CHARACTERIZATION AND REGULATION OF ORGANIC ANION TRANSPORTERS

By

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Prof. Guofeng You, Ph.D.

And approved by

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ABSTRACT OF THE THESIS

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Professor GUOFENG YOU

Human organic anion transporter 3 (hOAT3) belongs to a family of organic anion transporters that play critical roles in the body disposition of numerous clinically important drugs. Therefore, understanding the regulation of this transporter has profound clinical significance. In the current study, we investigated the short-term and long-term regulation of hOAT3 by protein kinase C (PKC). We showed that short-term activation of PKC by phobol 12-Myristate 13-Acetate (PMA) inhibited hOAT3 activity through accelerating its internalization from cell surface to intracellular recycling endosomes. The colocalization of hOAT3 with EEA1-positive recycling endosomes was demonstrated by immunolocalization with confocal microscopy. Furthermore, we showed that long-term activation of PKC resulted in the enhanced degradation of cell surface hOAT3. The pathways for hOAT3 degradation were further examined using proteasomal and lysosomal inhibitors. Our results showed that both proteasomal inhibitors and the

lysosomal inhibitors significantly blocked hOAT3 degradation. These results demonstrate that PKC plays critical roles in the trafficking and the stability of hOAT3.

I established five stable transfected cell lines. Those are COS-7 cells stably expressing hOAT3-c-myc, hOAT4-c-myc and HEK293 cells stably expressing hOAT1-c-myc, hOAT3-c-myc, hOAT4-c-myc. The stable transfected cell lines ensure long term and reproducible specific gene expression because it is accomplished by integration of transfected certain DNA of interest into cell's chromosome.

By using HEK293 cells stably expressing hOAT4-c-myc I established, I did anti-cancer drug library screening. The purpose of this experiment is the investigation of anti-cancer drug which has function to inhibit hOAT4.

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I am also grateful to Marianne Shen, Hui Pung and other colleagues in the Department of Pharmaceutics for their kind support and help, and all the faculty members in Department of Pharmaceutics, who provided useful academic courses.

DEDICATION

TO MY PRECIOUS PARENTS AND BROTHER

DONG-JIN SUH, MEE KYUNG PARK AND WONHYUNG SUH

FOR THEIR SINCERE SUPPORT THROUGHOUT MY LIFE

TO MY ADVISOR, PROFESSOR GUOFENG YOU,

WHO CONTINUED SUPPORT AND HAS GIVEN ME ENCOURAGEMENT, ADVICE AND SUGGESTIONS.

TO MY BELOVED WIFE, SUN YONG YOM

WHO ALWAYS HAS SUPPORTED ME AND ENCOURAGED ME FOR COMPLETING THIS WORK.

TO MY BELOVED DAUGHTER, YERIN SUH

TO MY BELOVED SON, BRANDON YEJOON SUH

WHO ALWAYS GIVE ME HAPPINESS.

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CHAPTER 1

Introduction

Drug transporters affect the disposition of drugs in human body through drug absorption, distribution, and elimination. These drug transporters are expressed in tissues and organs, such as kidney, brain, liver, and intestine. In addition, drug transporters play very critical roles in clinical pharmacology including drug bioavailability, oral absorption, drug resistance, which might be caused by unwanted drug transporters activities, drug elimination in the kidney and liver where drug transporters are tremendously expressed, drug toxicity which is caused by impaired drug elimination, and drug pharmacokinetics, pharmacodynamics where drug transporters may actively involve with. Accordingly, an understanding of drug transporters will accelerate the discovery and development of new drugs.

1.1 Organic anion transporter family

It is reported that ten OATs have been cloned and identified in various tissues and cell membranes according to their different expression level in certain organs. OATs are in the 22nd family of the solute carrier superfamily (SLC22). The Features of ten OATs are summarized in next table.

Transporter	Gene	Tissue Distrubution
OAT1	SLC22A6	Kidney, Brain, Placenta
OAT2	SLC22A7	Kidney, Liver
OAT3	SLC22A8	Kidney, Liver, Brain
OAT4	SLC22A11	Kidney, Placenta
OAT5	SLC22A19	Kidney, Liver
OAT6	SLC22A20	Nasal olfactory mucosa
OAT7	SLC22A9	Liver
OAT8	SLC22A9	Kidney(Rat)
OAT9	SLC22A9	Kidney, Liver(Mouse)
OAT10	SLC22A13	Kidney, Brain, Heart, Colon

OAT1: OAT1 is the first cloned OAT belongs a super family of OATs. OAT1s are predominantly expressed in the kidney. OAT1s are expressed in proximal tubule area of kidney, where also the drug elimination is going on. And also OAT1s are located on the apical side of the choroid plexus in the brain.

OAT2: OAT2s have species- and gender-related differences in expression. Human OAT2s (hOAT2s) and rat OAT2s (rOAT2s) are expressed mostly in the liver, less in kidney. But mouse OAT2s(mOAT2s) are chiefly expressed in kidney and less in liver. And OAT2s are expressed in female mice liver more than male mice liver.

OAT3: OAT3s are expressed in kidney, liver and brain. Same as OAT1s, OAT3s are located at the basolateral membrane of proximal tubular cells in the kidney. In the brain, OAT3s are expressed in the blood brain barrier and blood-cerebrospinal fluid barrier. OAT3s also have species-related differences in expression. hOAT3s are mostly expressed in the kidney, less in brain. mOat3s are mainly expressed in kidney, liver and at the apical side of the choroid plexus in

the brain. And rOat3s are largely expressed in the kidney and brain.

OAT4: OAT4s are expressed in the apical membrane of renal proximal tubule in the kidney. OAT4s are located in the syncytiotrophoblast cells in the placenta and are also located in the choroid plexus and brain micro-vessel epithelial cells in the brain.

In the kidney, hOAT1s and hOAT3s have the highest level of expression. hOAT4s are expressed thirdly.

1.2 Structure of Organic anion transporter

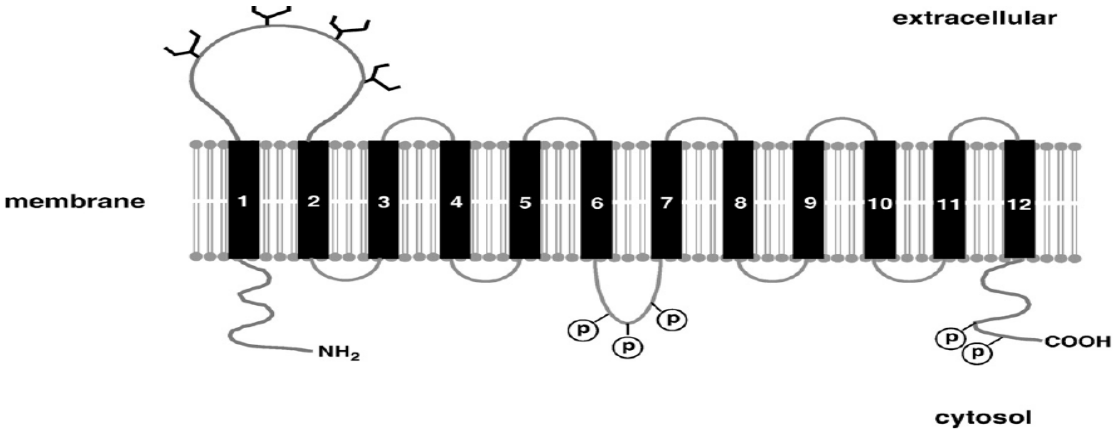


Figure 1-1. Predicted transmembrane topology of OAT family. Twelve trans-membrane domains are numbered from1–12. Potential glycosylation sites are denoted by tree-like structure and phosphorylation sites are labled P in circle. This figure is obtained from You, G., Medicinal Research Reviews, 2004. 24(6): p. 762-774

All of the cloned OATs share common structures. First, They have 12 trans-membrane domains (TMDs) with the intracellular localization of both amino and carboxyl termini; second, they have a large extracellular loop containing several glycosylation sites between the first and second TMDs; third, they have a large

intracellular loop containing multiple phosphorylation sites between the sixth and seventh TMDs.

1.3 Substrates for Organic anion transporter

There are a large number of potential substrates that can interact with or transported by OATs. These substrates are assorted by endogenous organic anions and exogenous organic anions in drugs and environmental chemicals.

Endogenous Organic Anions	
Cyclic nucleotides	cAMP, cGMP
Dicarboxylates	α-ketoglutarate, glutarate, succinate
Neurotransmitter metabolites	4-hydroxy-3-methoxymandelic acid, 3,4-dihydroxyphenylacetic acid,
Others	urate, folate, octanoate
Exogenous Organic Anions – Drugs	
Antibiotics	penicillin G, carbenicillin, amoxicillin, piperacillin, cloxacillin, nafcillin, cephaloridine, cefadroxil
Anti-viral drugs	azidothymidine, acyclovir, amantadine
Aiuretics	furosemide, bumetanide, ethacrynic acid, acetazolamide, benthiazide
ACE Inhibitors	captopril, enalapril, imidapril, delapril, benazapril, quinapril, ramipril
ATII antagonists	telmisartan, candesartan, valsartan, losartan
Anti-neoplastics	methotrexate, chlorambucil, 6-MP, thioguanine, dacarbazine, azathioprine, aclarubicin
Anti-epileptics	Valproate
Exogenous Organic Anions - Environmental Chemicals	
Mycotoxins	ochratoxin A, ochratoxin B, citreoviridin, citrinin,
Conjugated substances	zearalenol, fumonisin B1,
Sulphate conjugates	ostrone-S, p-nitrophenyl-S, 4-methylumbelliferyl-S, minoxidil-S, a-naphtyl-S,
Cysteine conjugates	S-benzyl-cys, CTFC, DCVC, N-acetyl-S-farnesyl-cys
Glucuronide conjugates	β-oestradiol 17-G, p-nitrophenyl-G, 4-methylumbelliferyl-G,
Glycine conjugates	PAH, o-hydroxyhippurate,

You, G., Medicinal Research Reviews, Vol. 22, No. 6, 602-616, 2002.

1.4 Transport mechanism and function of OATs

This figure shows the transport mechanism of OAT1and OAT3.

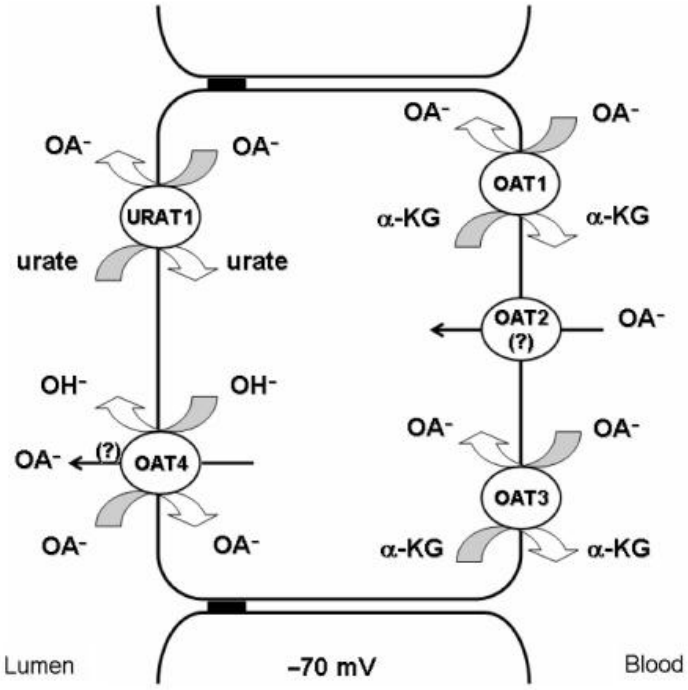


Figure 1-2. The transport mechanism of OAT1 and OAT3. This figure is obtained from Bobulescu IA., et al. Adv. Chronic. Kidney. Dis. 19 (6):358 -71
α-KG: α-Ketoglutarate

Na^+/K^+/ATPase creates the blood to cell directed Na^+ gradient at basolateral membrane. Dicarboxylates such as α-Ketoglutarate are then cotransported with Na^+ down its concentration gradient by a sodium dicarboxylate cotransporter. Finally, this cell to blood directed dicarboxylates gradient is utilized by organic anion transporters for exchanging organic anions (OAs) and results in its net uptake from blood to cell. Therefore, the organic anions are taken up by OATs across the basolateral membrane into the proximal tubule cells and subsequently exit across the apical membrane into the urine for elimination. From this mechanism and a plentiful expression of OAT1s and OAT3s in the kidney, it is expected that the function of OAT1s and OAT3s are controlling renal

elimination of endogenous and exogenous organic anions.

Different from OAT1s and OAT3s' mechanism, the transport of OAT2s is Na^+-independent. And the function of OAT2s is still unknown.

This figure shows the transport mechanism of OAT4.

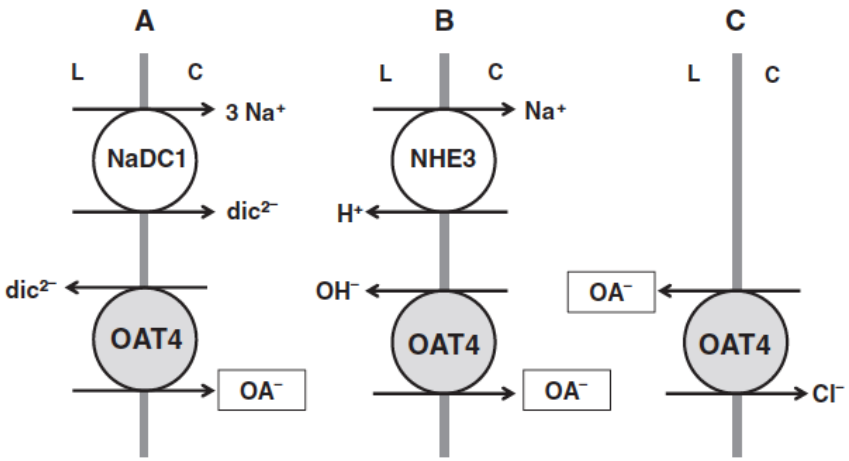


Figure 1-3. The transport mechanism of OAT4. This figure is obtained from Burckhardt, Pharmacology & Therapeutics 136 (2012) 106–130
OA^-**:** organic anions, NaDC1: sodium-dicarboxylate cotransporter 1, $dic2^-$: dicarboxylate, NHE3: Na^+/H^+ exchanger, L:lumen, C:cytosol.

It is reported that OAT4 can act through influx (A,B) and efflux (C) mechanism.

In "A" influx mode OAT4s uptake the OA^- such as estrone-3-sulfate with the efflux of the $dic2^-$ such as α-ketoglutarate. And also NaDC1 which locates in apical membrane of proximal tubule cells provides $dic2^-$. In "B" influx mode OAT4s uptake the OA^- with the efflux of hydroxyl ions(OH^-). This OH^- combine with H^+ secreted from the NHE3 which is also located in apical membrane to make water (H_2O). In "C" efflux mode, through OAT4s OA^-s are exchanged with extracellular chloride (Cl^-). OAT4s have function to transport endogenous substrates such as ES, dehydroepiandrosterone sulfate (DHEAS) and purine metabolite urate.

1.5 Regulation of Organic anion transporter

Regulation of transporter activity can be sorted into Long-term regulation and short-term regulation. Long-term regulations, also called the chronic regulation, of OATs are mainly mediated by gene expression such as transcription, translation and occur within hours and days. And short-term regulations, also called the acute regulation, are mainly post-translational modifications such as phosphorylation and glycosylation and occur within minutes or hours. A large number of studies concentrate on phosphorylation, glycosylation and protein-protein interactions of OATs, furthermore ubiquitination.

CHAPTER 2 Short-term and long-term effects of protein kinase C on the trafficking and stability of hOAT3

This chapter is based on my published paper.

2.1 Introduction

hOAT1 and hOAT3 have both shared and distinct properties. We previously showed that short-term activation of PKC inhibits OAT1 transport activity by reducing its surface expression through enhancing the rate of OAT1 internalization from cell surface to intracellular compartments [8]. However, whether OAT3 follows similar regulatory pathway is unknown. Moreover, the effects of long-term PKC activation on these transporters have never been investigated. These investigations are described in the current studies.

2.2 Materials and Methods

Materials

[3H] estrone sulfate was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA,USA). NHS-SS-biotin and streptavidin-agarose beads were purchased from Pierce Chemical(Rockford, IL, USA). Mouse anti-myc antibody was purchased from Roche. (Indianapolis, IN, USA). Mouse anti-EEA1 antibody was purchased from BD Biosciences (San Jose, CA, USA). Alexa Fluor® 488 goat

anti-mouse IgG (H+L) and Alexa Fluor® 555 goat anti-rabbit IgG (H+L) were purchased from Molecular Probes (Eugene, OR, USA). Goat anti-mouse IgG conjugated to horseradish peroxidase and SuperSignal West Dura extended duration substrate kit were purchased from Thermo Scientific (Waltham, MA, USA). All other reagents were from Sigma-Aldrich (St.Louis, MO, USA).

Cell cultures. Cells stably expressing hOAT3 [9] were maintained in DMEM medium supplemented with 0.2 mg/ml G418, 10 % fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml).

Transport measurement. Cells were plated in 48-well plates. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/ Ca^{2+} /Mg^{2+} (137 mM NaCl, 2.7 mM KCl, 4.3mM Na_2 HPO_4, 1.4 mM KH_2PO_4 , 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4) and [^{3}H] estrone sulfate. At the times indicated, the uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquotted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values are means ± S.D. (n = 3).

Cell surface biotinylation. Cell surface expression levels of hOAT3 were examined using the membrane-impermeant biotinylation reagent NHS-SS-biotin.

The cells were seeded onto six-well plates. After 24 hrs, the medium was removed and the cells were washed twice with 3 ml of ice-cold PBS, pH 8.0. The plates were kept on ice, and all solutions were kept ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of freshly made NHS-SS-biotin (0.5 mg/ml in PBS, pH 8.0) in two successive 20 min incubations on ice with very gentle shaking. Biotinylation was quenched by first briefly washing each well with 3 ml of 100 mM glycine and followed by incubation with 100 mM glycine on ice for 20min. The cells were then dissolved on ice for 40min in 400 µl of lysis buffer [10 mM Tris, 150mM NaCl, 1 mM EDTA, 0.1 % SDS, 1 % Triton X-100, and protease inhibitors (200 µg/ml phenylmethylsulfonyl fluoride and 3 µg/ml leupeptin),pH 7.4]. The unlysed cells were removed by centrifugation at 16,000 X g at 4 °C. Streptavidin-agarose beads were then added to the supernatant to isolate cell membrane protein. hOAT3 was detected in the pool of surface proteins by polyacrylamide gel electrophoresis and immunoblotting using an anti-myc antibody(1:100). Myc was tagged at the carboxyl terminus of hOAT3 for its immunodetection [9].

Internalization assay. We followed the procedure described previously by our laboratory [8]. hOAT3-expressing cells underwent biotinylation with 0.5 mg/ml sulfo-NHS-SS-biotin as described above. Following biotinylation, one set of cells was washed with PBS and kept at 4 °C to determine the total initial surface hOAT3 and stripping efficiency. To initiate internalization, cells in the duplicate plate were washed repeatedly with pre-warmed (37 °C) PBS containing either 1

μM of PMA or PBS only and incubated with the same solutions at 37 °C for indicated time periods. Residual cell surface biotin was stripped by incubating cells three times for 20 min with freshly prepared 50 mM MesNa in NT buffer (150 mM NaCl, 1 mM EDTA, 0.2 % bovine serum albumin, 20 mM Tris, pH 8.6). Stripping efficiency was determined for each experiment on biotinylated cells kept in parallel at 4 °C. Cells were lysed in lysis buffer with protease inhibitor cocktail. Biotinylated proteins were separated from non-biotinylated proteins by streptavidin-agarose resin similarly as we described above. Samples were then eluted from the beads by adding sample buffer and resolved by SDS-PAGE and analyzed by western blotting with anti-myc antibody. Relative hOAT3 internalized was calculated as % of the total initial cell surface hOAT3 pool.

Immunofluorescence analysis. hOAT3-expressing cells were grown on coverslips(22 mm) for 48 hrs, washed three times in PBS, and then fixed with 3% paraformaldehyde for 20 min at room temperature, permeabilized with 0.01% Triton x-100 for 5 min three times, and incubated with 5% dry milk at room temperature for 1 h. Afterwards, the cells were incubated with rabbit anti-c-Myc antibody (Sigma, 1:300) to label hOAT3-tagged with c-myc and with mouse anti-EEA1 antibody (1:250) at 4 °C overnight. The coverslips were then incubated with Alexa Fluor® 488 goat anti-mouse IgG(H+L) (1:500) or Alexa Fluor® 555 goat antirabbit IgG (H+L) (1:1000) at room temperature for 2 hrs. After washing, the coverslips were mounted on slides for image acquisition and analysis. Samples were visualized with a Zeiss LSM-510 laser-scanning microscope.

Degradation of cell surface hOAT3. hOAT3 expressing cells were plated in 35mm dishes. Each dish of cells was incubated with 1ml of cell membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin (1 mg/ml in PBS pH8.0, supplemented with 1mM Ca^{2+} and 1mM Mg^{2+} 1mM) in two successive 20 minutes incubations under trafficking impermissive condition (4°C) with very gentle shaking. The reagent was freshly prepared for each incubation. After biotinylation, each dish was rinsed with 2ml PBS containing 100mM glycine and then incubated with the same solution for 20 minutes on ice, to ensure complete quenching of the unreacted NHS-SS-biotin. The biotin-labeled cells were incubated in DMEM containing 1 μ M DMSO or 1 μ M PMA at 37°C. Treated cells were collected at 2, 4, and 6 hours and lysed in lysis buffer with protease inhibitor cocktail. The cell lysates were cleared by centrifugation at 16,000 Å~ g at 4 °C. 50μ l of streptavidinagarose beads were then added to the supernatant to isolate cell membrane proteins. Samples were loaded on 7.5% SDS-PAGE minigels and analyzed by immunoblotting with anti-myc antibody.

Inhibition of Proteases. Degradation of cell surface hOAT3 (described as above) was determined in the presence and absence of proteasome inhibitor MG132 (5μM), and lactacystin (5 μM) or lysosomal inhibitors leupeptin/pepstatin A (50, 2 μg/ml), and chloroquine (100μM) individually for specific time point as indicated in the figure legends.

Electrophoresis and immunoblotting. Protein samples (100 μg) were resolved

on 7.5% SDS-PAGE mini-gels and electroblotted onto polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5 % nonfat dry milk in PBS-0.05 % Tween, and incubated overnight at 4 °C with anti-myc antibody (1:100). The membranes were washed and then incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (1:5,000), and signals were detected using a SuperSignal West Dura extended duration substrate kit. Images were captured by Fluorchem ® 8800 system (Alpha Innotech, San Leandro, CA, USA). Density of bands was analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

Data analysis. Each experiment was repeated a minimum of three times. The statistical analysis given was from multiple experiments. Statistical analysis was performed using Student's paired t -tests. A P value < 0.05 was considered significant.

2.3 Results

2.3.1 Constitutive and PKC-regulated trafficking of hOAT3

We previously showed that hOAT1 constitutively internalizes from and recycles back to the plasma membrane and that inhibition of hOAT1 activity by short-term (30 min) activation of PKC results from a reduced surface expression of hOAT1 through accelerating internalization of the transporter [8]. hOAT1 and hOAT3 have shared and distinct properties. hOAT3 activity was also inhibited by

short-term activation of PKC with PMA and such inhibition was reversed in the presence of PKC inhibitor staurosporin (Figure 2-1). However whether the mechanism underlying PKC regulation of hOAT3 is similar to that hOAT1 is unknown.

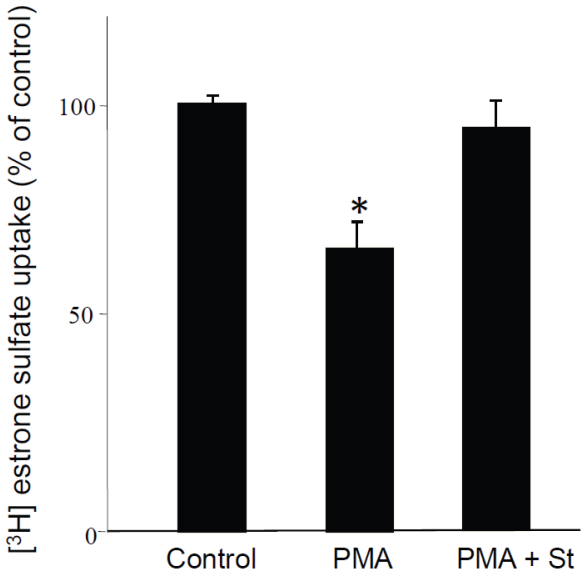


Figure 2-1. Effect of PKC on hOAT3 activity. COS-7 cells stably expressing hOAT3 were incubated for 30 min with 1 μM PKC activator PMA in the presence and absence of PKC inhibitor staurosporin (St, 2 μM) added directly to the culture media. After washing the cells, 3-min uptake of [^{3}H] estrone sulfate (0.1 μM) was measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. Values are mean ± S.D. (n = 3). Asterisks indicate values significantly different ($p < 0.05$) from that of control.

To address this issue, we took a biotinylation-based strategy. hOAT3 expressing cells were biotinylated with cell-impermeable biotinylation reagent sulfo-NHS-SS-biotin under trafficking-impermissive condition(4 °C). The labeled cells were then rewarmed back to trafficking-permissive condition (37 °C) to allow internalization to occur in the presence and the absence of PKC activator PMA. At indicated time points after initiation of internalization, biotin from biotinylated proteins remaining on the surface was removed by treatment with MesNa, a

nonpermeant reducing agent that cleaves disulfide bond and liberates biotin from biotinylated proteins at the cell surface. The amount of biotinylated proteins resistant (inaccessible) to MesNa treatment was defined as "the amount of protein internalized."

Our result (Figure 2-2) showed that under the basal condition, the amount of the surface-labeled hOAT3 internalized increasingly with time. Approximately 5%, 15%, or 33% of surface-labeled hOAT3 were detectable in the intracellular compartments after 5-, 15-, or 30-min initiation of internalization. Therefore, hOAT3 undergoes constitutive internalization in COS-7 cells.

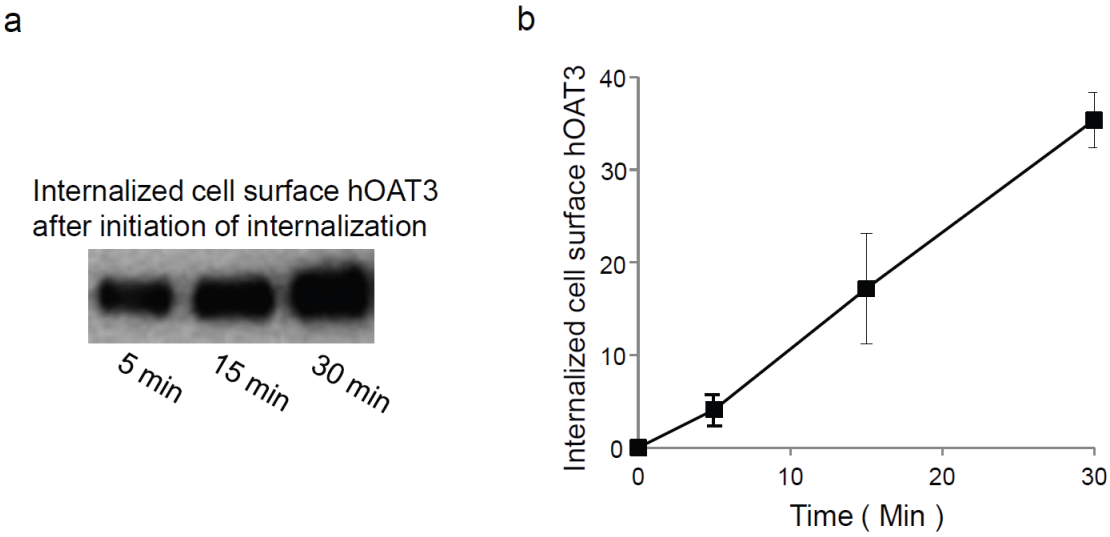


Figure 2-2. Biotinylation analysis of constitutive hOAT3 internalization in COS-7 cells. a. hOAT3 internalization was analyzed as described in “Materials and Methods” section followed by immunoblotting using anti-myc antibody (1:100). b. Densitometry plot of results from Figure 2-2a as well as from other experiments. Internalized hOAT3 was expressed as % of total initial cell surface hOAT3 pool. Values are mean ± S.E. (n = 3).

Furthermore, our result (Figure 2-3) showed that the amount of surface-labeled hOAT3 internalized in the presence of PMA was much greater than that in the

absence of PMA, suggesting that short-term activation of PKC by PMA inhibits hOAT3 transport activity through accelerating hOAT3 internalization into the intracellular compartments. Total expression of hOAT3 was not affected under such condition (not shown).

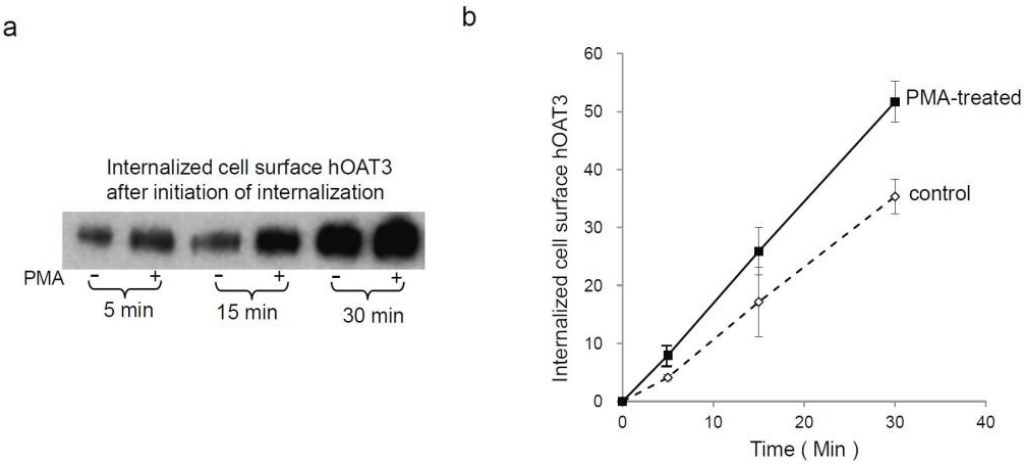


Figure 2-3. Biotinylation analysis of PKC-modulated hOAT3 internalization in COS-7 cells. a. hOAT3 internalization was analyzed in the presence and absence of PKC activator PMA (1 μM) as described in "Materials and Methods" section followed by immunoblotting using anti-myc antibody (1:100). b. Densitometry plot of results from Figure 2-3a as well as from other experiments. Internalized hOAT3 was expressed as % of total initial cell surface hOAT3 pool. Values are mean ± S.E. (n = 3).

2.3.2 Immuno-localization of hOAT3 and EEA1

The cellular distribution of hOAT3 was examined by immunofluorescence microscopy. We previously showed that hOAT1 constitutively traffics between plasma membrane and recycling endosomes [8]. To determine whether hOAT3 traffics through the same route, we immunolocalized hOAT3 and EEA1, a recycling endosome marker. The fluorescence images (Figure 2-4) showed that hOAT3 indeed partially colocalized with EEA1-positive recycling endosomes (shown as orange/yellow color).

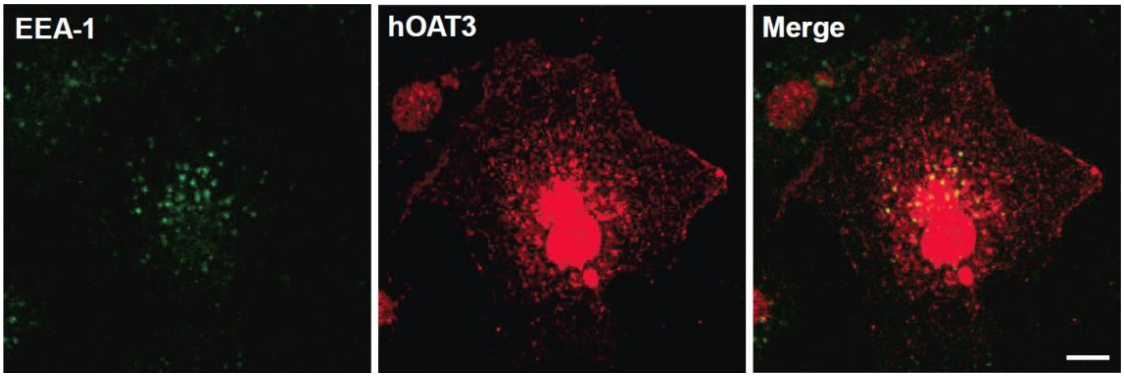


Figure 2-4. Immuno-localization of hOAT3 and EEA1. The cells were immunostained for hOAT3, and early endosome marker EEA1. Fluorescence images were taken for hOAT3 (red), and EEA1 (green). The merged image of hOAT3 and EEA1 was shown as orange/yellow. Bar = ~ 10 m.

2.3.3 Constitutive and PKC-regulated degradation of hOAT3

Our results above examined the effect of short-term activation of PKC (≤ 30 min) on cell surface hOAT3. The effect of long-term activation of PKC on any member of OAT family has never been investigated. We therefore examined the effect of long-term activation of PKC on cell surface hOAT3. hOAT3-expressing cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then treated with or without PMA at 37°C for 2, 4, and 6 hrs. Treated cells were lysed and cell surface proteins were isolated using streptavidin-agarose beads, followed by immunoblotting with anti-myc antibody. Our results (Figure 2-5) showed that the rate of cell surface hOAT3 degradation increased significantly after 2 hrs of treatment with PMA as compared to that of control.

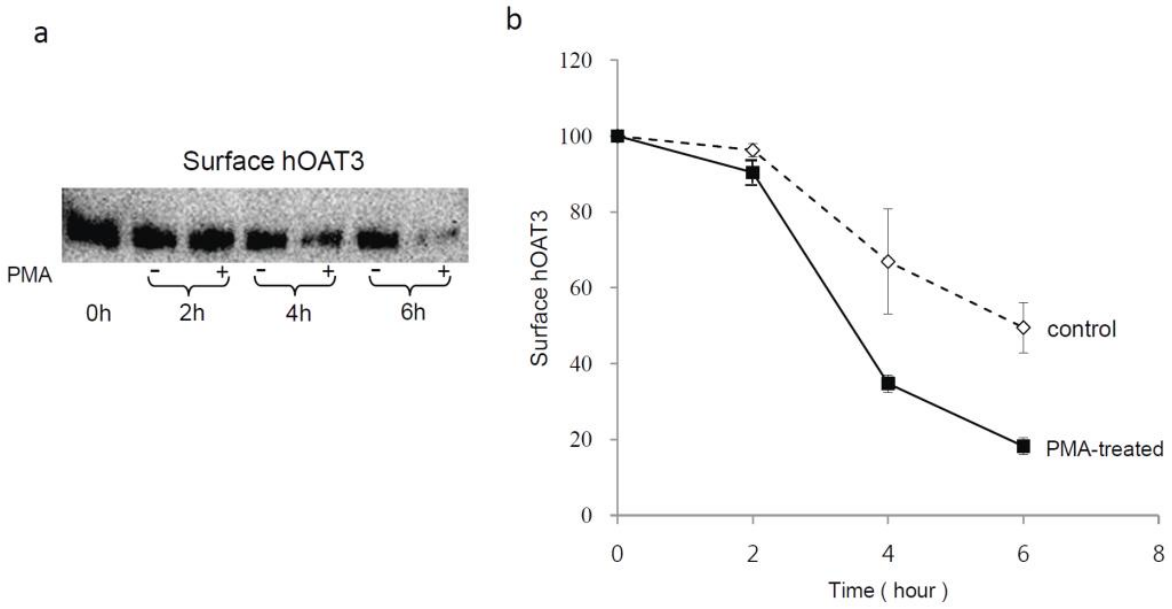


Figure 2-5. Biotinylation analysis of constitutive and PKC-modulated degradation of surface hOAT3. a. Cell surface hOAT3 degradation was analyzed in the presence and absence of PKC activator PMA (1 μM) as described in "Materials and Methods" section followed by immunoblotting using anti-myc antibody (1:100). b. Densitometry plot of results from Figure 2-5a as well as from other experiments. The amount undegraded cell surface hOAT3 was expressed as % of total initial cell surface hOAT3 pool. Values are mean ± S.E. (n = 3).

2.3.4 Degradation pathways of hOAT3

We next examined the degradation pathway of cell surface hOAT3 using a battery of different inhibitory reagents. Cells degrade proteins through two major systems, the proteasome and the lysosome. These different pathways of proteolysis can be determined by their sensitivity to different inhibitors. Degradation of polypeptides by the proteasome can be inhibited by MG132 and lactacystin, whereas lysosomal proteolysis can be inhibited by leupeptin, pepstatin A and chloroquine. For our experiment, hOAT3-expressing cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then treated with PMA at 37°C for 6 hrs in the presence of various protease inhibitors. Treated cells were lysed and cell surface proteins

were isolated using streptavidin-agarose beads, followed by immunoblotting with anti-myc antibody. Our results (Figure 2-6) showed that both proteasome inhibitors MG132 and lactacystin and lysosomal inhibitors leupeptin, pepstatin A and chloroquine significantly blocked hOAT3 degradation.

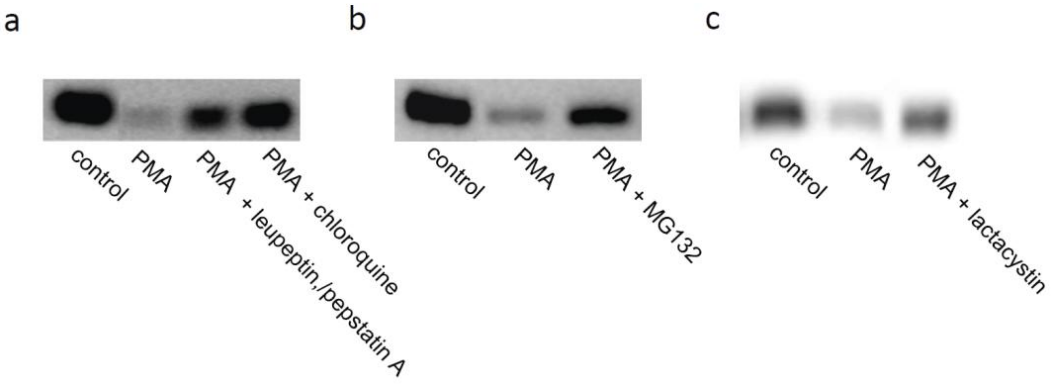


Figure 2-6. Effects of protease inhibitors on the degradation of cell surface hOAT3. Cell surface hOAT3 degradation was determined as described in "Materials and Methods" section in the presence of various protease inhibitors (6 hrs): a.lysosomal inhibitors leupeptin (50 μg/ml), pepstatin A (2 μg/ml) and chloroquine (100 **μ**M). b. proteasomal inhibitor MG132 (5 **μ**M). c. proteasomal inhibitor lactacystin (5 **μ**M).

2.4 Discussion

The organic anion transporter (OAT) family mediates the body disposition of a diverse array of environmental toxins, and clinically important drugs. Therefore, understanding the regulation of these transporters has profound clinical significance. In the current study, we investigated both the short-term and the long-term effects of PKC activation on cell surface hOAT3. Short-term regulation usually occurs when body has to deal with rapidly changing amounts of substances as a consequence of variable intake of drug, fluid, and meal as well as metabolic activity, whereas long-term regulation usually happens in situations in which the body undergoes massive changes, for example during development or

in diseases.

We choose COS-7 cells for our study because these cells offer several useful advantages for study of the cloned organic anion transporter. (i) These cells were directly derived from the kidney and have been very useful in understanding other renal transport processes and cellular functions, including organic cation transport [10, 11]. (ii) This cell line does not express endogenous OATs. Therefore, expression of OAT3 in COS-7 cells will allow us to dissect the transport characteristics of OAT3 in a relevant mammalian system without the possibly confounding effects of other organic anion transporters. (iii) They possess endogenous PKC and PKA signaling pathways and provide a good experimental model system for studying the regulatory mechanisms underlying many transport processes [12, 13]. (iv) The transport characteristics of OAT3 in COS-7 cells were in a good agreement with that observed in other systems [14-16]. The amount of OATs at the cell surface is critical for their drug transport activity. We previously established that hOAT1 undergoes constitutive internalization from and recycling back to cell surface and that acute activation of PKC inhibits hOAT1 activity by reducing hOAT1 cell surface expression through accelerating its internalization from cell surface to intracellular compartments without affecting the total expression of the transporter [8]. hOAT1 and hOAT3 have both shared and distinct properties. For example, these transporters have different substrate specificities. Our current study on the effect of short-term activation of PKC on cell surface hOAT3 demonstrated that hOAT1 and hOAT3 shared similar regulatory mechanisms: acute activation of PKC inhibits hOAT3 activity through accelerating its internalization from cell surface to intracellular compartments,

part of which were EEA1-positive recycling endosomes as demonstrated through our immunolocalization study (Figs. 3 and 4). The partial colocalization of hOAT3 with EEA1 could arise from the possibility that recycling endosomes are heterogeneous in their biochemical compositions, ion transport properties, and pH values [17]. Therefore, it may be possible that some of hOAT3 resides in a different subpopulation of recycling endosomes from that enriched in EEA1. It is also worth to note that hOAT3 is over-expressed in COS-7 cells. In such an over-expression system, it is not surprising to see that hOAT3 does not completely overlap with EEA1.

We then went further to investigate the effect of long-term PKC activation on cell surface hOAT3, an important issue, which has never been explored for any member of OAT family. We showed that prolonged treatment of hOAT3-expressing cells with PKC activator PMA resulted in an accelerated degradation of the transporter in both proteasome and lysosome (Figs. 5.and 6).

In conclusion, the major finding from the current study is that i) hOAT3 undergoes constitutive internalization, ii) short-term activation of PKC inhibits hOAT3 activity by accelerating hOAT3 internalization from cell surface to recycling endosomes without affecting the total expression of the transporter, and iii) long-term activation of PKC resulted in hOAT3 degradation in both lysosome and proteasome.

CHAPTER 3 Development of stable transfected cell lines

3.1 Introduction

The stably integrate genes into genome of mammalian cells have great potential for many biological, medical and pharmaceutical research areas and also for developing new medicines. The stably transfected cell expression is accomplished by integration of the gene of interest into specific target cell's chromosome. This integration change cell's fundamental DNA effectively.

The transient transfection has advantage for analyzing genes rapidly and small size protein producing. This transfected DNA is not passed from generation to generation during cell division and therefore the genetic alteration is not permanent.

Contrarily, stable transfection assures of long term, reproducible and specific gene expression. The critical applications for generation of stably transfected cell lines are the analysis of gene regulation, large size protein producing and discovery of new drugs.

I established five stable transfected cell lines. Those are COS-7 cells stably expressing hOAT3-c-myc, hOAT4-c-myc and HEK293 cells stably expressing hOAT1-c-myc, hOAT3-c-myc, hOAT4-c-myc.

3.2 Materials and Methods

Materials

[^{3}H] p-aminohippuric aci(PAH) and [^{3}H] estrone sulfate were purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA,USA). Geneticin(G418) and Lipofectamine 2000 reagent were purchased from Invitrogen (Carlsbad, CA).

Cell cultures. COS-7 cells and HEK293 cells were grown at 37°C and 5% CO_2 in DMEM supplemented with 10 % fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml). COS-7 cells stably expressing hOAT3-c-myc, hOAT4-c-myc were maintained at 37°C and 5% CO_2 in DMEM medium supplemented with 0.4 mg/ml G418, 10 % fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml). HEK293 cells stably expressing hOAT1-c-myc, hOAT3-c-myc, hOAT4-c-myc were maintained at 37°C and 5% CO_2 in DMEM medium supplemented with 0.8 mg/ml G418, 10 % fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml).

Development of stable transfected cell lines.

1. Plate 1.2 X 10^6 /well cells into 6-well or 35 mm dish
2. Confluent cells were transfected with 4 μg of DNA plasmids (hOAT1-c-myc, hOAT3-c-myc, hOAT4-c-myc) using Lipofectamine 2000 reagent.

3. 36-48hr after transfection split the transfected cells into two 100 mm dish and add medium containing 0.8 mg/ml G418.
4. Replacing the medium every two days until all the cells transfected with parental cells (COS-7 cells and HEK293 cells) are dead. (~99%)
5. Collect the rest cells transfected with hOATs plasmids and dilute cells with a concentration 10 cells/ml.
6. Dispense into 96-well plate at 100 μl/well. Culture at 37 °C in a humidified incubator. Clones should form in 7-14 days (From step 6 to Step 9, cell culture medium should contain G418 0.8mg/ml).
7. Incubate the cells at 37 **°C** until confluent and subculture into 24-well.
8. While cells are confluent in 24-well, plate 2X10^5 cells per well for 48 well plate and then screen the positive clone by uptake functional assay.

Transport measurement. Cells were plated in 48-well plates. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline/ Ca^{2+} /Mg^{2+} (137 mM NaCl, 2.7 mM KCl, 4.3mM Na_2 HPO_4, 1.4 mM KH_2PO_4 , 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4) and [^{3}H] p-aminohippuric aci(PAH) or [^{3}H] estrone sulfate. At the times indicated, the uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquotted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values are means ± S.D. (n = 3).

3.3 Results

3.3.1 COS-7 cells stably expressing hOAT3-c-myc

The hOAT3 function in COS-7 cells stably expressing hOAT3-c-myc (Passage 5) is investigated by uptake assay. The hOAT3s was analyzed for their ability to transport estrone sulfate, a substrate of hOAT3. (Figure 3-1.)

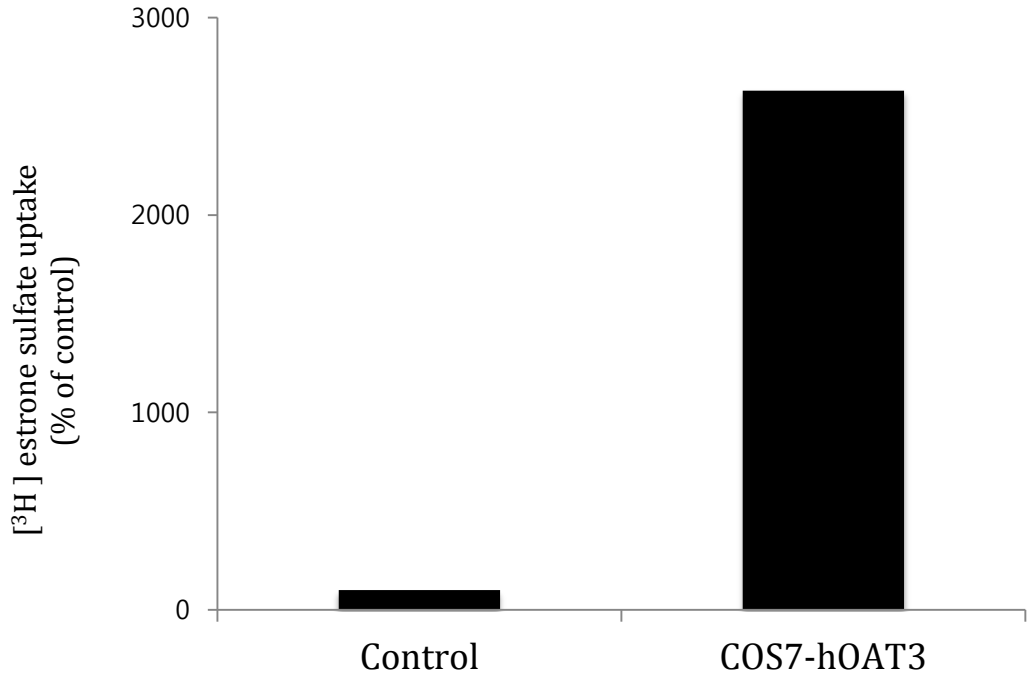


Figure 3-1. hOAT3 activity of COS-7 cells stably expressing hOAT3-c-myc. 4-min uptake of [^{3}H] estrone sulfate (0.1 μM) in parental COS-7 cells (control) and COS-7 cells stably expressing hOAT3-c-myc (COS7-hOAT3) was measured. Uptake activity was expressed as a percentage of the uptake measured in parental COS-7 cells.

3.3.2 COS-7 cells stably expressing hOAT4-c-myc

The hOAT4 function in COS-7 cells stably expressing hOAT4-c-myc (Passage 7) is investigated by uptake assay. The hOAT4s was analyzed for their ability to transport estrone sulfate, a substrate of hOAT4. (Figure 3-2.)

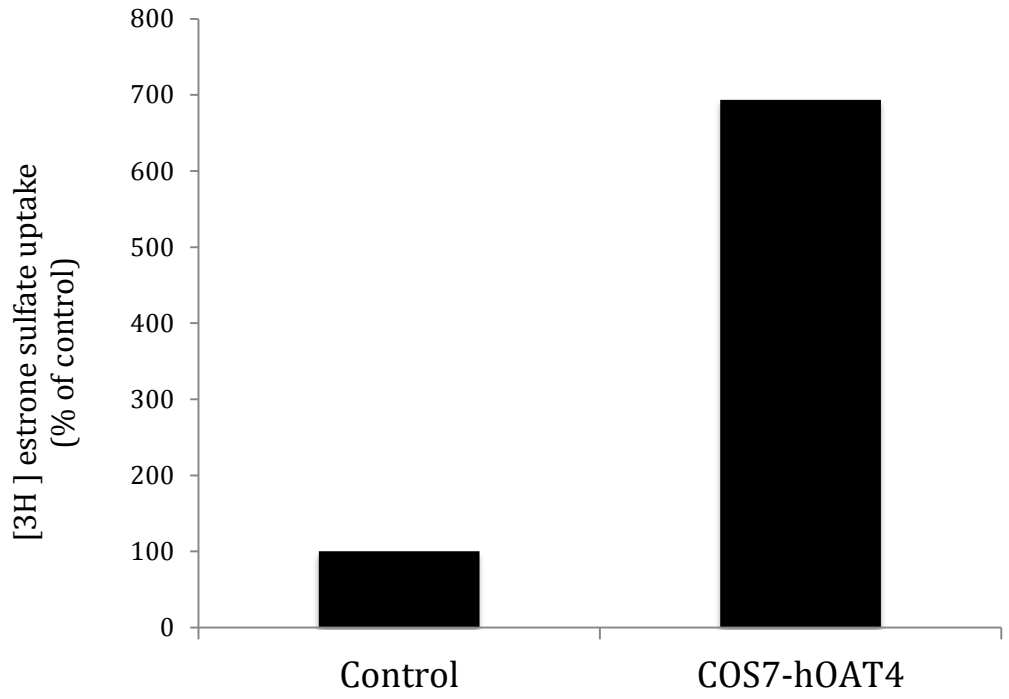


Figure 3-2. hOAT4 activity of COS-7 cells stably expressing hOAT4-c-myc. 4-min uptake of [^{3}H] estrone sulfate (0.1 μM) in parental COS-7 cells (control) and COS-7 cells stably expressing hOAT4-c-myc (COS7-hOAT4) was measured. Uptake activity was expressed as a percentage of the uptake measured in parental COS-7 cells.

3.3.3 HEK293 cells stably expressing hOAT1-c-myc

The hOAT1 function in HEK293 cells stably expressing hOAT1-c-myc (Passage 4) is investigated by uptake assay. The hOAT1s was analyzed for their ability to transport PAH, a substrate of hOAT1. (Figure 3-3.)

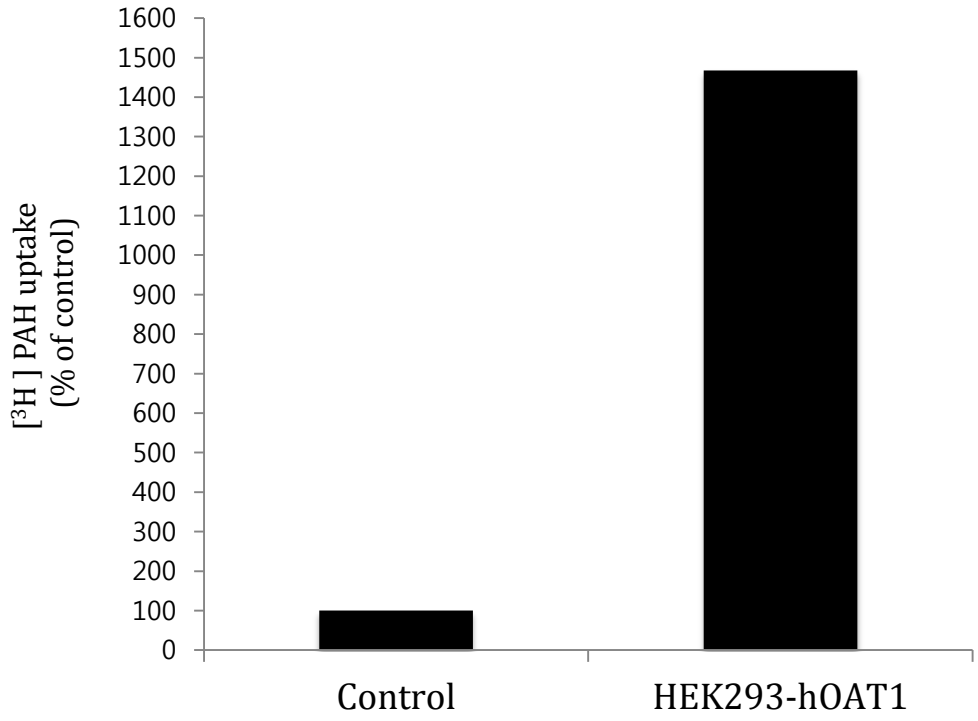


Figure 3-3. hOAT1 activity of HEK293 cells stably expressing hOAT1-c-myc. 3-min uptake of PAH (20 μM) in parental HEK293 cells (Control) and HEK293 cells stably expressing hOAT1-c-myc (HEK293-hOAT1) was measured. Uptake activity was expressed as a percentage of the uptake measured in parental HEK293 cells.

3.3.4 HEK293 cells stably expressing hOAT3-c-myc

The hOAT3 function in HEK293 cells stably expressing hOAT3-c-myc (Passage 4) is investigated by uptake assay. The hOAT3s was analyzed for their ability to transport estrone sulfate, a substrate of hOAT3. (Figure 3-4.)

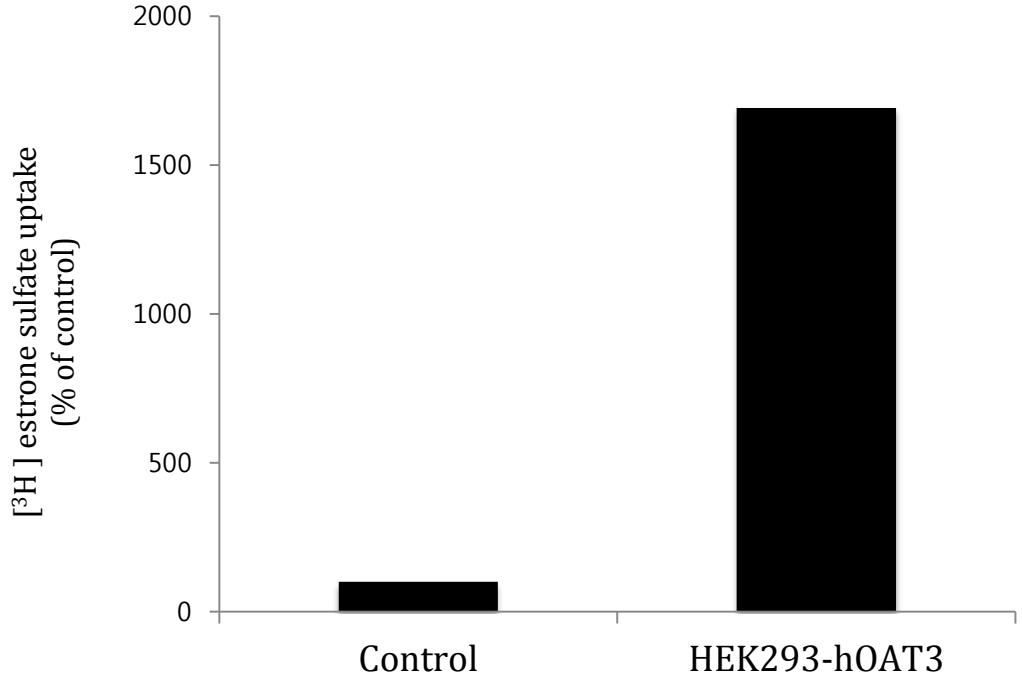


Figure 3-4. hOAT3 activity of HEK293 cells stably expressing hOAT3-c-myc. 4-min uptake of [^{3}H] estrone sulfate (0.1 μM) in parental HEK293 cells (Control) and HEK293 cells stably expressing hOAT3-c-myc (HEK293-hOAT3) was measured. Uptake activity was expressed as a percentage of the uptake measured in parental HEK293 cells.

3.3.5 HEK293 cells stably expressing hOAT4-c-myc

The hOAT4 function in HEK293 cells stably expressing hOAT4-c-myc (Passage 7) is investigated by uptake assay. The hOAT4s was analyzed for their ability to transport estrone sulfate, a substrate of hOAT4. (Figure 3-5.)

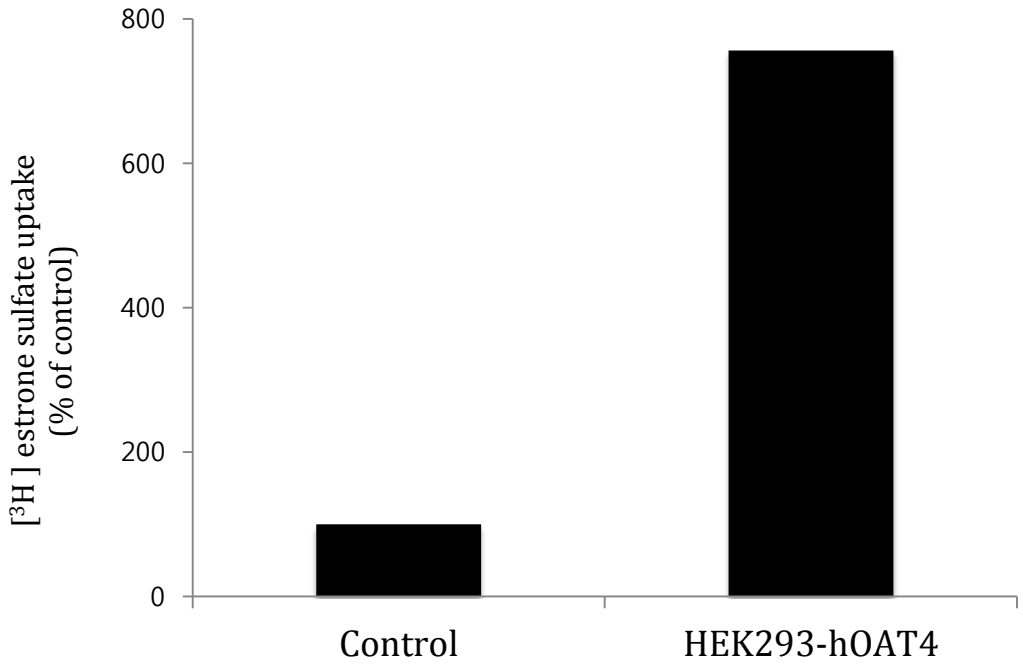


Figure 3-5. hOAT4 activity of HEK293 cells stably expressing hOAT4-c-myc. 4-min uptake of [^{3}H] estrone sulfate (0.1 μM) in parental HEK293 cells (Control) and HEK293 cells stably expressing hOAT4-c-myc (HEK293-hOAT4) was measured. Uptake activity was expressed as a percentage of the uptake measured in parental HEK293 cells.

3.4 Discussion

The generation of stably transfected cell lines is indispensable for a wide research area of applications, such as gene function studies, drug discovery and the production of proteins of interest.

Typically, transfected DNA is not passed generation during cell division and therefore the genetic alteration is not permanent. In other words, transfected DNA located in the cell wall or nucleus in the certain cell can replicate and perform certain functions, but it will be disappeared go through several

generations.

In contrast, the stable transfection ensure long term and reproducible specific gene expression because it is accomplished by integration of transfected certain DNA of interest into cell's chromosome. These cells continue to express transfected DNA even after many generations of cell divisions.

We are researching about trafficking and regulation of organic anion transporter actively by using five stable transfected cell lines I established. For example, the internalization assay of OATs, regulation of OATs by PKC and post-translational modifications such as ubiquitination, sumoylation.

CHAPTER 4 Anti-cancer drug library screening

4.1 Introduction

By using HEK293 cells stably expressing hOAT4-c-myc I established, I did anti-cancer drug library screening. The purpose of this experiment is the investigation of anti-cancer drug which has function to inhibit hOAT4.

4.2 Materials and Methods

Materials

[3H] estrone sulfate was purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA,USA). Dimethyl sulfoxide (DMSO) from Sigma (St. Louis, MO). Geneticin(G418) from Invitrogen (Carlsbad, CA).

Cell cultures. HEK293 cells stably expressing hOAT4-c-myc were maintained at 37°C and 5% CO_2 in DMEM medium supplemented with 0.8 mg/ml G418, 10 % fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml).

Transport measurement with anti-cancer drug. Plate $2X10^5$ cells per well for 48 well plate. For each well, uptake solution was added with drug. Working anti-cancer drug concentration is 10uM in DMSO. Only DMSO was added for control

cells. The uptake solution consisted of phosphate-buffered saline/ Ca^{2+} /Mg^{2+} (137 mM NaCl, 2.7 mM KCl, 4.3mM Na_2 HPO_4, 1.4 mM KH_2PO_4 , 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4) and [^{3}H] estrone sulfate. At the times indicated, the uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquotted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values are means ± S.D. (n = 3).

4.3 Results

Anti-cancer drug library screening (Total 101 drugs)

The hOAT4 function in HEK293 cells stably expressing hOAT4-c-myc (Passage 11) is investigated by uptake assay. The hOAT4s was analyzed for their ability to transport estrone sulfate, a substrate of hOAT4. (Figure 1) I checked probenecid uptake activity in HEK293 cells stably expressing hOAT4-c-myc with every group because probenecid had been most widely studied OATs inhibitor.

4.3.1 Anti-cancer drug group 1

Drug list: A2,B2,C2,D2,E2,F2,G2,H2,A3,B3,C3,D3,E3,F2,G3,H3,A4,B4,C4,D4

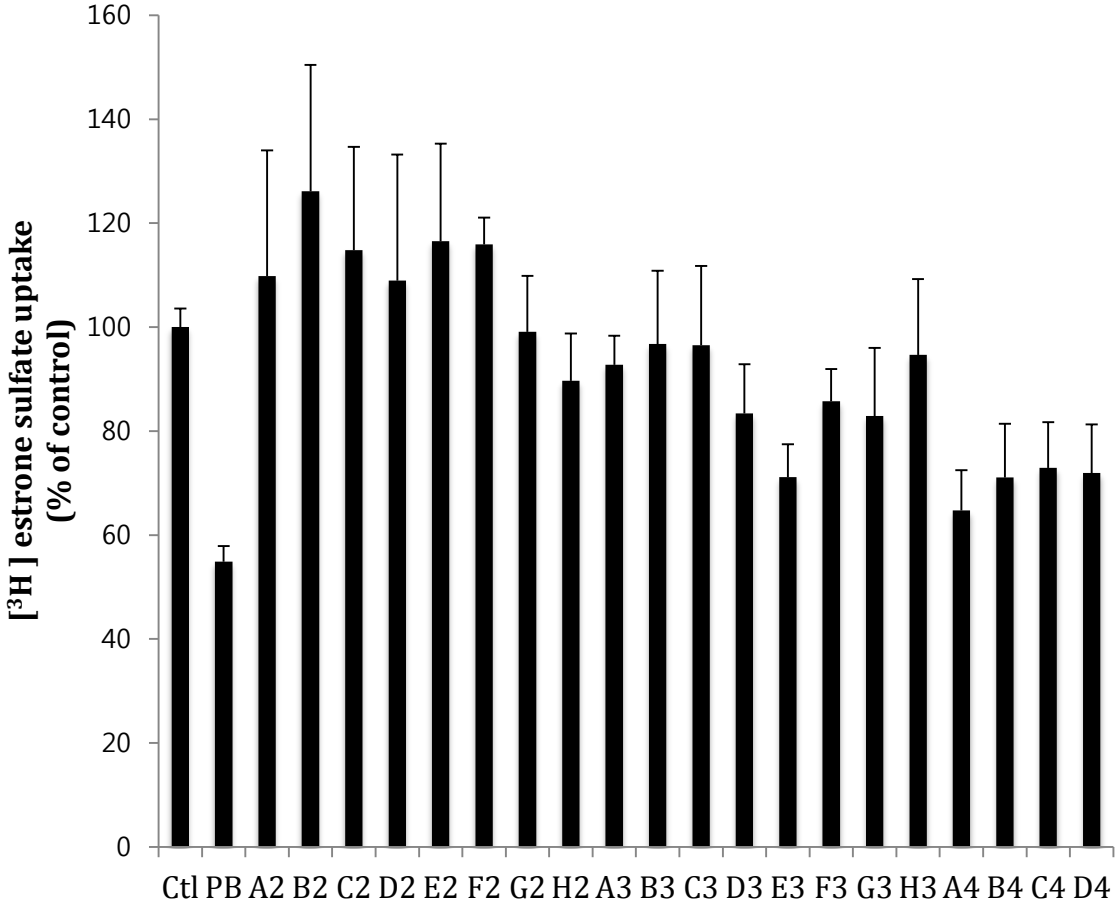


Figure 4-1. Effect of anti-cancer drug group 1 on hOAT4 activity. 4-min uptake of [^{3}H] estrone sulfate (0.1 μM) in HEK293 cells stably expressing hOAT4-c-myc was measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The result represents data from 3 experiments. The uptake values in parental HEK-293 cells were subtracted. Values are mean ± S.D. (n = 3).
Ctl: Control, PB: Probenecid

4.3.2 Anti-cancer drug group 2

Drug list: E4,F4,G4,H4,A5,B5,C5,D5,E5,F5,G5,H5,A6,B6,C6,D6,E6,F6,G6,H6

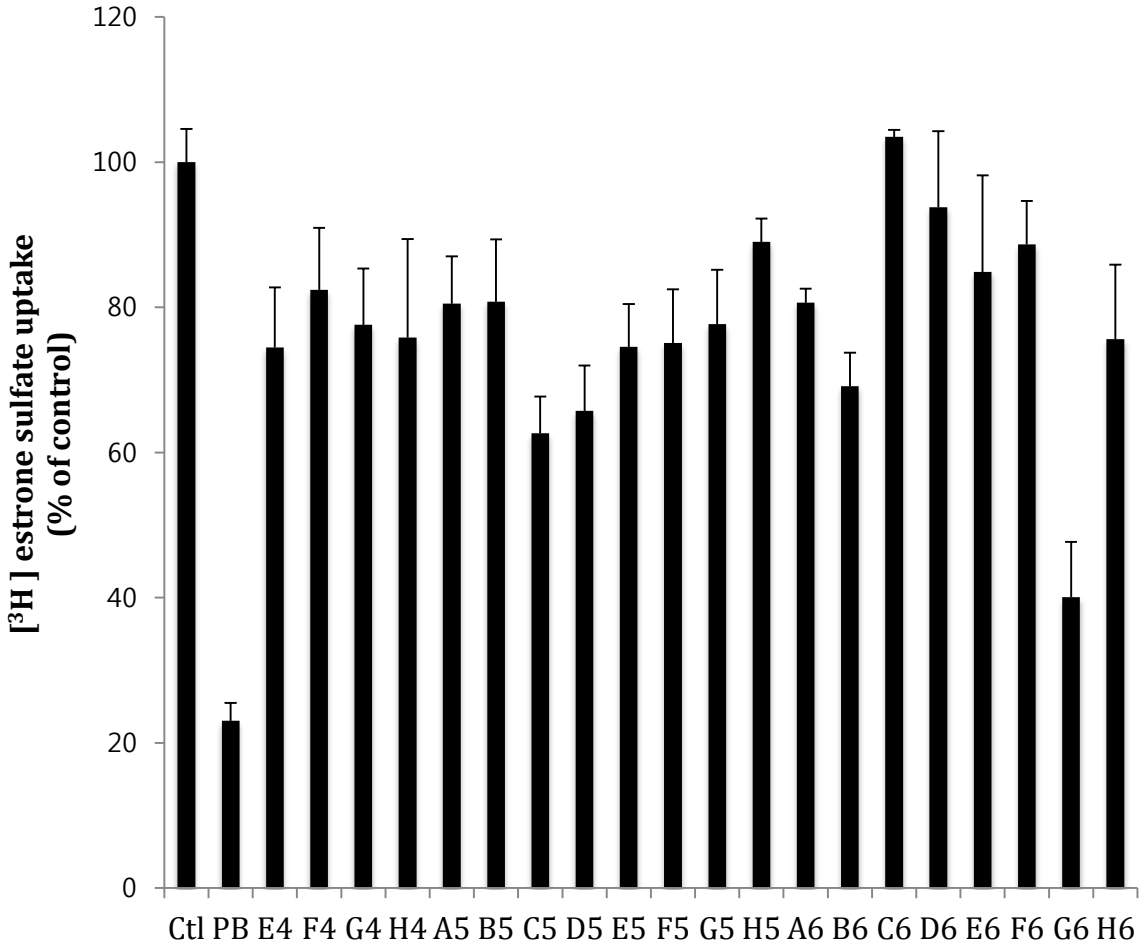


Figure 4-2. Effect of anti-cancer drug group 2 on hOAT4 activity. 4-min uptake of [^{3}H] estrone sulfate (0.1 μM) in HEK293 cells stably expressing hOAT4-c-myc was measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The result represents data from 3 experiments. The uptake values in parental HEK-293 cells were subtracted. Values are mean ± S.D. (n = 3).
Ctl: Control, PB: Probenecid

4.3.3 Anti-cancer drug group 3

Drug list: A7,B7,C7,D7,E7,F7,G7,H7,A8,B8,C8,D8,E8,F8,G8,H8,A9,B9,C9,D9

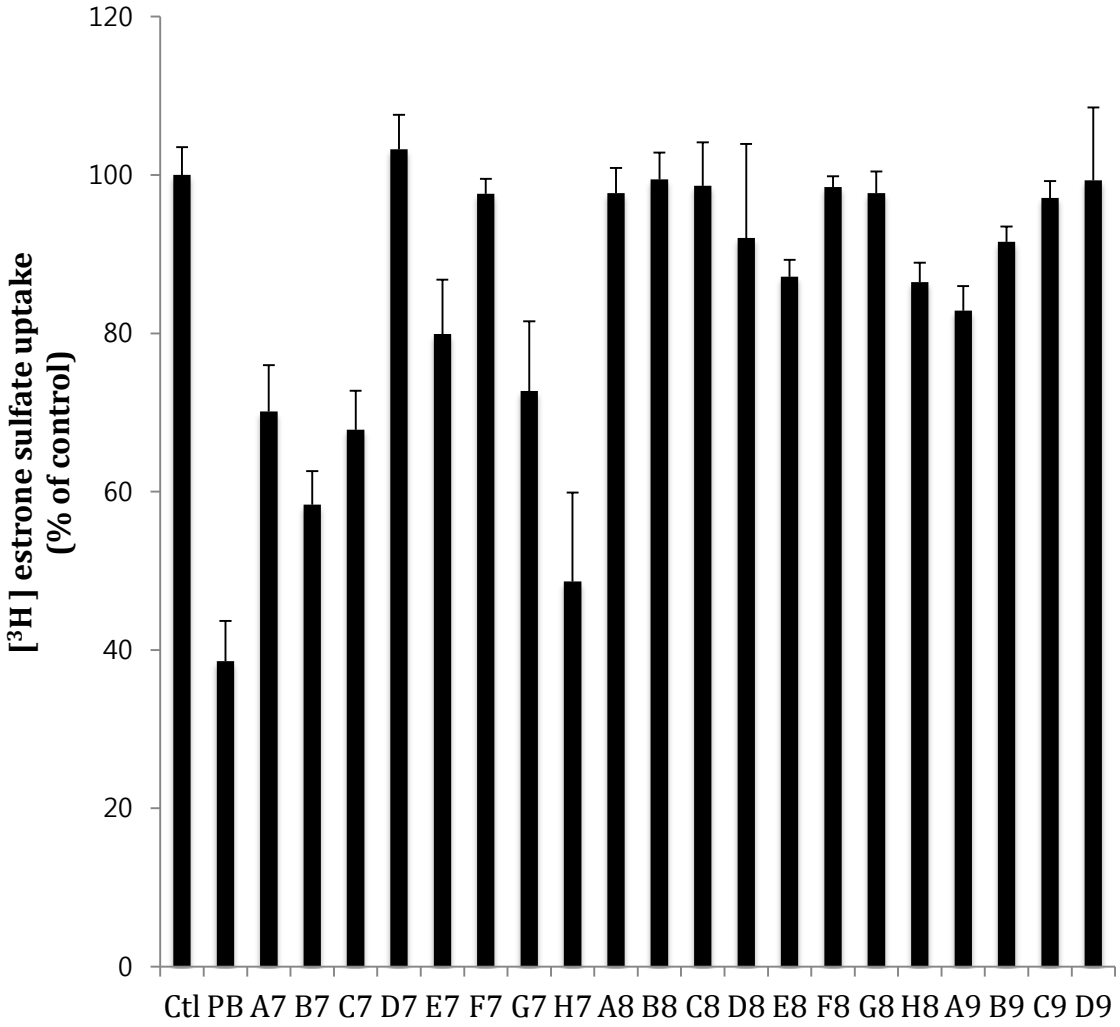


Figure 4-3. Effect of anti-cancer drug group 3 on hOAT4 activity. 4-min uptake of [^{3}H] estrone sulfate (0.1 μM) in HEK293 cells stably expressing hOAT4-c-myc was measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The result represents data from 3 experiments. The uptake values in parental HEK-293 cells were subtracted. Values are mean ± S.D. (n = 3).
Ctl: Control, PB: Probenecid

4.3.4 Anti-cancer drug group 4

Drug list:

E9,F9,G9,H9,A10,B10,C10,D10,E10,F10,G10,H10,A11,B11,C11,D11,E11,F11,G11,

H11

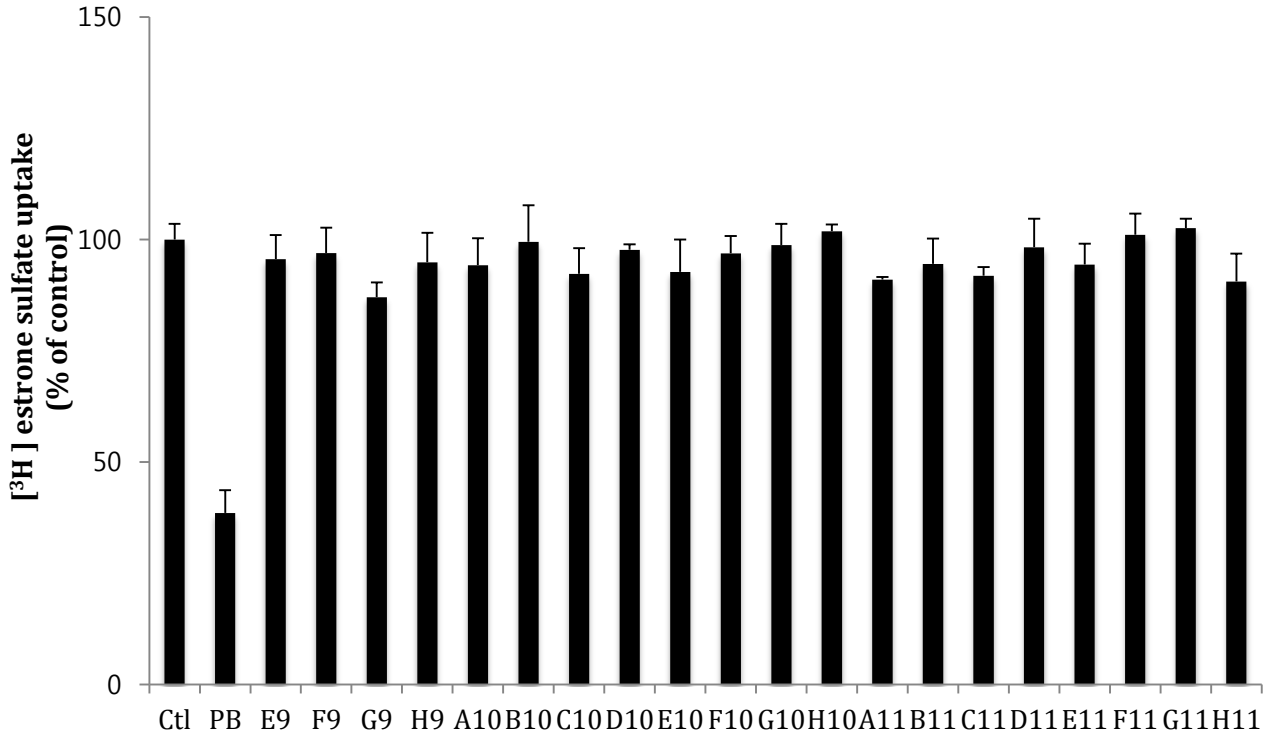


Figure 4-4. Effect of anti-cancer drug group 4 on hOAT4 activity. 4-min uptake of [^{3}H] estrone sulfate (0.1 μM) in HEK293 cells stably expressing hOAT4-c-myc was measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The result represents data from 3 experiments. The uptake values in parental HEK-293 cells were subtracted. Values are mean ± S.D. (n = 3).
Ctl: Control, PB: Probenecid

4.3.5 Anti-cancer drug group 5

Drug list:

A2',B2',C2',D2',E2',F2',G2',H2',A3',B3',C3',D3',E3',F2',G3',H3',A4',B4',C4,D4',E4'

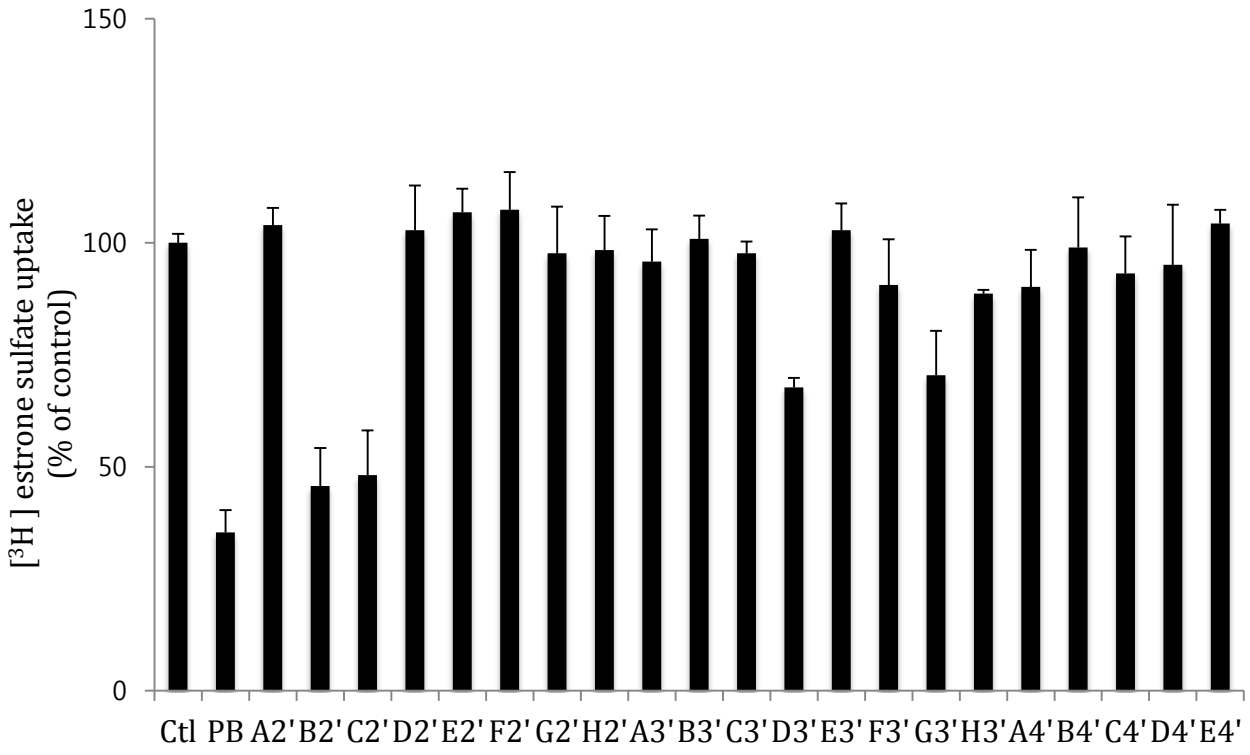
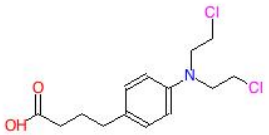


Figure 4-5. Effect of anti-cancer drug group 5 on hOAT4 activity. 4-min uptake of [^{3}H] estrone sulfate (0.1 μM) in HEK293 cells stably expressing hOAT4-c-myc was measured. Uptake activity was expressed as a percentage of the uptake measured in control cells. The result represents data from 3 experiments. The uptake values in parental HEK-293 cells were subtracted. Values are mean ± S.D. (n = 3).
Ctl: Control, PB: Probenecid

From these results, I investigated **G6, B7, H7, B2', C2'** anti-cancer drugs have function to inhibit hOAT4 by uptake assay. The information of these drugs is obtained from Developmental Therapeutics Program NCI/NIH website.

(http://dtp.nci.nih.gov/index.html)

G6

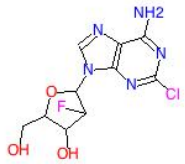


NSC 3088	CAS 305-03-3	PubChem SID 69624
Molecular Formula: C14H19Cl2NO2 (consistent with structure)	Molecular Weight: 304.0	PubChem BioAssay Data: Similar Compounds

Chemical Names

- Chlorambucil
- Leukeran
- Ambochlorin
- Phenylbutyric acid nitrogen mustard
- 4-(Bis(2-chloroethyl)amino)phenylbutyric acid
- p-(N,N-Di-2-chloroethyl)aminophenyl butyric acid
- CB 1348
- Elcoril
- Ecloril
- Cb l348

B7

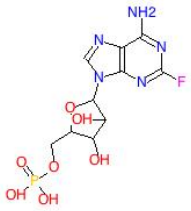


NSC 606869	CAS	PubChem SID 484621
Molecular Formula: C10H11ClFN5O3 (consistent with structure)	Molecular Weight: 304.0	PubChem BioAssay Data: Similar Compounds

Chemical Names

- Clofarabine
- Clolar
- Clofarex
- Adenosine, 2-chloro-2′-deoxy-2′-fluoro-
- CAFdA
- Adenine, 2-chloro-9-(2-deoxy-2-fluoro-.beta.-D-arabinofuranosyl)-

H7

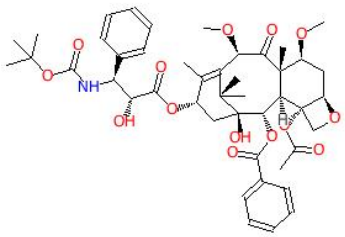


NSC 312887	CAS 75607-67-9	PubChem SID 455602
Molecular Formula: C10H13FN5O7P (consistent with structure)	Molecular Weight: 365.0	PubChem BioAssay Data: Similar Compounds

Chemical Names

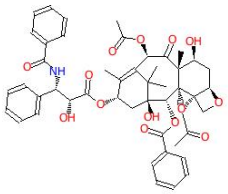
- Fludarabine phosphate
- Beneflur
- Fludara
- 9H-Purin-6-amine, 2-fluoro-9-(5-O-phosphono-.beta.-D-arabinofuranosyl)-

B2’



NSC 761432	CAS 183133-96-2	PubChem SID
Molecular Formula: C45H57NO14 (consistent with structure)	Molecular Weight: 835.9	PubChem BioAssay Data:
Chemical Names • Cabazitaxel • JEVTANA • XRP6258		

C2’



NSC 125973	CAS 33069-62-4	PubChem SID 418145
Molecular Formula: C47H51NO14 (consistent with structure)	Molecular Weight: 854.0	PubChem BioAssay Data: Similar Compounds
Chemical Names • Paclitaxel • Anzatax • Taxol • Taxol (Registered Trademark) • 7,11-Methano-5H-cyclodeca[3,4]benz[1,2-b]oxete,benzenepropanoic acid deriv. • Benzenepropanoic acid, .beta.-(benzoylamino)- .alpha.-hydroxy-, 6,12b-bis(acetyl oxy)-12-(benzoyloxy)-2a,3,4,4a,5,6,9,10,11,12,12a,12b,- dodecahydro-4,11- dihydroxy-4a,8,13,13-tetramethyl-5-oxo- 7,11-methano- 1H-cyclodeca[3,4]benz[1,2-b]oxet-9-yl ester, [2aR-[2a.alpha.,4.beta.,4a.beta.,6.beta.,9.alpha.(alpha. R*,.beta.S*),11.alpha.,12.alpha.,12a.alpha.,12b.alpha.]]- • Tax-11-en-9-one, 5.beta.,20-epoxy-1,2.alpha.,4,7.beta., 10.beta.,13.alpha.- hexahydroxy-, 4,10-diacetate 2- benzoate,13-ester with (2R,3S)-N-benzoyl-3-phenylisoserine		

4.4 Discussion

I showed hOAT4 activity was inhibited by some anti-cancer drugs through uptake assay. However the mechanism underlying this inhibition is not known.

We have previously shown that the activity of hOAT4 was down-regulated by activation of protein kinase C (PKC). For activating PKC, we used PKC activator phorbol 12-myristate 13-acetate (PMA). We explained that PKC activation inhibits hOAT4 activity by decreasing the surface expression of the transporter

was achieved by increasing hOAT4 internalization from cell surface to intracellular compartments through biotinylation assay.

Following this approach, we can try to do biotinylation assay with treatment of these anti-cancer drugs for discovering the hOAT4 activity inhibition mechanism.

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