model revealed that the elimination half life of [^{14}C] metformin (5 mg/kg, iv) from plasma of male F-344 rats was 2.3 h (Figure 6, Table 2).The volume of distribution was 484 ml and the systemic clearance was 3.4 ml/min. Since the liver metabolism/excretion of metformin is negligible, its systemic clearance reflects its renal clearance (Scheen, 1996). Infusion of NBuPy-Cl increased the plasma level of metformin in a dose-dependent manner (Figure 6). Thus, the plasma AUC_{0-180} for metformin was significantly increased due to the reduced renal clearance (Table 3). NBuPy-Cl had a minimal effect on the volume of distribution of metformin. This was further substantiated by the findings that tissue levels of metformin were not significantly elevated when they were harvested at the 3 h time point (Table 3). The small increases that were observed may reflect the presence of blood,

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which, in the NBuPy-Cl infused rats, had elevated concentrations of metformin. The only tissue that showed a small increase in the tissue to plasma ratio, indicative of actual increases in tissue levels, was the kidney, but this was not significant because of the large interanimal variations.

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Discussion

Organic cation transporters (OCTs) are widely expressed on the basolateral membrane of proximal tubule cells, where they function to transport a wide array of organic cations from plasma to the intracellular space of these cells (Choi and Song, 2008). OCT2 is richly expressed in human and rat kidneys, but OCT1 is weakly detected in human kidneys (Motohashi et al., 2002; Okuda et al., 1996). On the apical membrane of these tubular cells, organic cations are most likely exported to the urinary lumen by multidrug and toxic extrusion transporters (MATEs) (Nies et al., 2011). Mammalian MATEs include two members, MATE1 and MATE2-K. Human kidneys express both homologs, whereas rodent kidneys express only MATE1 (Terada et al., 2006). Recently, we observed that three ILs, NBuPy-Cl, Bmim-Cl and BmPy-Cl, were excreted rapidly in the urine of rats as the parent compounds (Sipes et al., 2008; Cheng et al., 2009; Knudsen et al., 2009). Because the rate of clearance from plasma exceeded glomerular filtration rate it was proposed that these three ILs, which are organic cations, undergo transport mediated secretion.

NBuPy-Cl and BmPy-Cl were significantly accumulated in CHO cells expressing hOCT2 following 10 min incubation, compared to naive CHO cells (Cheng et al., 2009; Knudsen et al., 2009). The transport kinetics of these two ILs,

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as assessed by K_t values (K_t, 18 µM for NBuPy-Cl; 37 µM for BmPy-Cl), were similar to that of the model substrate of hOCT2, TEA (K_t, 40 µM). NBuPy-Cl, and BmPy-Cl were also well transported in CHO cells expressing rOCT1 or rOCT2 (data unpublished). Results of subsequent experiments performed after the disposition studies on Bmim-Cl were published revealed that Bmim-Cl was also transported by hOCT2, rOCT1 and rOCT2 (data not presented). These in vitro results suggest that OCTs could contribute to the rapid renal elimination of these ILs.

The results presented in this manuscript demonstrate that, in addition to serving as substrates, NBuPy-Cl, BmPy-Cl, and Bmim-Cl are also potent inhibitors of rOCT1/2 and hOCT2. The IC_{50} values for inhibition of hOCT2 mediated TEA transport by these three ILs ranged from 0.5 to 2.3 µM. These inhibitory values are similar to those observed for MPP (2 µM) and tetrapentylammonium (10 µM), compounds considered to be potent inhibitors of hOCT2 (Suhre et al., 2005). When metformin, the widely prescribed Type 2 antidiabetic drug, was used as the probe substrate for hOCT2, these ILs demonstrated even stronger inhibitory effects. This observation was also noted when tetraalkylammonium compounds were used to inhibit hOCT2 mediated MPP and metformin transport, i.e., transport of metformin was inhibited to a

greater degree (Dresser et al., 2002; Choi et al., 2007). Why the different inhibitory effects were observed for the two probe substrates is still not clear. The transport characteristic of NBuPy-Cl by hOCT2 (Kt, 18 μM) is also significantly different from its inhibitory potency on hOCT2 mediated metformin uptake (IC_{50}, 0.7 μM). These might be related to different binding sites of two chemicals within the large binding surface of OCTs and/or the influence of non-competitive or other allosteric interactions (Koepsell et al., 2007).

ILs are called “designable chemicals” because they can be customized structurally to fit specific applications. These alterations in structure will change not only the chemical and physical properties of ILs, but may also alter their disposition, transport and toxicity. As demonstrated by the SAR results presented here, the inhibitory effects of the pyridinium-based ILs changed dramatically as the length of the alkyl side chain was altered. The IC_{50} values decreased significantly with an increase in the number of carbons on the alkyl chain. This SAR has also been reported for other chemical classes (Ullrich, 1997; Bednarczyk et al., 2003; Suhre et al., 2005; Choi et al., 2007). It was suggested that the hydrophobicity of the ILs increased with increasing the alkyl chain length (Ranke et al., 2007). This increased hydrophobicity could facilitate their

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interaction on the phamacophore of OCTs, and thus contribute to the enhanced inhibitory activity (Bednarczyk et al, 2003).

Results presented here show that NBuPy-Cl is also a potent inhibitor of hMATE1 and hMATE2-K. Since both OCTs and MATEs play critical roles in the renal secretion of organic cations, disruption of any of these transporters may alter the renal elimination profile of certain chemicals. For example, renal secretion of TEA was reduced in both rats and mice with impeded OCT1/2 function (Jonker et al., 2003; Matsuzaki et al., 2008). Similarly, plasma concentrations of metformin were increased in MATE1 knock-out mice, and its urinary excretion significantly reduced (Tsuda et al., 2009). Thus, it is not surprising that infusion of NBuPy-Cl markedly altered the plasma pharmacokinetics of metformin. It has the capacity to inhibit the transport processes related to both the uptake of metformin into proximal tubules as well as its extrusion from these cells into the tubular lumen.

Metformin is a comparatively low affinity substrate for hOCT1, hOCT2, hMATE1 and hMATE2k, with K_t values of 1.47, 0.99, 0.78 and 1.98 mM, respectively (Koepsell et al., 2007; Tanihara et al., 2007). As determined here, NBuPy-Cl blocks both OCTs and MATEs with IC_{50} values in the low micromolar range. Based on the equation for calculating steady-state plasma concentration following i.v. infusion (Rowland and Tozer, 1995), the estimated steady-state concentration of NBuPy-Cl in plasma is 0.8 µg/ml (4.7 µM), with an infusion dose of 2 mg/kg/h. Therefore, it was not surprising that renal clearance of metformin was reduced in the presence of this compound. Indeed, the NBuPy-Cl inhibitory

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profile suggests that a plasma concentration of ~5 μM would block about half of the rOCT1 (IC_{50}, 4.7 μM) and rOCT2 (IC_{50}, 3.8 μM) activity. Due to negative intracellular potential (~70 mV) of renal proximal tubule cells, NBuPy-Cl, the positively charged moiety, could well reach substantially higher levels intracellularly and inhibit MATEs more extensively. Thus, exit of metformin from renal proximal tubule cells may have been rate limiting under these conditions. In fact, the exit step has been shown to be rate-limiting in renal secretion of organic cations (Schäli et al, 1983), which is consistent with the accumulation of metformin in kidney tissue associated with the highest dose of NBuPy-Cl.

Since metformin is primarily eliminated in the urine as parent compound and at a clearance rate that exceeds GFR (Scheen, 1996), the increased plasma AUC of metformin in NBuPy-Cl infused animals relates, at least in part, to NBuPy-Cl's inhibitory effects on OCTs and/or MATEs. In our previous publication, we reported that NBuPy-Cl, at a single i.v. dose of 5 mg/kg, did not alter the plasma clearance of inulin in unanesthetized animals. This indicated that NBuPy-Cl at this dose did not affect rat GFR. It should be cautioned, however, that the GFR could have been decreased in animals co-administered NBuPy-Cl and the anesthetics that were used. For example, pentobarbital has been reported to decrease GFR (Walker et al., 1986). Based on the clearance of metformin in unanesthized rats (1.4 ml/min/100g) reported by Choi et al. (2010) and that of the control rats (1.0 ml/min/100g) reported here, anesthesia could account for up to 30% of the reduced metformin clearance. Another factor that could reduce GFR is impairment of renal function because of renal toxicity.

However, in studies with Bmim-Cl, a closely related ionic liquid, no evidence of renal toxicity was observed (as assessed by both serum chemistry and histopathology) at oral doses up to 50 mg/kg. This dose, which was >50% bioavailable, maintained blood levels of Bmim-Cl at ~2 µg/ml for several hours, close to the estimated steady state blood levels of NBuPy-Cl reported here. Thus, the in vivo data presented here support the hypothesis that ionic liquids have the potential to inhibit renal transporters and alter the pharmacokinetics of substrates of these transporters.

It should be noted that the doses of NBuPy-Cl infused into rats were high. It is unlikely that such blood levels would be achieved and maintained in humans exposed orally and/or dermally to environmental/occupational levels of NBuPy-Cl or other ILs. In rats, dermal absorption of these three ILs was less than 35 % of the applied dose (5 mg/kg, 125 µg/cm^2) and the absorbed dose was readily eliminated. Dermal absorption in humans is not expected to exceed that observed for rats. Clearly, additional studies are needed to focus how alterations in structure affect absorption of ILs after oral dosing or dermal application. More lipophilic ILs may achieve higher internal concentrations. This coupled with greater inhibitory effects on OCTs could influence their pharmacokinetic parameters, as well as drug chemical interactions.

In summary, in in vitro studies, NBuPy-Cl strongly inhibited OCTs and MATEs. In in vivo studies with rats, this inhibition resulted in a reduction in the plasma clearance of metformin. The structurally related ILs, Bmim-Cl and BmPy-Cl, and pyridinium-based ILs with increasing alkyl chain length, were also

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inhibitors of OCTs. The inhibitory effect increased as the length to the alkyl chain increased.

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Authorship Contributions

Participated in research design: Cheng, Wright, Kuester, and Sipes.

Conducted experiments: Cheng, and Martinez-Guerrero.

Contributed new reagents or analytic tools: Hooth.

Performed data analysis: Cheng, Sipes and Martinez-Guerrero .

Wrote or contributed to the writing of the manuscript: Cheng, Sipes, Hooth and Wright.

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FOOTNOTES

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Legends for Figures

Figure 1: Chemical structures of N-Butylpyridinium Chloride (NBuPy-Cl) and other structure related ionic liquids.

Figure 2: Characterization of the function of OCTs and MATEs in transfected CHO cells.

A: [^{3}H]TEA uptake by rOCT1 (■), rOCT2(▲) or hOCT2(●) over time in the absence (solid symbols) or presence (empty symbols) of 5 mM of TEA. B: [^{3}H]MPP uptake by hMATE1 (■) or hMATE2-K (●) over time in the absence (solid symbols) or presence (empty symbols) of 1 mM of MPP. N=3, mean±SEM.

Figure 3: Inhibitory effects of NBuPy-Cl on OCTs.

Intracellular uptake of [^{3}H]TEA by CHO_rOCT1 (A, 120s); [^{14}C]metformin by CHO_rOCT2 (B, 120 s) and CHO_hOCT2 (C, 60 s) in the presence of increasing concentrations of NBuPy-Cl (N=3-5, mean±SEM).

Figure 4: Influence of alkyl chain length on the inhibitory effects of pyridinium based ILs on OCTs.

A: Intracellular uptake of [^{14}C]metformin (120 s) by CHO_rOCT2 in the presence of increasing concentrations of RPy-Cl (RPy-Cl: Py-Cl, EtPy-Cl, NBuPy-Cl and HePy-Cl; N=3-5, mean±SEM); B: Relationship between inhibitory effects (IC_{50}) and alkyl chain length (number of carbons) of RPy-Cl (N=3-5, mean±SEM). The statistical analysis compared the Log(IC_{50}) using one way ANOVA with newman-Keuls post test.

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Figure 5: inhibitory effects of NBuPy-Cl on hMATE1 and hMATE2-K.

Intracellular uptake of [^{3}H]MPP by CHO_hMATE1 (A) or CHO_hMATE2-K (B) in 2 min with the presence of increasing concentrations of NBuPy-Cl (N=3-6, mean±SEM).

Figure 6: Effects of NBuPy-Cl on the pharmacokinetics of metformin.

Plasma concentrations of [^{14}C]metformin (n=3-4, mean±SEM) following a single i.v. administration (5 mg/kg) to male F344 rats with saline (▲, 2 ml/h) or NBuPy-Cl (■, 0.5 mg/kg/h; ●, 2 mg/kg/h) infusion through jugular vein.

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	IC_{50} (μM)			
	hOCT2		rOCT1	rOCT2
ILs	TEA	metformin	TEA	metformin
BmPy-Cl	0.48±0.05**	0.15±0.01*#	4.70±1.37	1.80±0.26**
Bmim-Cl	1.50±0.43	0.44 ±0.04	7.53±0.97	1.81 ±0.08**
Py-Cl	790±51**	555±40**#	762±254*	671±92**
EtPy-Cl	36.7±3.7*	20.1±2.2*#	72.1±13.3*	14.4±0.9**
NBuPy-Cl	2.29±0.64	0.69±0.08#	4.74±0.66	3.78±0.23
HePy-Cl	0.35±0.03**	0.10±0.01*#	2.21±0.23*	0.10±0.01**

Table 1

Inhibitory effects (IC_{50}) of NBuPy-Cl and structurally related ILs on OCTs mediated TEA or metformin transport (mean±SEM, n=3-5). *: $p<0.05$, **: $p<0.01$ when compared with NBuPy-Cl; #: $p<0.05$ when compared with the probe substrate of TEA for hOCT2.

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		AUC0_180 min*μg/ml	T1/2 h	CL ml/min	Vss ml
NBuPy-Cl	0 (saline)	358±47	2.3±0.6	3.4±1.0	484±47
	0.5 mg/kg/h	391±37	2.8±0.4	2.3±0.2	473±22
	2 mg/kg/h	687±86*	4.5±1.2	1.2±0.4*	334±22

Table 2

Kinetic parameters of metformin in male F-344 rats following i.v. administration (5mg/kg) with NBuPy-Cl or saline infusion (n=3-4, mean±SEM). *: $p<0.05$. AUC0_180: area under the curve from 0 to 180 min; T1/2: elimination half life; CL: systemic clearance; Vss: volume of distribution. Predicted parameters were calculated using non-compartment model analysis.

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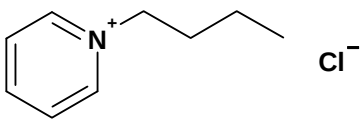
NBuPy-Cl	Metformin concentration µg/g tissue [tissue/plasma]¶		
	0 (saline) N=4	0.5 mg/kg/h N=4	2 mg/kg/h N=4
Adipose	4.2±2.3 [4.6]	1.8±0.4 [1.5]	7.3±0.9 [3]
Heart	41.3±4.4 [56]	31.6±2.5 [25]	45.8±8.4 [19]
Kidneys	204±73 [213]	151±47 [121]	756±192 [266]
Liver	16.9±2.9 [21]	13.7±0.3 [11]	44.9±11.1 [16]
Lung	14.5±2.7 [19]	15.0±0.8 [12]	28.7±4.8 [11]
Muscle	7.1±2.0 [8.8]	5.8±0.4 [4.7]	12.8±1.2 [5.4]
Spleen	8.8±1.6 [11]	8.5±0.5 [6.8]	18.3±3.4 [7]
Testes	3.9±0.3 [5.3]	4.6±0.4 [3.7]	7.4±1.2 [2.9]

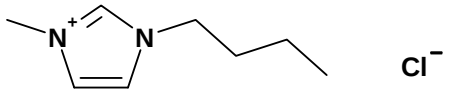
Table 3

Metformin concentration in selected tissues (µg/g) of F-344 rats following i.v. administration of metformin (5 mg/kg) to male F-344 rats infused with saline or NBuPy-Cl (Mean±SEM). ¶: ratio of metformin concentration in tissue (g) versus plasma (ml).

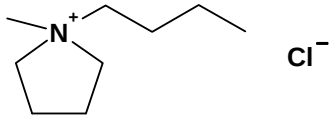
Figure 1



N-butylpyridinium chloride
(NBuPy-Cl, $C_9H_{14}ClN$)



1-butyl-3-methylimidazolium chloride
(Bmim-Cl, $C_8H_{15}ClN_2$)



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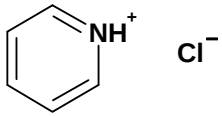
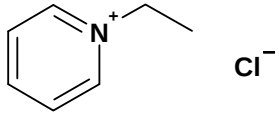
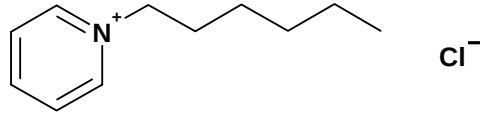
(BmPy-Cl, $C_9H_{20}ClN$)



Pyridine hydrochloride
(Py-Cl, C_5H_6ClN)



1-ethylpyridinium chloride
(EtPy-Cl, $C_7H_{10}ClN$)



1-hexylpyridinium chloride
(HePy-Cl, $C_{11}H_{18}ClN$)

Figure 2-A

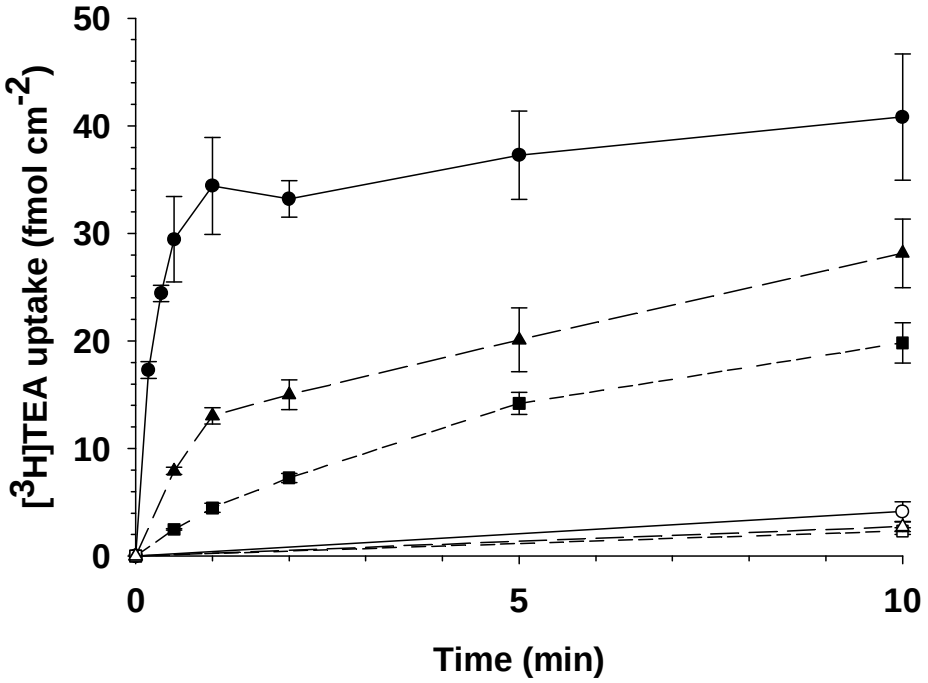


Figure 2-B

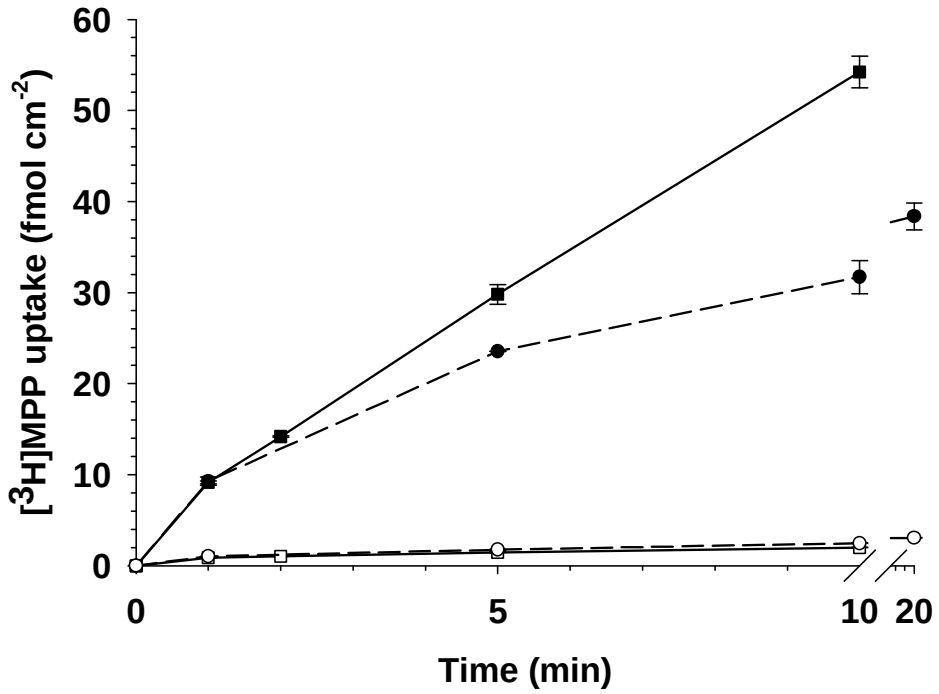


Figure 3-A

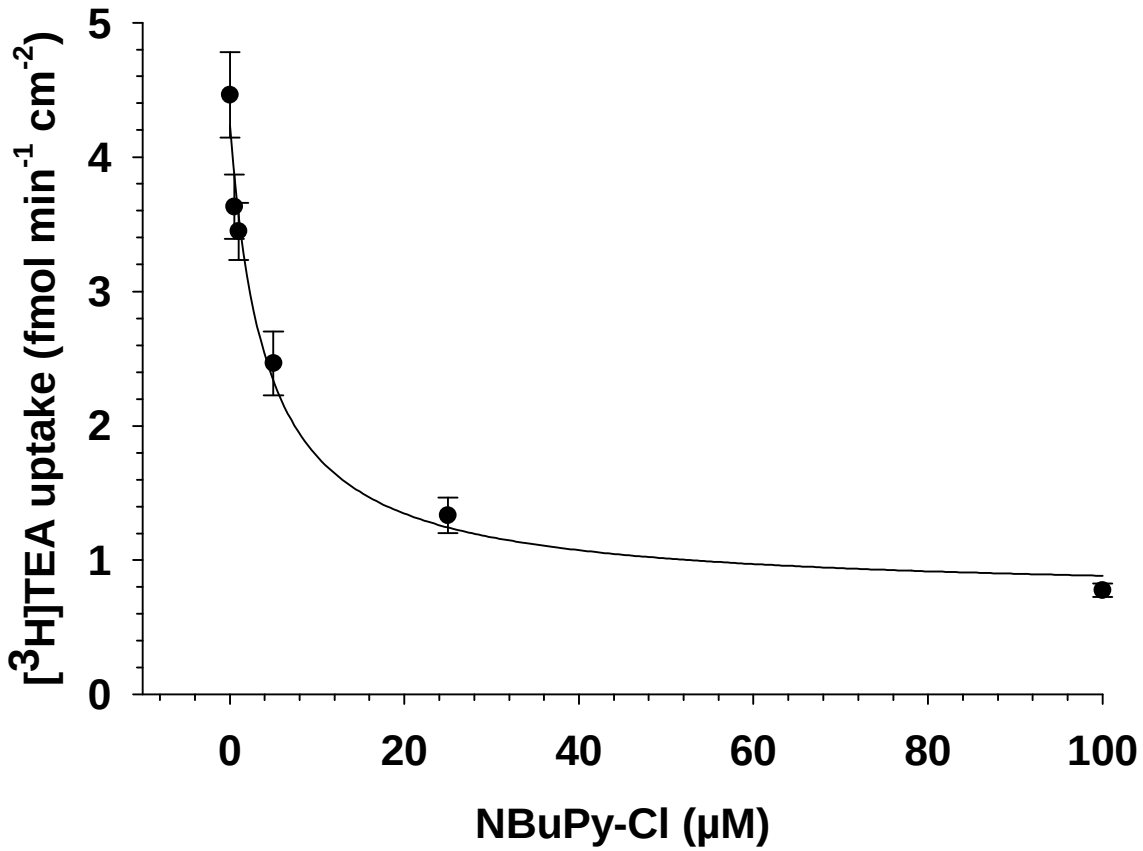


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Figure 3-B

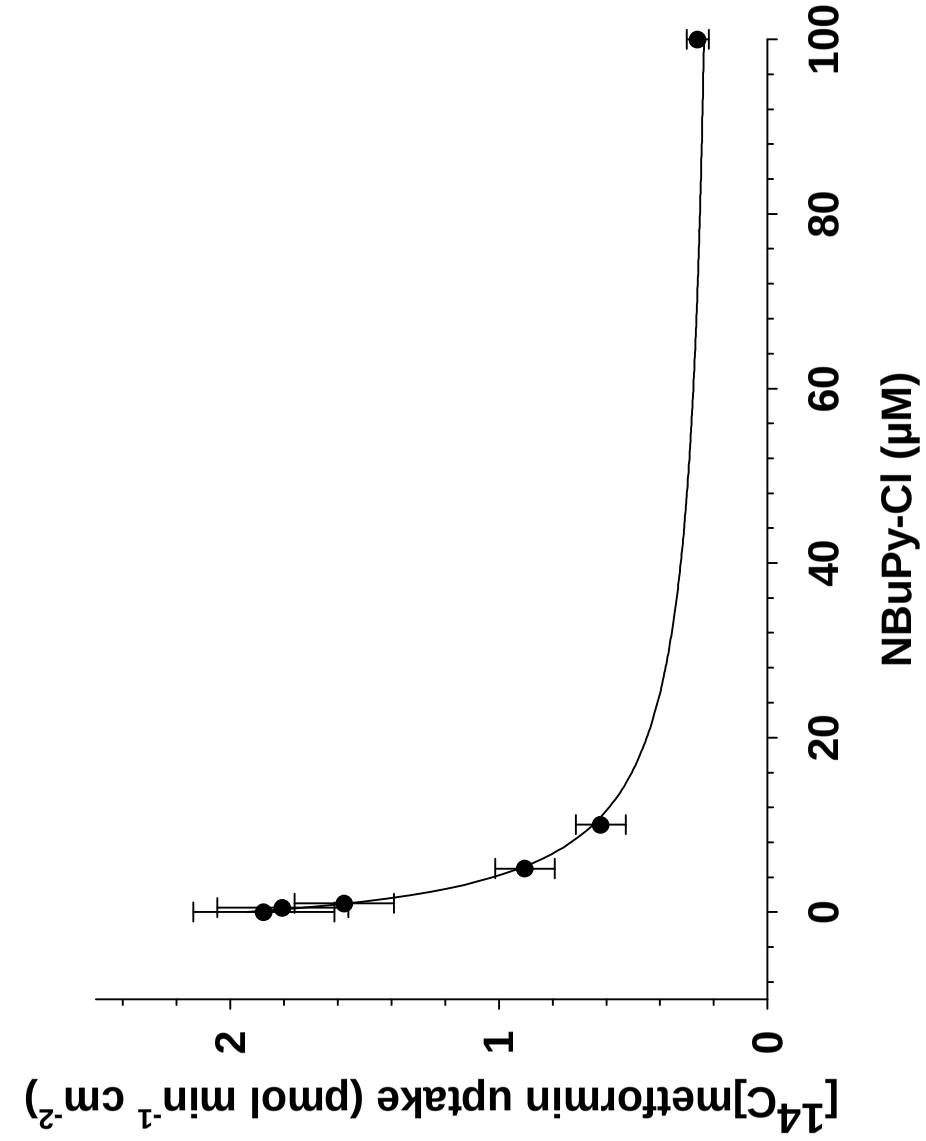

Figure 3-C

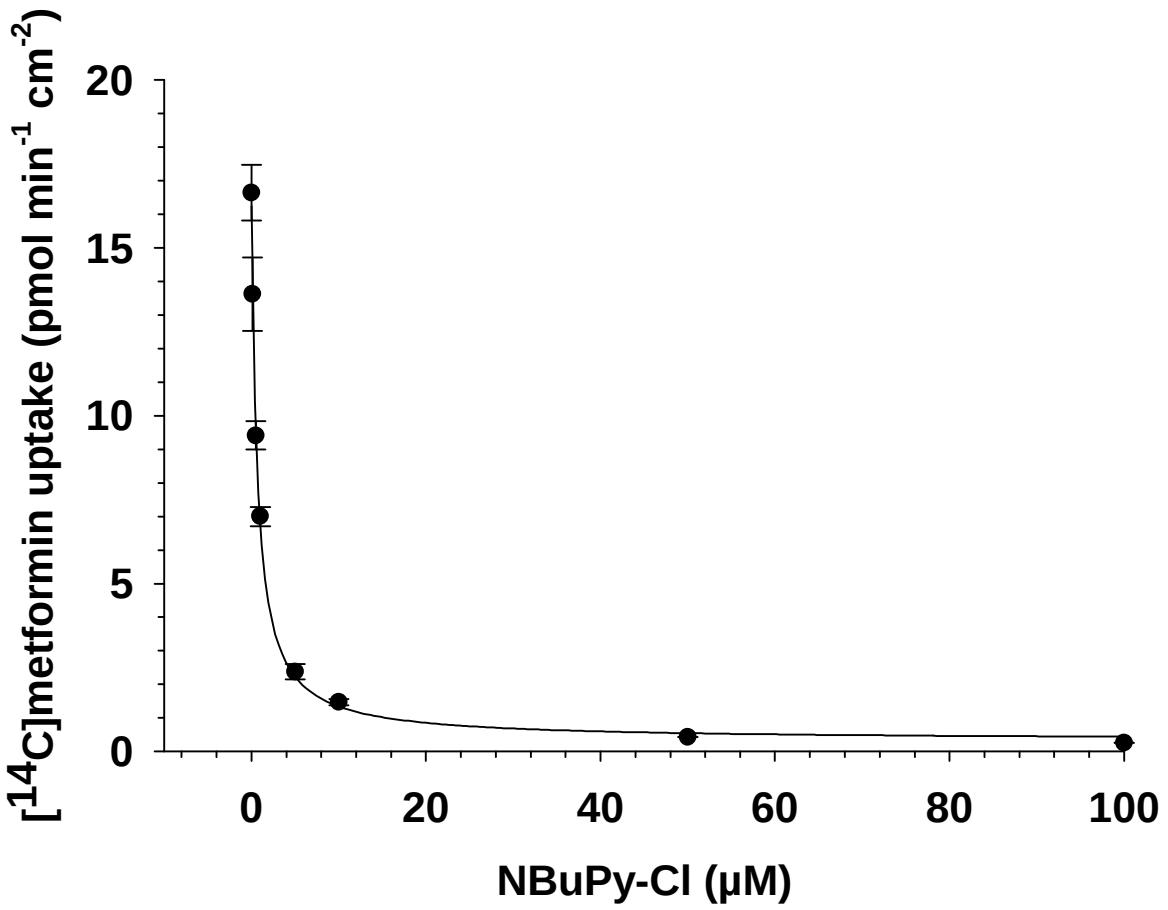

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Figure 4-A

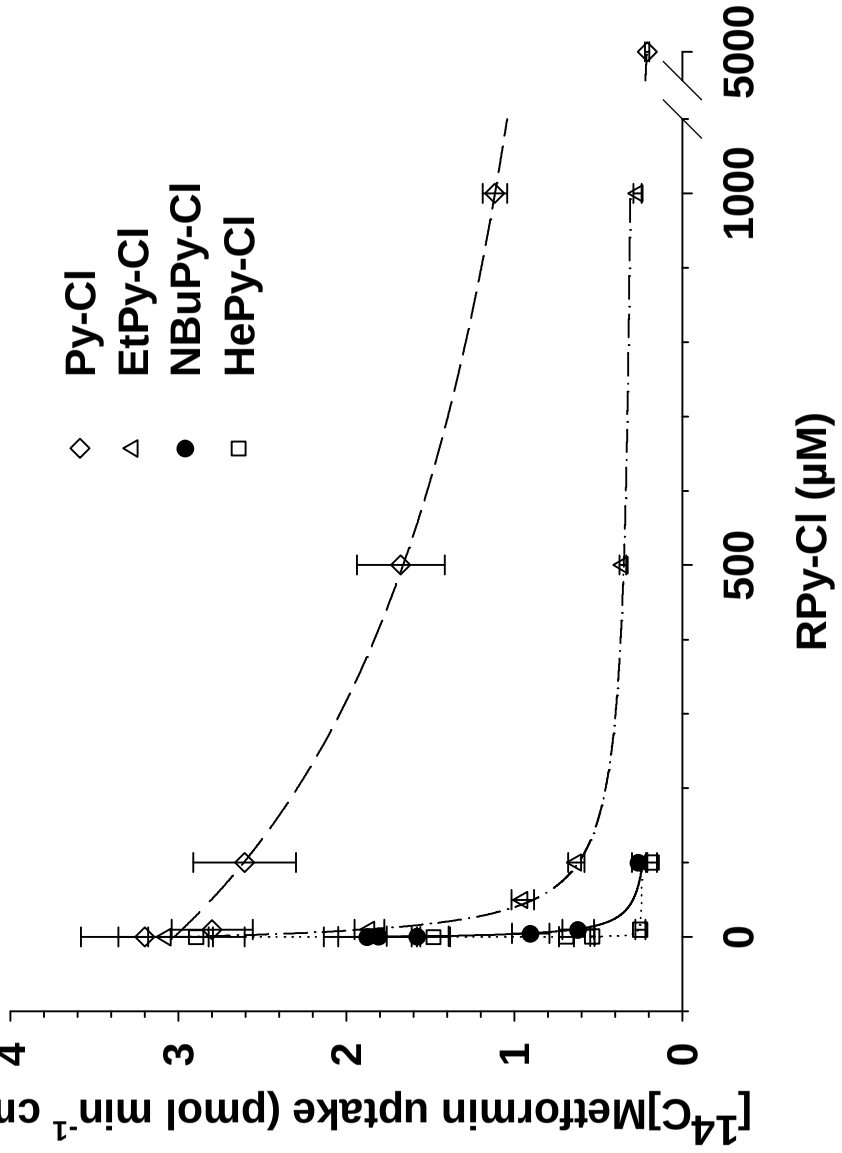

Figure 4-B

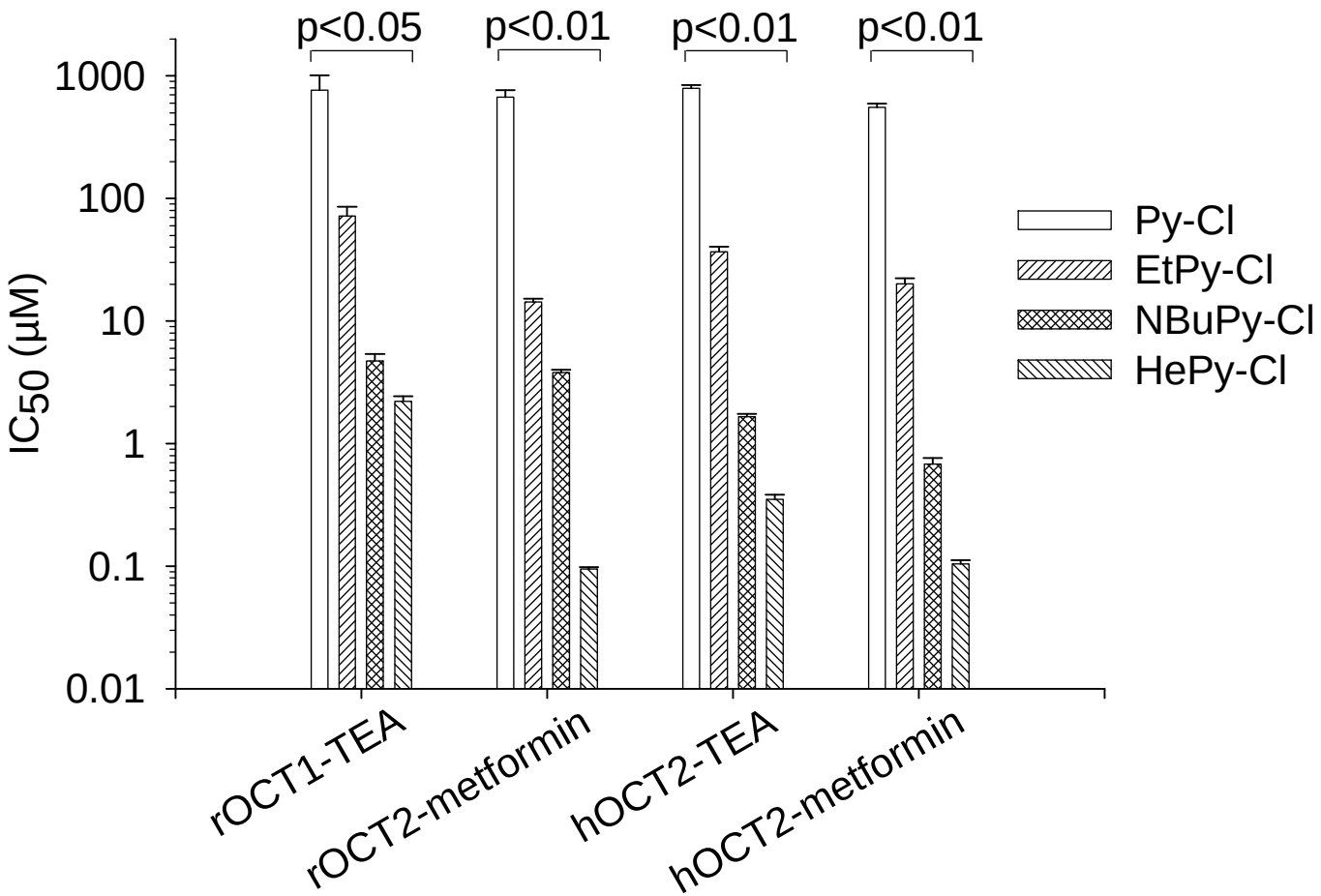

Figure 5-A

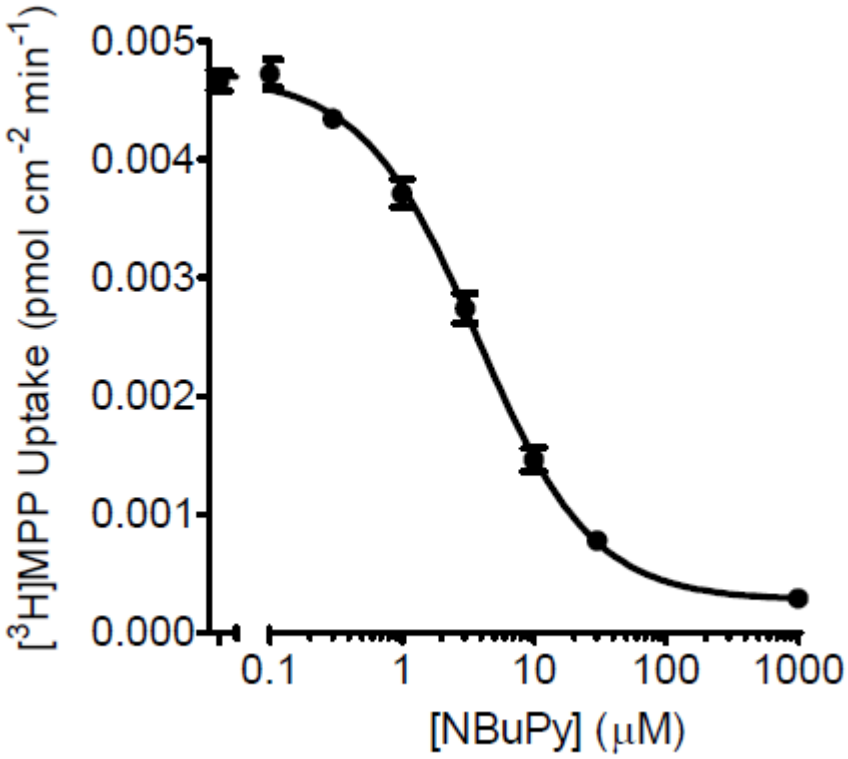

Figure 5-B

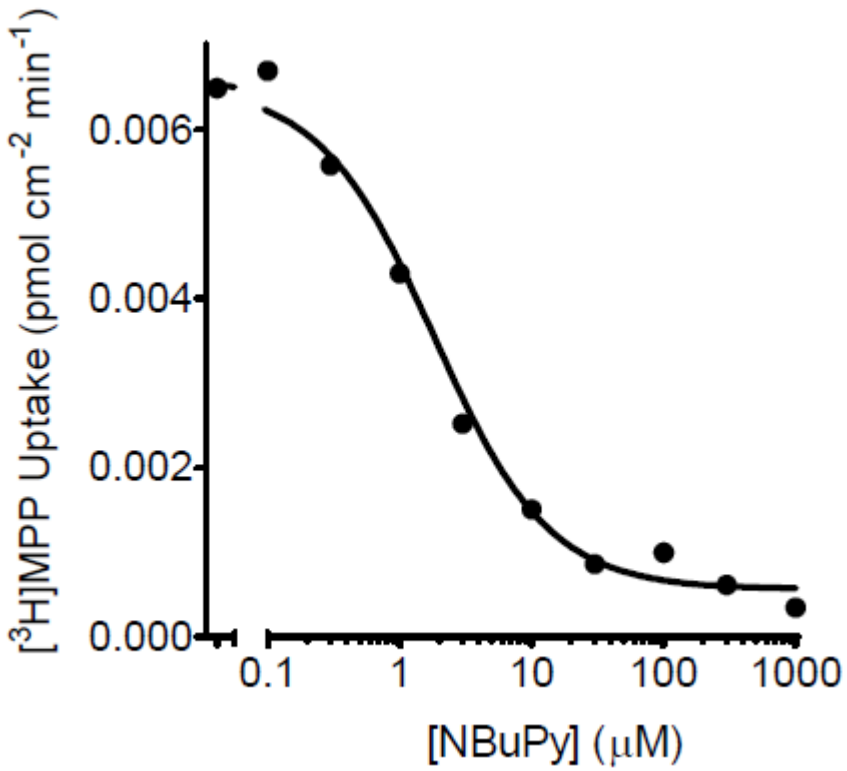

Figure 6