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ORGANIC ANION TRANSPORTER 2 (*SLC22A7*) IS A FACILITATIVE TRANSPORTER OF cGMP

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Abbreviations used: hOAT2, human organic anion transporter 2; cGMP, guanosine 3'5'-cyclic monophosphate; SLC, solute carrier superfamily; ABC, ATP-binding cassette transporter superfamily; GC, guanylate cyclase; PDE, cyclic nucleotide phosphodiesterase; PAH, para-aminohippurate; EV, empty vector.

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Abstract

The second messenger, cGMP, mediates a host of cellular responses to various stimuli, resulting in the regulation of many critical physiologic functions. The existence of specific cGMP transporters on the plasma membrane that participate in the regulation of cGMP levels has been suggested in a large number of studies. In this study, we identified a novel plasma membrane transporter for cGMP. In particular, we show that hOAT2 (*SLC22A7*), a member of the solute carrier (SLC) superfamily, is a facilitative transporter for cGMP and other guanine nucleotides. hOAT2, which is ubiquitously expressed at high levels in many cell types, was previously thought to primarily transport organic anions. Among purine and pyrimidine nucleobases, nucleosides and nucleotides, hOAT2 showed the greatest preference for cGMP, which transported cGMP with a K_m value of 88 $\pm$ 11 μM and exhibited between 50- and 100-fold enhanced uptake over control cells. Our data revealed that hOAT2, is a bidirectional facilitative transporter that can control both intracellular and extracellular levels of cGMP. Additionally, we observed that a common alternatively spliced variant of hOAT2 demonstrated a complete loss of transport function as a result of a low expression level on the plasma membrane. We conclude that hOAT2 is a highly efficient, facilitative transporter of cGMP and may be involved in cGMP signaling in many tissues. Our study suggests that hOAT2 represents a potential new drug target for regulating cGMP levels.

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The cyclic nucleotide cGMP is a second messenger involved in mediating cellular response to various stimuli in numerous cell types. cGMP signaling through the activation of cGMP-dependent protein kinases regulates a wide variety of intracellular functions, and the potential involvement of extracellular cGMP in a number of biological processes has been suggested (Sager 2004; Poulopoulou and Nowak 1998). Because of its critical roles, proteins that regulate intracellular cGMP levels have been the subject of many studies. Such proteins include guanylate cyclases (GC), which play key roles in cGMP production, and phosphodiesterases (PDEs), involved in cGMP degradation.

The existence of plasma membrane transporters capable of transporting cGMP into as well as out of cells has been suggested in a large number of studies in a variety of cell types (Sager 2004), although the molecular identities of such transporters have remained unclear. Recently, three members of the multidrug resistance protein (MRP) family (part of the ATP binding cassette (ABC) superfamily of transporters), MRP4, MRP5, and MRP8 have been shown to transport cGMP (Jedlitschky et al., 2000; Chen et al., 2001; Guo et al, 2003). These transporters may play a role in the extrusion of cGMP from cells; however, these transporters do not participate in the transmembrane influx of cGMP since they are exclusively efflux pumps.

The solute carrier (SLC) superfamily is a major class of transporters responsible for the cellular influx and efflux of a great variety of endogenous substances including amino acids, peptides, sugars, organic ions, and nucleosides as well as a multitude of xenobiotic drugs. Although nucleoside transporter families exist within the SLC superfamily, (i.e., the concentrative nucleoside transporter (CNT) family (*SLC28A*) and the equilibrative nucleoside

transporter (ENT) family (*SLC29A*), none of these have been shown to transport cGMP (Gray et al., 2004; Baldwin et al., 2004).

In humans, four organic anion transporters, OAT1 (*SLC22A6*) (Cihlar et al., 1999; Koepsell and Endou 2004), OAT2 (*SLC22A7*) (Sun et al., 2001), OAT3 (*SLC22A8*) (Cha et al., 2001), and OAT4 (*SLC22A11*) (Cha et al., 2000), have been functionally characterized and have been shown to mediate the facilitative transport of various structurally diverse organic anions with partly overlapping substrate specificities (Burckhardt and Burckhardt, 2003). Among the OAT family, only hOAT2 has ubiquitous expression pattern with abundant expression in many tissues, whereas the other OATs are primarily expressed in the kidney. The specificity of hOAT2 has not been studied in great detail, although it has been thought to be an organic anion-preferring transporter with substrate specificity overlapping that of the other OATs.

While performing a routine screen to identify substrates of hOAT2, we discovered that the guanine nucleoside analog, acyclovir, vigorously interacted with hOAT2 (unpublished data). This finding led us to explore the interaction of other guanine nucleotides with hOAT2. The data presented in this study suggest that an important function of hOAT2 is to transport naturally occurring nucleobases, nucleosides and nucleotides, with a particular preference for guanine analogs. The transport of cGMP is the most avid of the substrates yet identified for hOAT2 and our data indicate that hOAT2 functions as a bidirectional facilitative cGMP transporter.

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Materials and Methods

Materials

All standard chemicals were purchased from Sigma. [^{3}H]-PAH and [^{3}H]-estrone sulfate (ES) were obtained from American Radiolabeled Chemicals, and all radiolabeled nucleobases, nucleosides, and nucleotides were from Moravek. Cell culture materials were supplied from UCSF Cell Culture Facility. Human total RNA and cDNA were purchased from Clontech. All primers were obtained from Invitrogen and probes for TaqMan assays were from Applied Biosystems.

Bioinformatics

Full length reference sequences for all proteins (OAT1-7, ENT 1-4, and CNT1-3) were obtained from NCBI. A multiple sequence alignment and neighbor-joining tree were created using ClustalX 1.83 using the default parameters (Thompson et al., 1997). The resulting dendrogram was created using TreeView 1.6.6 (Page, 1996).

Cloning and Transient and Stable Expression of Human OATs

cDNAs coding human OAT2 (hOAT2 or hOAT2-546aa, GenBank accession number NM_006672) and its splice variant (hOAT2-548aa, GenBank accession number NM_153320) were cloned by RT-PCR from human liver and kidney cDNA (Clontech, CA), respectively. Primers used were 5'-CCAGAGTCCAAGGGTCTATGT-3' (sense) and 5'-ATCAAGGATGGATGAGCAGAG-3' (antisense). Human OAT4 cDNA (GenBank accession number NM_018484) was cloned from kidney. cDNA clones for human OAT1 (GenBank accession number NM_004790) and OAT3 (GenBank accession number NM_004254) were obtained similarly as described above. Each cDNA

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was subcloned into pcDNA5/FRT (Invitrogen) and used to generate transient or stable HEK293 cell lines using the Flp-In® system (Invitrogen) as described previously (Erdman et al., 2006). To generate stable cell lines, Flp-In-293 cells were plated at a density of 6 x 10^5 cells per well in six-well tissue culture treated plates using antibiotic-free media and incubated overnight. Cells reached 95% confluence at 24 h after seeding, at which point cells were transfected with 0.4µg of hOAT2 cDNA or pcDNA5/FRT (empty vector), 3.6 µg of pOG44 DNA, and 20 µg of Lipofectamine 2000. Two days after transfection, cells were trypsinized and split 1:4 into new six-well plates and selected for stable transfectants by addition of hygromycin B (75 µg/mL) to the growth media (DMEM containing 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin). After 10 to 14 days under selection colonies were pooled and expanded in 25-cm^2 flasks.

Functional Assays

Human embryonic kidney (HEK293) cells were cultured in the growth media described above at 37ºC, 95% humidity, and 5% CO_2. Cells were transiently transfected with 1 µg of hOAT2 or other transporter and 3 µg of Lipofectamine 2000 (Invitrogen) in each well according to the manufacturer's protocol. Cells were seeded at a density of 4 x 10^5 cells per well in poly-D-lysine-coated 24-well plates (Becton Dickenson, MA) and were grown overnight. Uptake assays with transiently transfected cells were conducted 18-24h after transfection. For uptake studies, the cells were rinsed with pre-warmed uptake buffer (128 mM NaCl, 4.73 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 5 mM HEPES, pH 7.4), and then incubated in 0.3 mL of pre-warmed buffer containing radiolabeled test compounds in the presence or absence of 10 µM nitrobenzylthioinosine (NBMPR), which was

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added to inhibit background ENT-mediated uptake of nucleosides and nucleobases. At the indicated time points, the reaction was terminated by washing with ice-cold buffer. Test substrates were quantified by liquid scintillation counting and the uptake amounts were normalized to total protein in each well. cGMP levels were measured radiometrically (Figs. 1, 2A, 3, and 5A) or enzymatically (Figure 4). For the enzymatic measurement of cGMP, stably transfected empty vector and hOAT2 cells were rinsed with 0.5 mL uptake buffer and then incubated with 0.25 mL of test solution. After a 1 hour incubation, an aliquot of an extracellular sample was immediately aspirated. The remaining portion was subsequently washed twice with ice-cold buffer. Afterward, 0.5 mL of lysis reagent was added and shaken for 1 hour to retrieve the intracellular cGMP sample according to the manufacturer's protocol (cGMP Enzymeimmunoassay Biotrak (EIA) System; Amersham Biosiences). The determined concentration of intracellular and extracellular cGMP was corrected as a cGMP level per cells.

RNA Extraction, Reverse Transcription, and Real-time PCR Assay

The expression of the organic anion tranpsorters (OAT1, OAT2, OAT3 and OAT4) was quantified by real-time reverse transcription-PCR, using TaqMan® Gene Expression Assays and an ABI 7500 Fast sequence detection system (Applied Biosystems, Foster City). Human GAPDH was used as an endogenous control to normalize expression.

Total RNA was extracted 48 h after transfection using the RNAqueous® system (Ambion Inc., Texas). One microgram of each RNA preparation was reverse-transcribed by random priming using High Capacity cDNA synthesis kit (Applied Biosystems, Foster City). Real-time PCR was performed using a 4.5 µL aliquot of the total

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cDNA sample using the TaqMan® Gene Expression Assays Hs00537914_m1 for human OAT1, Hs00198527_m1 for human OAT2, Hs00188599_m1 for human OAT3 and Hs00218486_m1 for human OAT4. Human GAPDH endogenous control (Hs99999905_m1) was used as an internal standard for sample normalization. Relative levels of the human OAT1, OAT2, OAT3 and OAT4 mRNAs were calculated using the $\Delta\Delta C_T$ (comparative threshold cycle) method. Each test was performed as a duplicate and all experiments were repeated three times. The levels of the human OAT1, OAT2, OAT3 and OAT4 mRNAs were expressed relative to the HEK293 Flp-In cell lines transfected with pcDNA5FRT vector only, which was normalized to 1.

Tissue distribution of an alternative splice form of hOAT2 was detected using a TaqMan® real-time PCR custom assay was performed with an ABI Prism 7700 Sequence Detector system.

Thin Layer Chromatography (TLC)

After allowing uptake to proceed for 1.5 minutes in OAT2-transfected and corresponding untransfected HEK cell lines in a 24-well plate, cells were lysed with 1% pentadecafluorooctanoic acid/ammonium salt buffer for an hour. The lysate was diluted to twice its volume with acetonitrile to precipitate all protein content. An aliquot of supernatant was evaporated and redissolved in 10 µL of water. Silica gel TLC of each uptake sample processed as described above was carried out with $CHCl_3$/MeOH/H_2O (6:6:1) as the mobile phase and compared with a standard sample of cGMP. The identity of the compound was confirmed by analysis using a PerSeptive Mariner ESI-TOF mass spectrometer. In addition, the amount of cGMP present in uptake samples was determined by measuring the radioactivity of the cGMP spot on the TLC plate and comparing it with the measured uptake.

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Construction for GFP-tagged Proteins and Microscopic Studies

Plasmids containing GFP-fused proteins were constructed as described previously (Erdman et al., 2006). The resulting fusion constructs were used to generate stable HEK293 cell lines using the Flp-In System, and analyzed by confocal microscopy as described previously (Urban et al., 2006).

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Results

hOAT2 transports nucleobases and nucleosides

hOAT2 was capable of transporting a wide range of purine and pyrimidine nucleobases, nucleosides and nucleotides (Fig. 1, Table 1). Among the nucleobases and the nucleosides, cGMP was the preferred hOAT2 substrate (approximately 60-fold uptake over empty vector (EV) transfected cell lines in the absence of NBMPR; 4.11 $\pm$ 1.65 pmol/mg protein/min for hOAT2 vs. 0.0710 $\pm$ 0.0315 pmol/mg protein/min for EV). When ENT-mediated background transport was inhibited with 10 μM NBMPR, hOAT2 mediated transport of cGMP was approximately 86-fold uptake over EV transfected cell lines; 3.26 ± 0.819 pmol/mg protein/min for hOAT2 vs. 0.0380 ± 0.0196 pmol/mg protein/min for EV. 2'-deoxyguanosine was also shown to be a hOAT2 substrate in the presence of NBMPR (approximately 24-fold uptake over EV; 74.0 ± 26.2 fmol/mg protein/min for hOAT2 vs. 3.13 ± 0.745 fmol/mg protein/min for EV). To determine whether the compounds tested were also substrates of hENT1 and hENT2, we compared their relative uptake in empty vector transfected HEK cells in the presence and absence of NBMPR (Table 1). Gemcitabine, a known substrate of ENTs, served as a positive control. As shown, cGMP was not transported by ENTs, whereas some of the nucleobases, nucleosides, and nucleotides were. Additional experiments with TLC and mass spectrometry confirmed that >90% of the intracellular [^{3}H]-cGMP in hOAT2 stably transfected cells at 1.5 minutes was intact (data not shown).

hOAT2 is distinct from other hOATs in substrate selectivity and structure

Although the other hOAT transporters showed efficient uptake of model substrates (61-fold uptake of adefovir by

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hOAT1, 25-fold uptake of estrone sulfate by hOAT3, and 21-fold uptake of estrone sulfate by hOAT4), they exhibited less uptake of cGMP compared to hOAT2 (Fig. 2B). In contrast, hOAT2 exhibited a great ability to transport cGMP (50-fold enhanced uptake) compared to its model substrate PAH (Fig. 2B). However, surface expression of these proteins is not known, and differences in surface expression may have influenced the comparative uptake values of cGMP. Multiple sequence analysis indicated that hOAT2 is distinct from other organic anion transporters and in spite of its functional similarities compared to nucleobase transporters, it does not appear to be related to ENT and CNT transporters at the amino acid level to any greater extent than the other members of the organic anion transporter family (Fig. 2C).

hOAT2 facilitates the transport of cGMP and 2'-deoxyguanosine

Multiple time course studies of cGMP and 2'-deoxyguanosine confirmed that 1.5 minutes was in the linear range of transport (data not shown); therefore, 1.5 minutes was used for cGMP and 2'-deoxyguanosine kinetics studies. The transport kinetics of cGMP and 2'-deoxyguanosine were saturable, with K_m estimates of 88 ± 11 μM for cGMP and 128 ± 17 μM for 2'-deoxyguanosine, and V_{max} values of 3373 ± 126 pmol/mg protein/1.5min for cGMP and 7225 ± 332 pmol/mg protein/1.5min for 2'-deoxyguanosine (Fig. 3A and 3B). cGMP was shown to have the lowest K_m among the guanine derivatives tested.

hOAT2-mediated transport of cGMP was inhibited by 2'-deoxyguanosine with a K_i value of 93 ± 12 μM, which was approximately equal to the K_m value for hOAT2-mediated 2'-deoxyguanosine transport in the presence of NBMPR (Fig. 3C).

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Intracellular and extracellular levels of cGMP are modulated by hOAT2

Since our preliminary data indicated that hOAT2 can mediate either uptake or efflux, we were interested in whether the transporter served primarily in the influx or efflux of cGMP. We determined the extracellular levels of endogenously produced cGMP extruded from EV- and hOAT2-transfected HEK293 cells treated with or without 750 μM sodium nitroprusside (SNP, a guanylate cyclase stimulator) plus 750 μM 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor). Fig. 4A shows that basal extracellular levels of cGMP in hOAT2-transfected cells were 6-fold lower than those in EV-transfected cells, suggesting that hOAT2 mediates cGMP influx under these conditions. That is, the net effect of hOAT2 under basal conditions is in the influx direction. In contrast, when intracellular cGMP levels were increased by 1 hr-treatment with SNP and IBMX, extracellular cGMP levels were 3-fold higher in hOAT2- transfected cells in comparison to EV-transfected cells (Fig. 4C). In this case, when a steep outwardly-directed cGMP concentration gradient was generated, the net effect of hOAT2 was in the efflux direction. In both EV and hOAT2 expressing cells, the intracellular pools of cGMP were a small fraction of the total, suggesting endogenous cGMP efflux activity in both cell lines. However, the intracellular cGMP amounts qualitatively and inversely reflected extracellular cGMP levels (Fig. 4B and 4D). This is consistent with hOAT2 acting in the influx of cGMP (in opposition to the efflux transporters) under basal conditions, and in the efflux of cGMP when its concentration gradient was directed outwardly by SNP and IBMX. Taken together, the data suggest that hOAT2, as a bidirectional facilitative transporter, can modulate cellular levels of cGMP.

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Additionally, timed efflux studies in hOAT2 and EV transiently transfected HEK cells preloaded with [^{3}H]-cGMP (2 µM) demonstrated that the extracellular concentrations of [^{3}H]-cGMP increased with time in the hOAT2 expressing cells (16.4 ± 3.20 pmol/mg protein at 10 minutes versus 21.8 ± 0.988 pmol/mg protein at 30 minutes) indicating that the transporter mediated [^{3}H]-cGMP efflux. Concentrations of [^{3}H]-cGMP in the extracellular media of empty vector transfected cells did not increase with time (data not shown). Mass spectrometric analysis of the extracellular media, indicated that cGMP was transported in the efflux direction by hOAT2, i.e., we detected substantial [^{3}H]-cGMP concentrations in the extracellular media at 10 minutes.

hOAT2 has two splice variants that differ in terms of two amino acids (hOAT2-546 and hOAT2-548)

The hOAT2 cDNA used in the previous experiments (hOAT2-546aa) contained a 1638 bp open reading frame encoding a 546 amino acid protein, which corresponds to the NCBI-registered reference sequence for *SLC22A7* (NM_06672). During our initial procedures, we cloned another hOAT2 cDNA (hOAT2-548aa), which has been listed as a related sequence to SLC22A7 in the NCBI data base (NM_153320) from a human kidney cDNA library. The sequence analysis revealed that the only difference between the two clones is a 6 bp-insertion (TCCCAG) between exon 1 and exon 2 of the reference *SLC22A7* gene. This additional nucleotide sequence is found in intron 1 of the reference SLC22A7 gene, and is flanked by the consensus sequence for intron/exon boundaries, suggesting that the two cDNAs of hOAT2 are alternatively spliced variants.

Our studies indicated that hOAT2-546aa was capable of transporting cGMP, whereas the hOAT2-548aa isoform exhibited a complete loss of cGMP uptake (Fig. 5A). Mutagenesis analysis demonstrated that deletion of two

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amino acid residues from hOAT2-548aa restored function, whereas insertion of two amino acid residues into hOAT2-546aa caused a complete loss of function (data not shown). Additionally, cellular localization via fluorescence and confocal microscopy of GFP (control), hOAT2-546aa-GFP (functional isoform), and hOAT2-548aa-GFP (non-functional isoform) revealed that hOAT2-546aa-GFP was localized to the plasma membrane. In contrast, hOAT2-548aa-GFP remained exclusively in the intracellular compartment with markedly lower fluorescence (Fig. 5B). The lack of surface expression of the hOAT2-548aa isoform most likely results in the observed loss of its transport function. Quantitative RT-PCR assays specific for each isoform showed that each hOAT2-expressing tissue contained approximately equal levels of the mRNA species of the two splice forms (Fig. 6).

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Discussion

Our studies indicate that hOAT2 exhibits a robust transport function for a wide array of naturally occurring nucleobases, nucleosides, and nucleotides (Fig. 1 and Table 1) with a particularly, unique role for cGMP.

cGMP is a second messenger involved in the intracellular signal transduction of a variety of extracellular stimuli in many tissues. Intracellular levels of cGMP are thought to be determined primarily by synthesizing and catabolizing enzymes, in the GC and PDE families. cGMP signaling can be modulated pharmacologically by nitric oxide (GC activator) and sildenafil (PDE5-specific inhibitor) (Friebe and Koesling, 2003; Dousa, 1999). Our data suggest that hOAT2 works in concert with these enzymes in regulating intracellular cGMP levels. The interaction of cGMP with PDE5 has a lower K_m (2-5 μM) in comparison to its interaction with hOAT2 determined in this study (K_m = 88 μM). These interaction kinetics support the notion that both hOAT2 and PDE5 are determinants of intracellular levels of cGMP. Although speculative, it is possible that hOAT2 acts to eliminate excess intracellular cGMP that is not enzymatically inactivated by PDE5. Intracellular cGMP levels vary widely depending upon the expression levels and activities of transporters and enzymes involved in its production and breakdown. Some reports include values as low as 1 μM and as high as 300 μM (Andric, et al. 2006). The K_m for OAT2 obtained in our study is consistent with reported intracellular concentrations of cGMP. In particular, we observed that under control conditions, hOAT2 mediates the influx of cGMP, whereas under conditions in which GC is stimulated and PDE is inhibited, hOAT2 functions in the efflux of cGMP from cells (Fig. 4A-4D). Additional experiments in our lab reveal that ENTs do not transport cGMP. Also, CNTs have not been demonstrated to accept nucleotide

monophosphates of any kind. Sequence comparisons indicate that OAT2 is about 40% identical to other OATs and only 15 to 20% identical to the ENTs and CNTs. Such sequence comparisons may not provide much information on the substrate specificities of various transporters since small differences in sequence can result in noticeable differences in protein structure.

To our knowledge, only one other influx transporter has been shown to transport cGMP (Sekine et al., 1997). This transporter, OAT1, is almost exclusively expressed in the kidney and takes up cGMP much less avidly than hOAT2 (Fig. 2A). Because hOAT2 is expressed at high levels in many tissues, our data suggest that hOAT2 plays a critical role in transmembrane influx of cGMP in many cell types.

Three efflux transporters for cGMP in the ABC superfamily have been identified and are members of the multidrug resistance protein family with K_m values between 2-10 μM (MRP4, MRP5, and MRP8) (Jedlitschky et al., 2000; Chen et al., 2001; Guo et al., 2003; Andric et al., 2006). Because hOAT2 is a facilitative transporter, it will have a distinct role from the MRPs, which, as efflux transporters, will function to actively eliminate cGMP from cells in tissues in which these transporters are expressed. In contrast, hOAT2 may facilitate both the uptake and efflux of cGMP in hOAT2-expressing tissues. Based on its wide tissue distribution, we speculate that hOAT2 is a key transporter for modulating intracellular cGMP levels in many tissues. In addition, there are several studies suggesting a role for cGMP as an extracellular effector molecule (Poulopoulou et al., 1998), particularly in brain (Erceg et al., 2006; Montoliu et al., 1999; Wang et al., 2004) and kidney (Chevalier et al., 1996; Neant and Bailly, 1993), tissues in which hOAT2 is abundantly expressed. hOAT2 may be involved in modulating the effects of

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extracellular cGMP in these tissues.

Structure activity studies indicated that the addition of a monophosphate, rather than a cyclic monophosphate, at the 5'-position reduced both the affinity and the capacity of hOAT2-mediated transport (Fig. 1B). Addition of one (GDP) or two (GTP) phosphate groups caused a further reduction in hOAT2 transport activity. These results suggest that cyclic 5', 3' monophosphate increases substrate-hOAT2 interaction and further suggest that the primary substrate of hOAT2 is cGMP.

To date, alternatively spliced variants in the OAT family have been identified for hOAT1 (Bahn et al., 2004). Two of four hOAT1 isoforms are non-functional due to deletion of transmembrane domain 11 and a portion of transmembrane 12. The OAT2 splice variant, hOAT2-548aa, is unusual because it contains such a small exon, and does not result in a frameshift. Nevertheless, the small difference between the two isoforms of hOAT2 produced a clear distinction in the activity of hOAT2, with hOAT2-548aa resulting in complete loss of transport function. Furthermore, mRNA expression studies demonstrating that the 6 bp insertion did not affect mRNA stability suggest that alternative splicing of this small exon is a stochastic event.

We determined that the mechanism for the loss of function of hOAT2-548aa was related to a reduced expression level of the protein on the plasma membrane (Fig. 5A). Similar to other OATs, the predicted secondary structure of hOAT2 consists of 12 TMDs with a large hydrophilic loop between TMD1 and TMD2. hOAT2-548aa contains a two-amino acid insertion**,** Ser and Gln**,** between Glu-131 and Trp-132 in the large extracellular loop 1 of hOAT2-546aa. It is likely that addition of these two amino acids results in reduced stability of the 548 form. In particular,

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hOAT2-548aa-GFP-transfected cells displayed a much lower GFP-derived signal in comparison with those transfected with hOAT2-546aa-GFP (Fig. 5B). This suggests that the lower levels of hOAT2-548aa protein on the plasma membrane are the result of a generalized increased rate of degradation of the 548 aa form. In fact, the 548 aa form was not present in Western blots using a GFP-specific antibody; whereas the 546 form was present in abundance (data not shown). It is also possible that these two amino acids disrupt a motif responsible for proper trafficking to the plasma membrane. It is noteworthy that the amino acid sequence in the vicinity of Glu131 is highly conserved among members of the OAT family suggesting that this region may contain a motif(s) that is/are essential for stability or trafficking of the protein.

In summary, our study revealed a novel functionality of hOAT2 to transport guanine nucleosides and in particular, cGMP in a bidirectional manner. This activity was present in one splice form, hOAT2-546aa, but not in an alternatively spliced form, hOAT2-548aa, which appeared to exhibit reduced stability in cells. By regulating intracellular as well as extracellular levels of cGMP, hOAT2, together with guanylate cyclases and phosphodiesterases, may play a key role in cGMP mediated signaling. Small molecule inhibitors may be used as tools to understand the role of hOAT2 in the context of regulation of intracellular and extracellular cGMP levels.

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Legends for Figures

Figure 1. Uptake of naturally-occurring nucleotides, nucleobases, and nucleosides by hOAT2

hOAT2 (solid bars)-transiently transfected HEK293 cells were incubated for 1.5 min with each radiolabeled compound (2 μM) in the presence of 10 μM NBMPR. Uptake of p-aminohippurate (PAH), a typical substrate of organic anion transporters, is shown for comparison. Each value represents the mean uptake via hOAT2 divided by the mean respective uptake in empty vector transfected cells. Data are from three independent experiments performed on separate days. The uptake of each compound was tested in triplicate samples in each independent experiment.

Figure 2. Specificity of cGMP and model substrate transport among hOAT family members (A, B) and dendrogram of the human OAT, ENT, and CNT transporter families (C)

(A) HEK cells transiently transfected with each hOAT isoform were incubated for 1.5 min with 2 μM cGMP. Only hOAT2-transfected cells showed substantially increased rate of uptake of cGMP above empty vector (EV)-transfected controls. cGMP transport by hOAT1 and hOAT3 was 6-fold and 5-fold greater than control, respectively, and was not transported by hOAT4 (< 2 fold). hOAT2 was the most selective for cGMP (50-fold enhanced uptake). Each bar represents the mean ± S.D. from triplicate samples in a representative experiment. **(B)** A 1.5 minute uptake following a transient transfection of each hOATs' respective model substrate (black bars) tested simultaneously with EV (white bars). The model substrates are as follows: adefovir for hOAT1, PAH for hOAT2,

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and estrone sulfate for hOAT3 and hOAT4. **(C)** A multiple sequence alignment using reference sequences for each transporter was performed using ClustalX; the dendrogram was generated from the ClustalX alignment output.

Figure 3. Concentration-dependent transport of (A) cGMP and (B) 2'-deoxyguanosine by hOAT2 and (C) 2'-deoxyguanosine inhibition of hOAT2-mediated cGMP transport

Empty vector-stably transfected and hOAT2-stably transfected HEK293 cells were incubated for 1.5 minutes with varying concentrations (5, 15, 25, 50, 125, 250, 375, and 500 μM) of cGMP **(A)**, or 2'-deoxyguanosine **(B)**. 2'-deoxyguanosine transport rate was measured in the presence of 10 μM NBMPR to inhibit background uptake. hOAT2-specific uptake was determined by subtracting uptake in empty vector-transfected cells from that in hOAT2-transfected cells after correcting for total protein. Kinetic parameters were estimated by fitting hOAT2-specific uptake rates to a Michaelis-Menten equation by nonlinear regression. Data are shown as the mean ± S.D. from triplicate samples in a representative experiment. **(C)**, Empty vector-transfected and hOAT2-transfected HEK293 cells were incubated for 1.5 minutes with 2 μM radiolabeled cGMP and varying concentrations (0, 3, 30, 100, 300, and 1000 μM) of 2'-deoxyguanosine. NBMPR (10 μM) was added to all of the 2'-deoxyguanosine reaction mixes to inhibit ENT1 transport. hOAT2-specific uptake was determined by subtracting uptake by EV-transfected cells from that in hOAT2-transfected cells after correcting for total protein. IC_{50} and K_i parameters were estimated by nonlinear regression. Uptake results shown in the graphs are from a representative experiment. Each point represents the mean ± S.D. of triplicate samples.

Figure 4. Extracellular and intracellular cGMP levels in HEK293 cells stably transfected with hOAT2

Extracellular (**A, C**; solid bar) and intracellular (**B, D**; open bar) cGMP levels in cells stably transfected with empty vector (EV) or hOAT2 were determined after 1 hr incubation in the absence (**A, B**) or presence (**C, D**) of sodium nitroprusside (750 μM) and IBMX (750 μM). Data are the mean ± S.D. of three independent experiments. Each experiment was conducted in duplicate samples.

Figure 5. cGMP uptake (A) and intracellular localization in HEK293 cells expressing two alternatively spliced variants of hOAT2 (B)

(A) HEK293 cells were transiently transfected with empty vector, hOAT2-546aa-GFP, or hOAT2-548aa-GFP. Cells were incubated for 1.5 min with 2 μM cGMP. Each bar represents the mean ± S.D. from triplicate samples in a representative experiment. Results were confirmed in two independent experiments. **(B)** Cells stably expressing GFP, hOAT2-546aa-GFP, or hOAT2-548aa-GFP were visualized by confocal microscopy. Plasma membrane was stained using AlexaFluor594 wheat germ agglutinin. Cells transfected with GFP alone show diffuse expression throughout the intracellular space. hOAT2-546aa-GFP localized specifically to the plasma membrane, whereas hOAT2-548aa-GFP showed diffuse localization throughout the cytoplasm with lower fluorescence intensity.

Figure 6. Tissue distribution of alternative splice forms of hOAT2

mRNA expression of hOAT2-546aa (solid bars) and hOAT2-548aa (open bars) was determined by real-time PCR

using TaqMan primers and probes specific to each isoform. Sensitivity of each assay for its target sequence was >10,000-fold greater than for the alternative target. Expression level of each isoform was normalized to GAPDH expression. Each bar represents the mean ± S.D. from quadruplicate assays of two independent cDNA sources. BLQ = below the limit of quantification.

Table 1. Uptake of naturally-occurring nucleotides, nucleobases and nucleosides in empty vector and hOAT2 transfected cells

Compound	Uptake Rate in the Absence of NBMPR (pmol/mg protein/min)		Uptake Rate in the Presence of NBMPR (pmol/mg protein/min)		ENT Transport Fold
	EV	hOAT2	EV	hOAT2	
cGMP	0.0710 ± 0.0315	4.11 ± 1.65	0.0380 ± 0.0196	3.26 ± 0.819	1.87
Guanine	0.263 ± 0.119	0.804 ± 0.513	0.0859 ± 0.0292	0.604 ± 0.313	3.06
Adenine	0.412 ± 0.0578	1.30 ± 0.239	0.262 ± 0.0530	1.10 ± 0.212	1.57
Guanosine	0.109 ± 0.0575	0.226 ± 0.169	0.0775 ± 0.0110	0.225 ± 0.163	1.41
GMP	0.00935 ± 0.00813	0.0196 ± 0.0190	0.00113 ± 0.000545	0.0185 ± 0.0185	8.27
GDP	0.00638 ± 0.000838	0.0110 ± 0.00328	0.00218 ± 0.0000522	0.00892 ± 0.00271	2.93
GTP	0.00724 ± 0.000970	0.0113 ± 0.00135	0.00143 ± 0.000502	0.00901 ± 0.00216	5.06
PAH	0.0228 ± 0.00326	0.0344 ± 0.00699	0.0265 ± 0.00684	0.0312 ± 0.00568	0.860
2’-deoxyguanosine	0.0551 ± 0.0234	0.0790 ± 0.0314	0.00313 ± 0.000745	0.0740 ± 0.0262	17.6
Uracil	0.0280 ± 0.00870	0.0411 ± 0.00825	0.0198 ± 0.00725	0.0258 ± 0.00333	1.41
Uridine	1.14 ± 0.242	1.34 ± 0.102	0.115 ± 0.0305	0.151 ± 0.0224	9.91
Hypoxanthine	0.351 ± 0.0523	0.403 ± 0.0351	0.123 ± 0.0171	0.203 ± 0.0304	2.85
Thymine	0.00774 ± 0.00451	0.00845 ± 0.00544	0.00585 ± 0.00296	0.00707 ± 0.00394	1.32
Adenosine	0.108 ± 0.0353	0.126 ± 0.0133	0.0295 ± 0.00581	0.0521 ± 0.0132	3.66
Thymidine	0.306 ± 0.0694	0.331 ± 0.0306	0.0338 ± 0.00415	0.0391 ± 0.00254	9.05
Cytidine	0.0548 ± 0.0177	0.0571 ± 0.00903	0.00194 ± 0.000239	0.00463 ± 0.00145	28.2
Cytosine	0.0101 ± 0.00552	0.0112 ± 0.00124	0.00768 ± 0.00512	0.00704 ± 0.00176	1.32
Inosine	0.306 ± 0.0904	0.282 ± 0.0174	0.0193 ± 0.00159	0.0354 ± 0.00524	15.8
Gemcitabine	0.699 ± 0.0958	N/A	0.0998 ± 0.0343	N/A	7.00

A table displaying the mean uptake values of nucleotides, nucleobases, and nuclesides in the absence and presence of NBMPR (10 µM). The uptake of PAH (a known hOAT2 substrate) and gemcitabine (a known ENT substrate), are shown for comparison. Each value represents the mean ± S.D. ENT fold transport was calculated by dividing empty vector transport in the absence of NBMPR divided by empty vector transport in the presence of NBMPR.

Figure 1

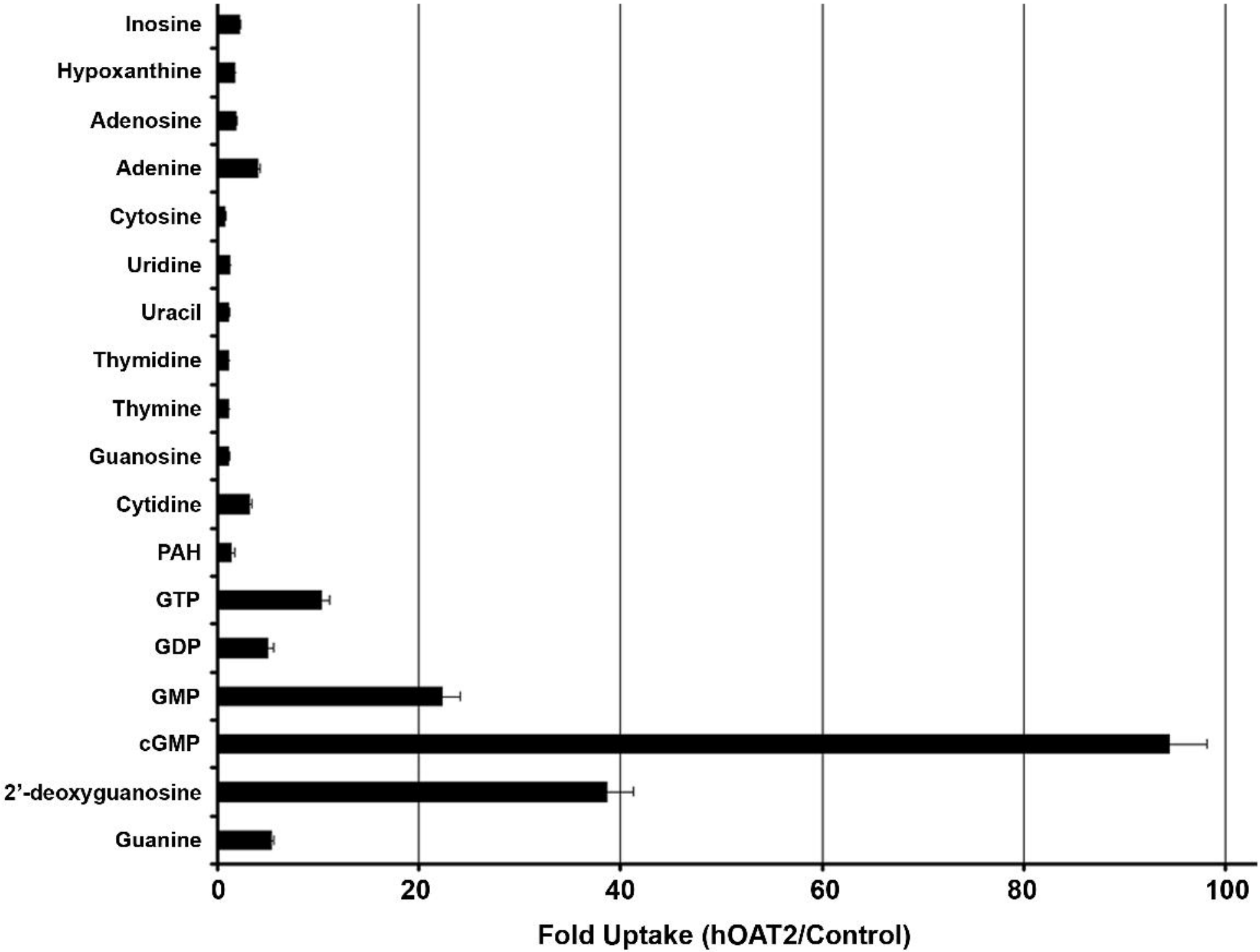

Inosine
Hypoxanthine
Adenosine
Adenine
Cytosine
Uridine
Uracil
Thymidine
Thymine
Guanosine
Cytidine
PAH
GTP
GDP
GMP
cGMP
2'-deoxyguanosine
Guanine
0
20
40
60
80
100
Fold Uptake (hOAT2/Control)

Figure 2

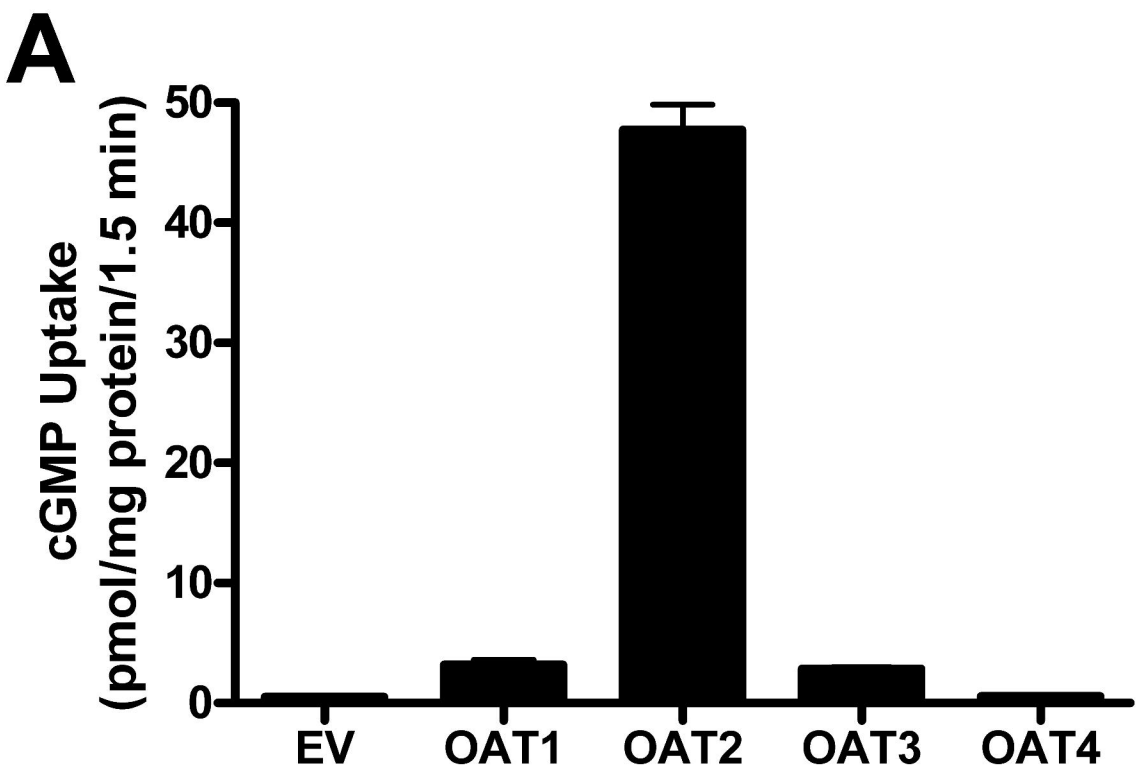

A
cGMP Uptake
(pmol/mg protein/1.5 min)
50
40
30
20
10
0
EV
OAT1
OAT2
OAT3
OAT4

Figure 2

B

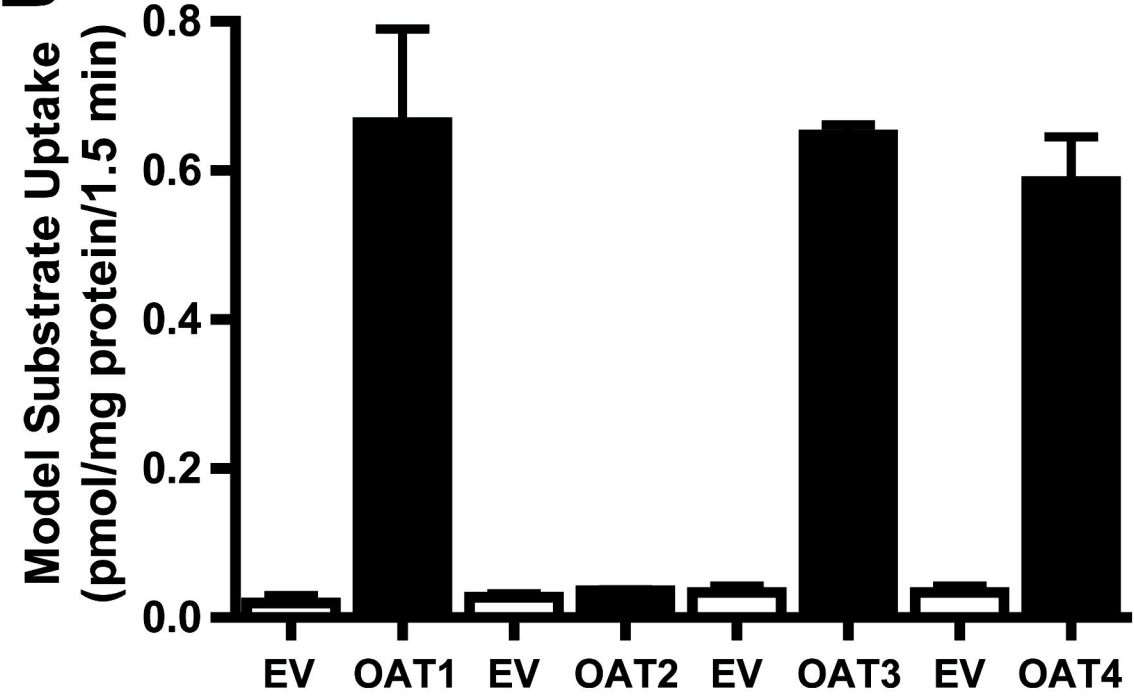

Model Substrate Uptake
(pmol/mg protein/1.5 min)
0.8
0.6
0.4
0.2
0.0
EV
OAT1
EV
OAT2
EV
OAT3
EV
OAT4

Figure 2

C

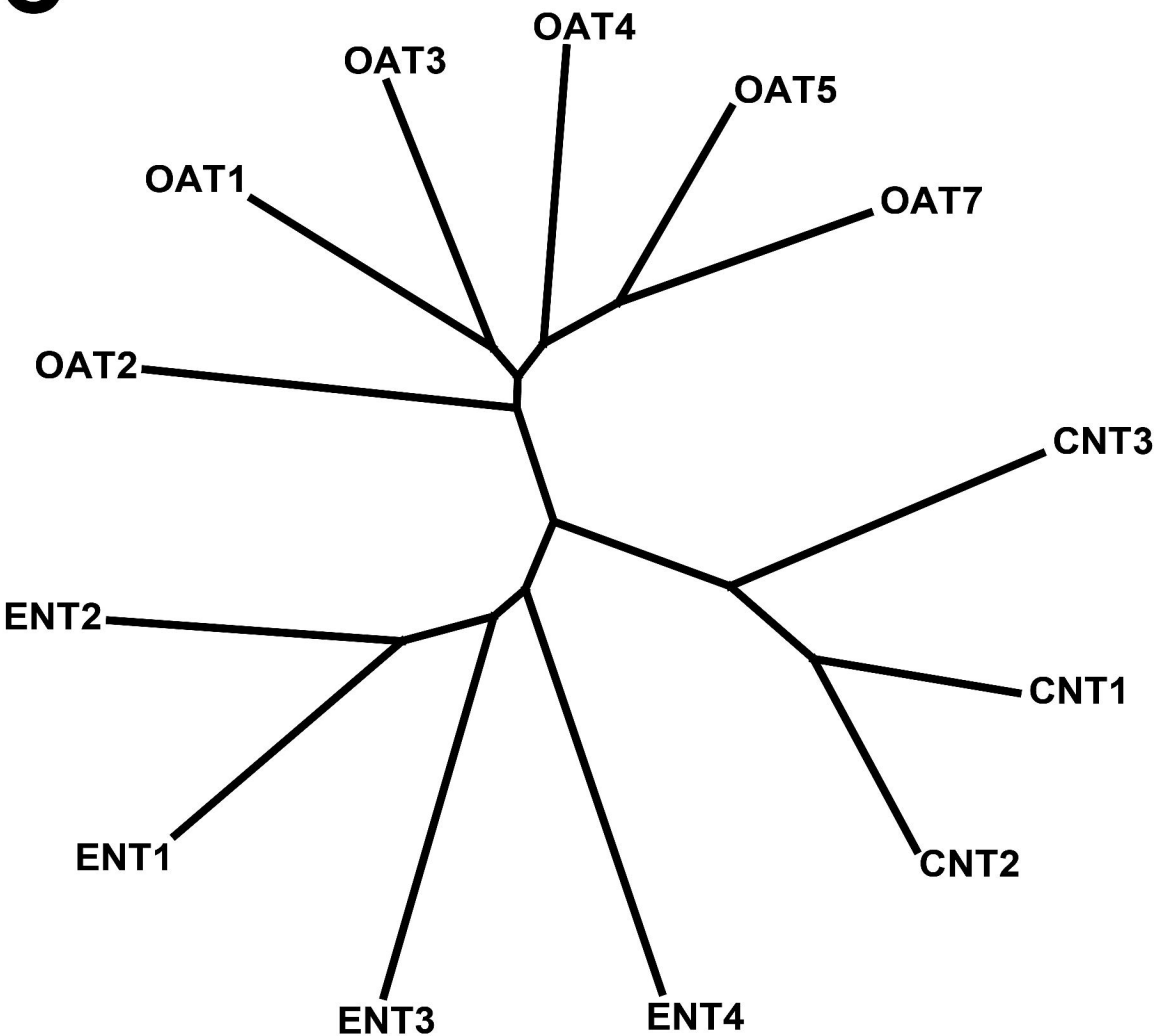

OAT4
OAT3
OAT5
OAT1
OAT7
OAT2
CNT3
ENT2
CNT1
ENT1
CNT2
ENT3
ENT4

Figure 3

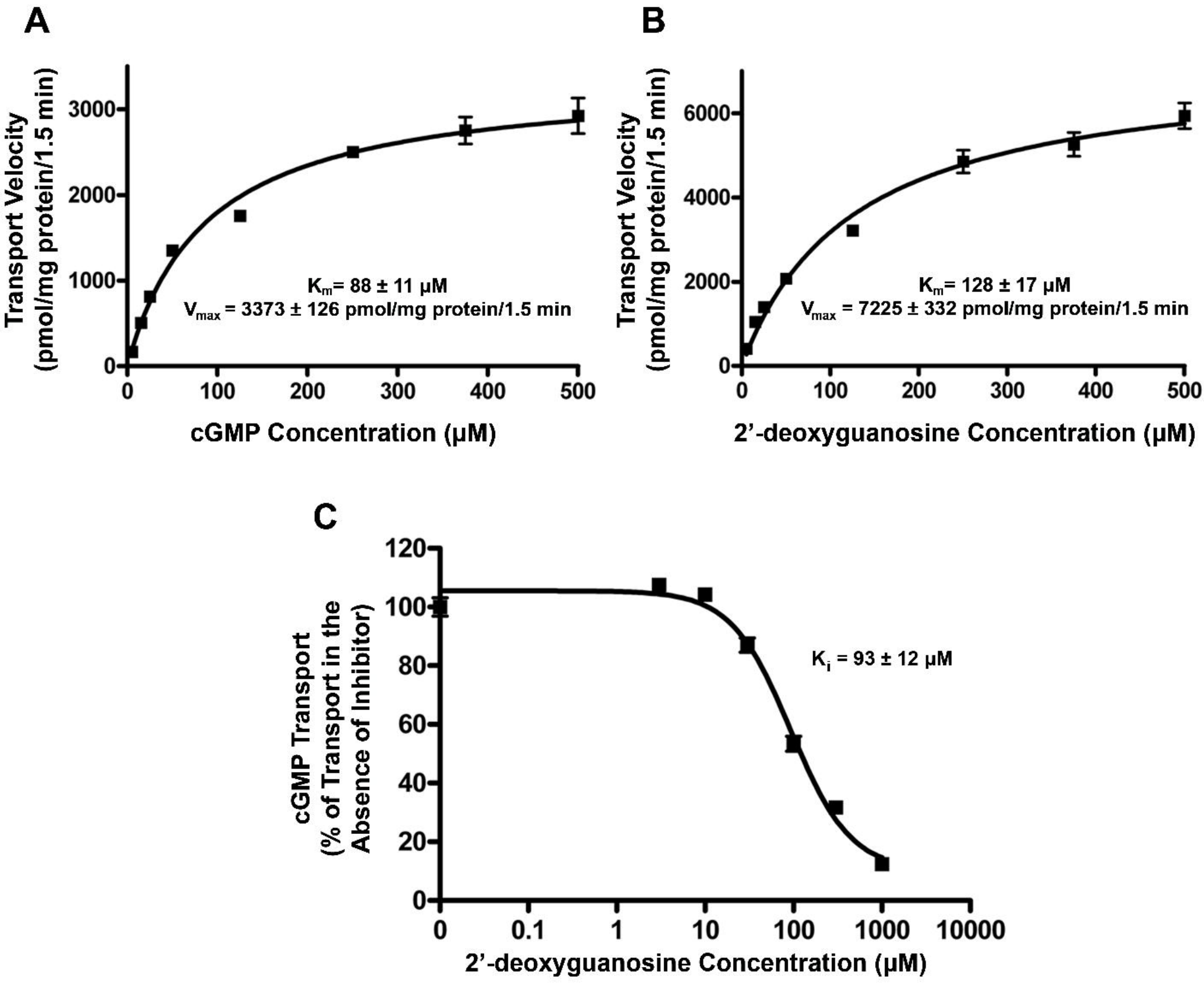

A
Transport Velocity (pmol/mg protein/1.5 min)
3000
2000
1000
0
0
100
200
300
400
500
cGMP Concentration (μM)
K_m= 88 ± 11 μM
V_{max} = 3373 ± 126 pmol/mg protein/1.5 min
B
Transport Velocity (pmol/mg protein/1.5 min)
6000
4000
2000
0
0
100
200
300
400
500
2'-deoxyguanosine Concentration (μM)
K_m= 128 ± 17 μM
V_{max} = 7225 ± 332 pmol/mg protein/1.5 min
C
cGMP Transport (% of Transport in the Absence of Inhibitor)
120
100
80
60
40
20
0
0
0.1
1
10
100
1000
10000
2'-deoxyguanosine Concentration (μM)
K_i = 93 ± 12 μM

Figure 4

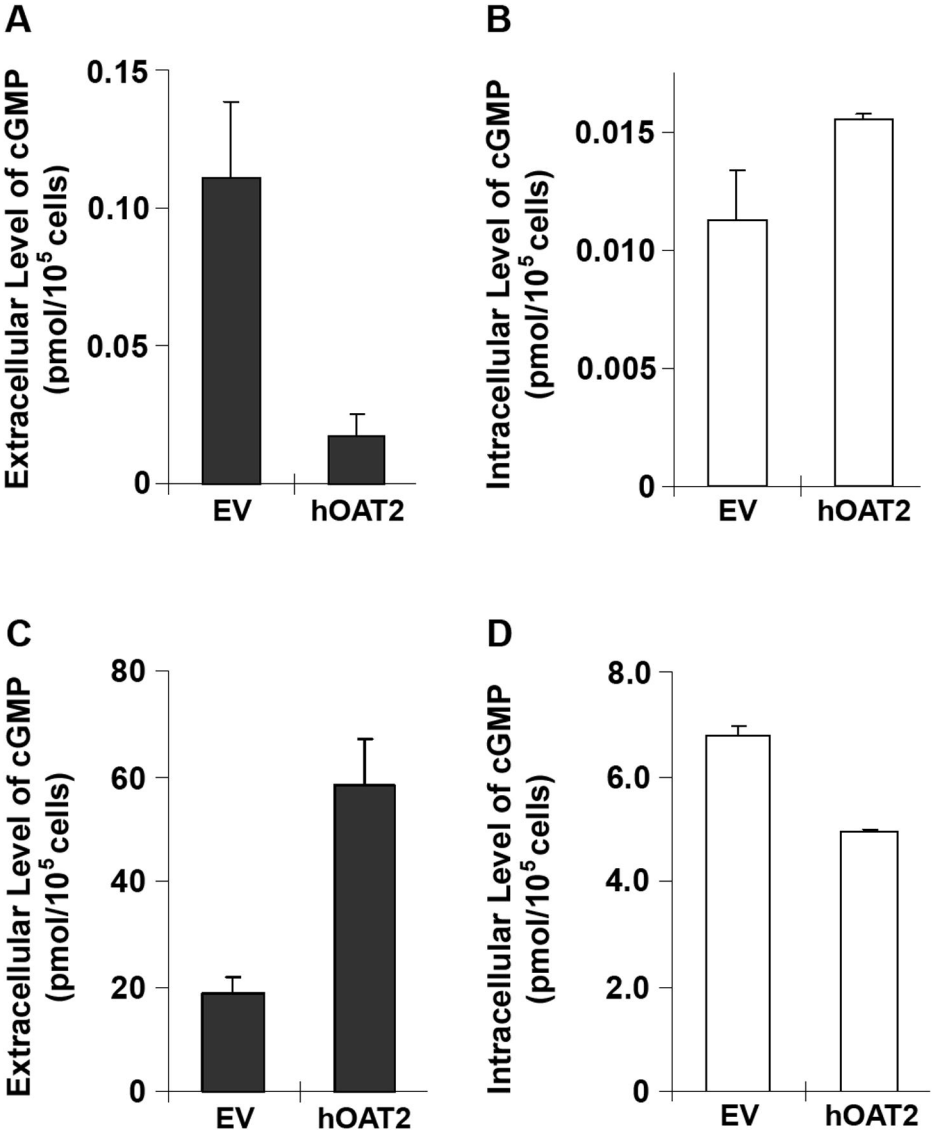
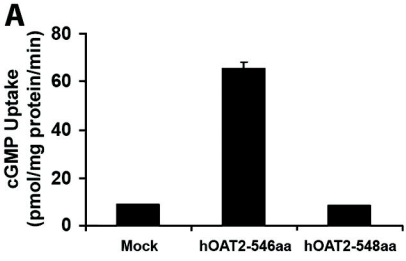

A
Extracellular Level of cGMP (pmol/10^5 cells)
0.15
0.10
0.05
0
EV
hOAT2
B
Intracellular Level of cGMP (pmol/10^5 cells)
0.015
0.010
0.005
0
EV
hOAT2
C
Extracellular Level of cGMP (pmol/10^5 cells)
80
60
40
20
0
EV
hOAT2
D
Intracellular Level of cGMP (pmol/10^5 cells)
8.0
6.0
4.0
2.0
0
EV
hOAT2

Figure 5


A
cGMP Uptake
(pmol/mg protein/min)
80
60
40
20
0
Mock
hOAT2-546aa
hOAT2-548aa

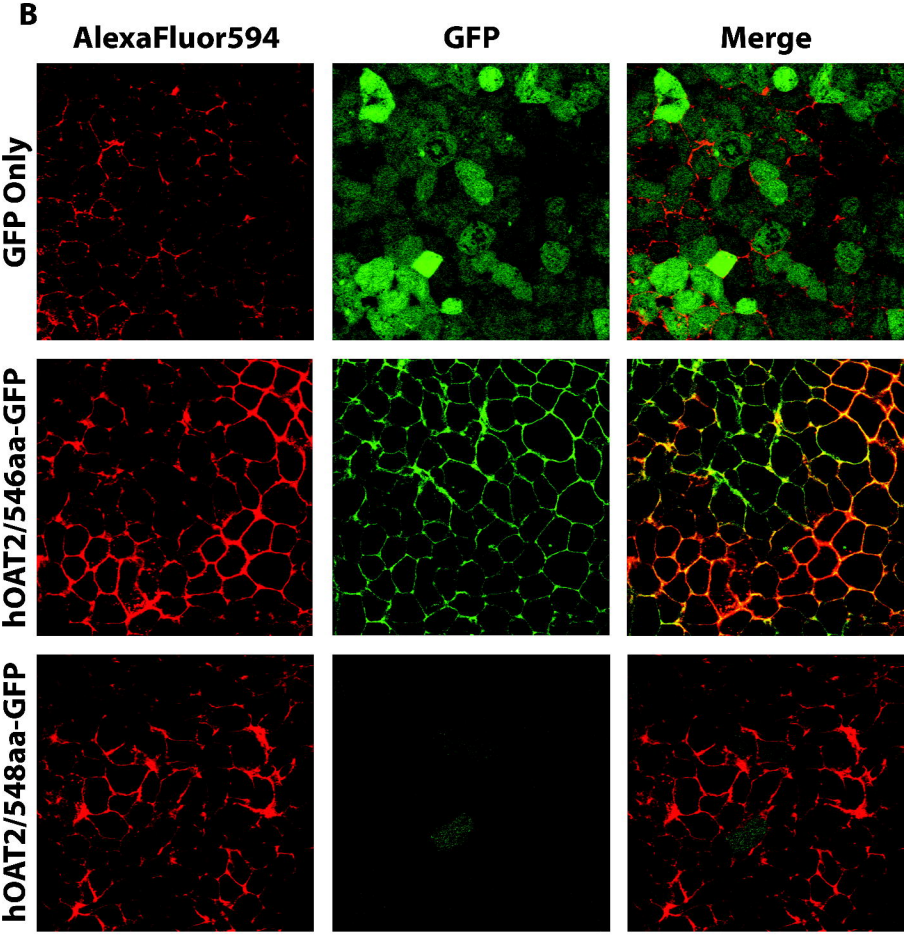
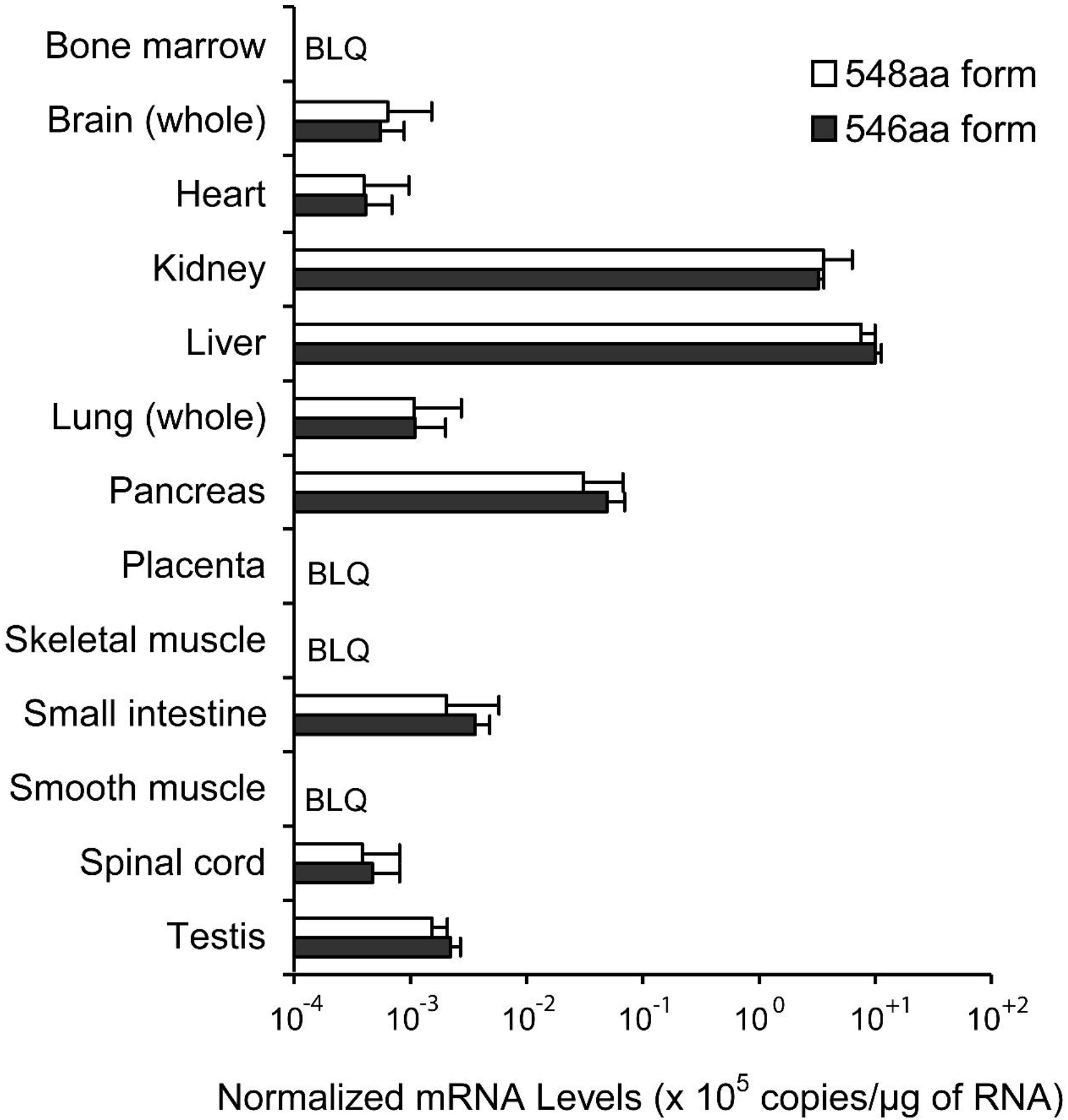

B
AlexaFluor594
GFP
Merge
GFP Only
hOAT2/546aa-GFP
hOAT2/548aa-GFP

Figure 6


Bone marrow
BLQ
Brain (whole)
Heart
Kidney
Liver
Lung (whole)
Pancreas
Placenta
BLQ
Skeletal muscle
BLQ
Small intestine
Smooth muscle
BLQ
Spinal cord
Testis
548aa form
546aa form
10-4
10-3
10-2
10-1
100
10+1
10+2
Normalized mRNA Levels (x 10^5 copies/µg of RNA)