

## Introduction

*In vivo* studies have shown that kidney membrane transporters play a key part in drug disposition and renal clearance. One such transporter is OAT1 (SLC22A6), which is critical for maintaining homeostasis of endogenous substances. This makes OAT1 a good transporter to assay for drug interactions with the kidney. Unfortunately, primary cells lose OAT1 expression in culture, and transiently expressed OAT1 has great variations between production lots, which make data hard to interpret. In our study we have generated HEK 293T/17 cells that stably overexpress the OAT1 gene driven by the human elongation factor-1 alpha (EF1 $\alpha$ ) promoter. After confirming the mRNA expression by RT-PCR, we performed immunostaining that indicated OAT1 is correctly trafficked to the membrane. Most importantly, we validated that the overexpressed OAT1 transporter has normal transport activities by using 5-carboxyfluorescein (5-CF) and para-aminohipurate (PAH; data not shown) uptake assays, and that the uptake can be inhibited by the well-known inhibitors probenecid and novobiocin. Both inhibitors responded in a dose dependent manner for 5-CF uptake with IC<sub>50</sub> values between 5-16  $\mu$ M. Even at higher passages, the cell line retained the functionality of OAT1. Overall, our data has shown that this modified cell line is a very useful *in vitro* tool for testing regulation of OAT1 membrane transporter activity in kidney cells.

## Materials and Methods

### Cell line generation (OAT1-HEK, ATCC® CRL-11268G-1™)

HEK 293T/17 (ATCC® CRL-11268™) cells were transfected with a plasmid expressing the full length sequence of human OAT1 (NM\_004790) under the control of the EF1 $\alpha$  promoter using TransfeX™ Transfection reagent (ATCC® ACS-4005). Transfected cells were grown under antibiotic selection and individual positive clones were identified and verified by RT-PCR, immunofluorescence and Sanger sequencing. A clone negative for OAT1 or the parental line was used as a control.

### Cell culture and immunocytochemistry (ICC)

Cell culture medium consisted of DMEM (ATCC® 30-2002) supplemented with 10% FBS (ATCC® 30-2020). OAT1-HEK transfectants were seeded onto poly-L-ornithine coated chamber slides and cultured at 37°C/5% CO<sub>2</sub>. After 24 hours, cells were fixed and stained with a rabbit monoclonal antibody to OAT1 (Abcam) and a goat anti-rabbit 488 secondary antibody then visualized using a fluorescent microscope.

### 5-CF uptake assay

OAT1-HEK or control cells were seeded at 10<sup>5</sup> cells/well in black walled 96-well plates. After 24h the cells were washed three times in warm HBSS (ATCC® 30-2213) and incubated for 10 minutes at 37°C/5% CO<sub>2</sub>. Cells were then incubated with 150  $\mu$ M 5-CF (Sigma) for 20 min at 37°C/5% CO<sub>2</sub>. After incubation, the reaction was terminated by washing the cells three times with cold HBSS. Cells were then lysed with M-Per lysis buffer (Thermo Fisher Scientific) and read immediately on a fluorescent plate reader at 490ex/530em or visualized by fluorescent microscopy.

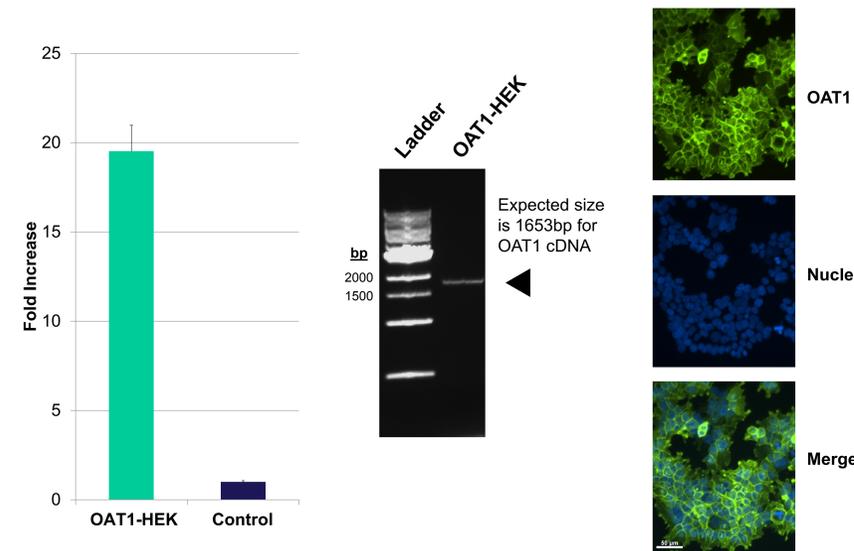
### Drug inhibition assay and IC<sub>50</sub> determination

For the inhibition assay OAT1-HEK cells were incubated with 5-CF and either probenecid (Sigma) or novobiocin (Sigma) for 10 minutes in 96-well plates, then uptake was measured as described above.

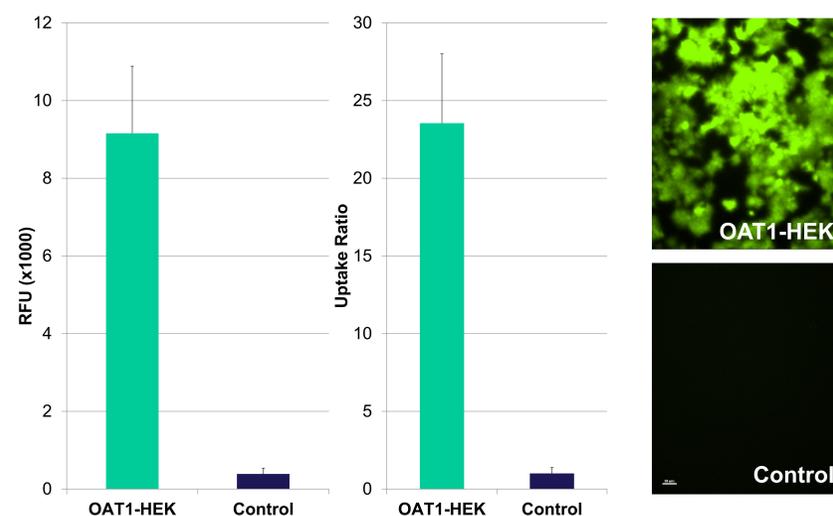
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## Results

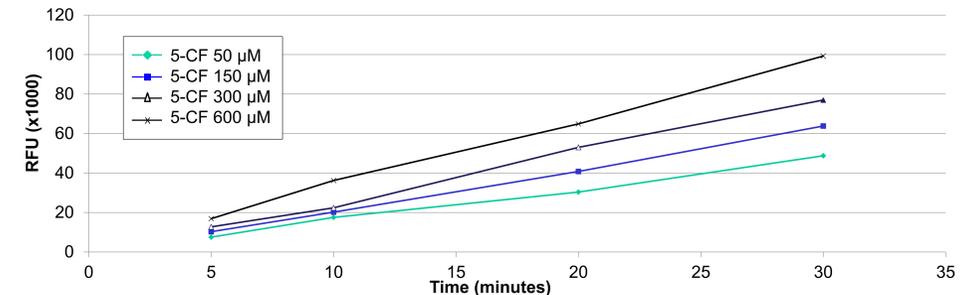
Sanger sequencing confirmed that the OAT1-HEK line expressed the complete human OAT1 gene with no mutations (data not shown). Copy number was determined via Droplet Digital™ PCR (Bio-Rad) to be 6 copies per cell (data not shown).



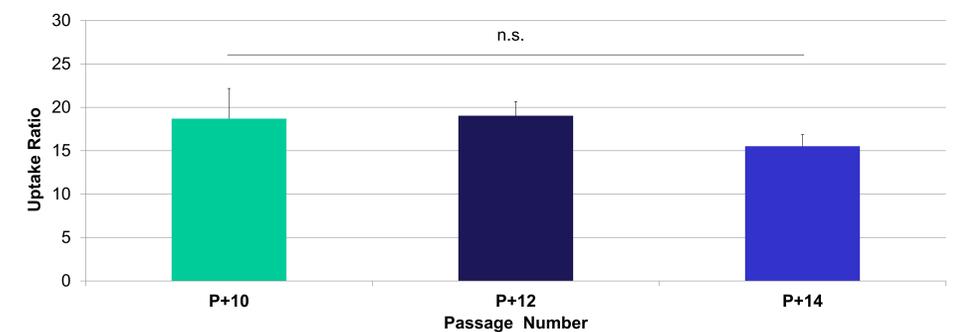
**Figure 1. Confirmation of OAT1 expression in OAT1-HEK by RT-PCR and ICC.** RT-PCR revealed a ~20-fold increase in human OAT1 relative to whole kidney lysate. N=3 (left). The RT-PCR product was analyzed by gel electrophoresis and found to be of the expected size of the human OAT1 sequence (middle). Immunofluorescent staining with a human anti-OAT1 antibody in cultured OAT1-HEK cells revealed the cells were ~90% positive for membrane localized OAT1 (right).



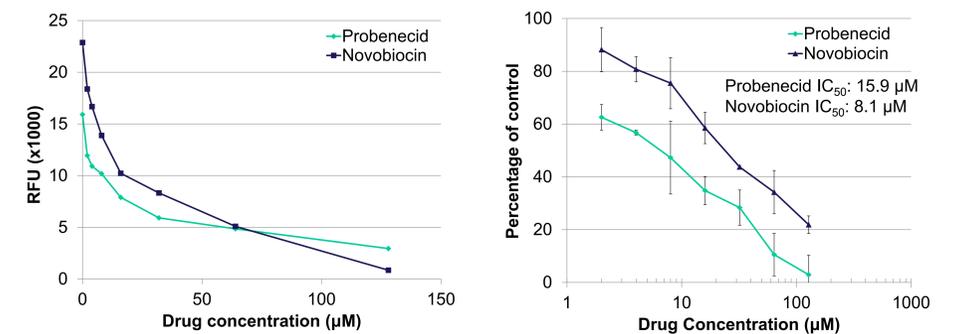
**Figure 2. OAT1 mediated uptake of fluorogenic substrate in OAT1-HEK cells.** OAT1-HEK or control cells were incubated with the fluorogenic substrate 5-CF as described in the materials and methods. Mean RFUs for the control line was <400 while the signal from the OAT1-HEK line was >9000. n=3 (left). This indicated an uptake ratio of 23 relative to control. N=3 (middle). Fluorescent microscopy revealed that ~90% of OAT1-HEK, but not control cells, exhibited uptake and accumulation of 5-CF (right).



**Figure 3. Time- and concentration-dependent uptake of 5-CF in OAT1-HEK cells.** OAT1-HEK cells incubated with 5-CF for the indicated times and concentrations demonstrated a linear increase in RFUs ( $r^2 \geq 0.99$ ) up to 30 minutes. N=4.



**Figure 4. OAT1 mediated 5-CF uptake is stable over extended culture in OAT1-HEK cells.** 5-CF uptake was determined as described in the materials and methods and expressed relative to the control line. No significant decrease in uptake was seen up to at least 14 passages ( $p = 0.77$ ). N=4.



**Figure 5. Dose-dependent inhibition of 5-CF uptake by OAT1 inhibitors probenecid and novobiocin in OAT1-HEK.** OAT1-HEK cells were incubated with the indicated compounds as described in the materials and methods and tested for 5-CF uptake. The results were graphed as raw signal (left) or as percentage uptake relative to non-treated control cells (right). N=3.

## Summary

- HEK293T/17 were engineered to overexpress the human OAT1 transporter to provide an *in vitro* model that better mimics the *in vivo* environment for renal toxicity studies.
- OAT1-HEK stably expressed high levels of OAT1 mRNA and membrane-localized protein.
- Functionality of the overexpressed OAT was demonstrated by time- and dose-dependent uptake of the fluorogenic substrate 5-CF.
- OAT1 activity by dose-dependent inhibition of 5-CF uptake by probenecid and novobiocin generated IC<sub>50</sub> values similar to those previously reported (e.g., Takeda, *et al.* Eur J Pharmacol, 2001 and Duan, *et al.* Drug Metab Dispos, 2009).
- OAT1-HEK cells and the 5-CF uptake assay serves as a useful alternative to LC-MS or radioactive assays for sensitive and high-throughput screening of OAT1 modulators.