

Study Report

Inhibitory Potential of BYI08330-enol and BYI08330-desmethyl-enol as Inhibitors on hOAT1, hOAT3 and hOAT4 In Transfected HEK-Cells

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We hereby declare that the work described in this report was performed under our supervision in accordance with the methods described herein and that the report reflects the results obtained fully and faithfully.

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List of Abbreviations

(v/v)	volume/volume
(w/v)	weight/volume
°C	degree Celsius
BSA	bovine serum albumin
c	concentration
Ci	curie
DMSO	dimethyl sulfoxide
dpm	decays per minute
ES	estrone 3-sulfate
x g	earth's gravitational acceleration (9,81 m/s ²)
h	hour
HBSS	Hanks' salt solution
HEPES	Hydroxyethylpiperazinylethansulfonsäure
HEK	human embryonic kidney cells
L	liter
M	molar
min	minute
MW	molecular weight
NaOH	sodium hydroxide solution
OAT	Organic Anion Transporter
P	protein amount
PAH	P-aminohippuric acid
PBS	phosphate-buffered saline
SA	specific activity
SLC	solute carrier
t	time

1. Summary

1.1 Objective

In order to investigate whether a chemical is a substrate or an inhibitor of hOAT1, hOAT3 and hOAT4, transporter assays using hOAT-transfected cell lines were performed. There is evidence from toxicological studies in rats that BYI08330-enol (“E”) and BYI08330-desmethyl-enol (“DME”) may interact with OATs. Transfected cell lines with rat organic anion transporter 1 and 3 are not available to date; therefore human OATs are used in this study. The aim of this orientating study is to characterize whether E and DME are interacting with hOAT1-mediated p-aminohippuric acid (PAH) uptake and with hOAT3- as well as hOAT4-mediated estrone sulfate (ES) uptake, respectively, using hOAT transfected HEK-cell lines.

1.2 Methods

To perform uptake experiments, hOAT-transfected and vector-transfected cells were harvested, plated into 24-well-plates at a density of 2×10^5 cells/well and were cultured for 3 days [1]. The [^3H]-uptake of the probe substrate for hOAT1, hOAT3 and hOAT4 was performed at two concentrations. To determine the inhibitory effect of E and DME, each test substance was added in two different concentrations to the [^3H] probe substrate. [^3H] content was measured in a scintillation counter.

1.3 Results

The hOAT1-mediated [^3H]PAH uptake was only significantly inhibited by 200 μM E by a maximum value of $36 \pm 2\%$ (100 μM PAH). The second test substance DME showed no interaction with OAT1. The hOAT3-mediated [^3H]ES uptake was significantly inhibited by both concentrations of E by a maximum value of $34 \pm 2\%$ (20 μM) and $76 \pm 1\%$ (200 μM). The test substance DME had a much lower inhibitory effect on the OAT3-mediated ES-uptake (1 μM) with values up to a maximum of $10 \pm 8\%$ (20 μM) and $24 \pm 4\%$ (200 μM). The hOAT4-mediated [^3H]ES uptake was significantly reduced by both test substances. The inhibitory effect was concentration dependent with a maximum value at the lower ES concentration (1 μM) of 73 ± 1 (200 μM E) and $62 \pm 0.3\%$ (200 μM DME), respectively.

2. Introduction

The human organic anion transporters (hOATs) belong to the solute carrier family (SLC22A) and accept a huge variety of chemically unrelated endogenous and exogenous organic anions. hOAT1 and hOAT3 are highly expressed on the basolateral side of proximal tubule cells of kidney, facilitating the uptake of several endogenous and exogenous organic anions into the cell and are consequently the first step of renal secretion of these compounds [2, 3]. The human OAT4 is highly expressed on the luminal site of proximal tubular cells of kidney [4, 5]. In the kidney hOAT4 is involved in the apical release as well as in the reabsorption process of several endogenous and exogenous organic anions [3]. Interestingly the gene of hOAT4 is detected only in primates and dogs, but not in rodents. However, it was reported that the human OAT2 is expressed in the basolateral membrane of proximal tubules whereas the rat and mouse orthologs were localized to the luminal membrane [6, 7]. Therefore, the OAT2- transporter could take over the function of OAT4 in rats and eliminate organic anions from the proximal tubule cells, which were taken up in the first step by OAT1 and OAT3 from the basolateral side of the renal epithelial cells.

Membrane transporter proteins involved in the renal elimination of xenobiotics may become saturated after exaggerated high experimental doses. This will lead to a transition from first order kinetics to zero-order kinetics, implying that the elimination rate is no longer proportional to the drug concentration. Thus, identification of transporter interactions may help in predicting the pharmacokinetics of drugs and explaining its toxic effects at high dose levels.

3. Materials and Methods

3.1 Reagents, Cell Culture Material and Equipment

Hanks' salt solution (HBSS)	Biochrom AG, Berlin, Germany
HEPES-buffer solution (1M)	Biochrom AG Berlin, Germany
PBS buffer (1 x Dulbeccos pH 7.4)	AppliChem, Darmstadt, Germany
Hgromycine	PAA Laboratories GmbH, Pasching, Österreich
Sodium hydroxide solution (NaOH) (1N)	AppliChem, Darmstadt, Germany
Quantum 286 for epithelial cells with L-Glutamin	PAA Laboratories GmbH, Pasching, Österreich
Penicillin/streptomycin	PAA Laboratories GmbH, Pasching, Österreich
DMSO for molecular biology	Sigma-Aldrich, Deisenhofen, Germany
Trypsin-EDTA	PAA Laboratories GmbH, Pasching, Österreich
poly-D-lysine hydrobromide	Sigma-Aldrich, Deisenhofen, Germany
Ultrapure DNase/RNase-free distilled water	Invitrogen, Karlsruhe, Germany
Mycoplasma Detection Kit	Minerva biolabs, Berlin, Germany
24-well plates	Sarstedt, Nümbrecht-Rommelsdorf, Germany
6 mL Röhrchen	PerkinElmer
Roti@ecoplus	CarlRoth, Karlsruhe, Germany
Dishes for cell culture 150 mm	Sarstedt Nümbrecht-Rommelsdorf, Germany
Dishes for cell culture 100 mm	Sarstedt Nümbrecht-Rommelsdorf, Germany

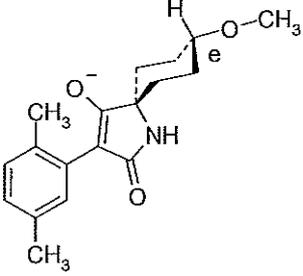
Equipment:

Laminar flow	Microflow, Nunc GmbH, Wiesbaden, Germany
Beckmann LS6000 Szintillations Counter	Beckmann, Germany
Infrared CO ₂ incubator	Labotect, Göttingen, Germany
Accurate scales (Feinwaage 2002 MP1)	Sartorius, Göttingen, Germany
Centrifuge (Labofuge 400R)	Heraeus, Osterode, Germany

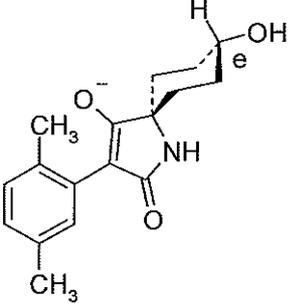
3.2 Test Items and Reference Compounds

3.2.1 Chemical Structure and Characterization of the Test Items

3.2.1.1 Test Item: BYI08330-enol

Structural formula: 	BYI08330-enol ("E"):	
	Internal code	AE 1302944
	Appearance	Beige powder
	Molecular weight:	301 g/mol
	Storage condition:	20 to 30 °C
	Water Solubility	2.7 g/L (buffer pH 7); 28 g/L (buffer pH 8) thus, at pH 7.4 WS is significantly greater than 2.7 g/L
	Acute oral toxicity:	LD50, rat > 2000 mg/kg bw

3.2.1.2 Test Item: BYI08330-desmethyl-enol

Structural formula: 	BYI08330-desmethyl-enol ("DME"):	
	Internal code	BCS-CN63407
	Appearance	white solid
	Molecular weight:	287g/mol
	Storage condition:	10 to 30 °C
	Water Solubility	2.7 g/L (buffer pH 7); ≥ 28 g/L (buffer pH 8)
	Acute oral toxicity:	LD50, rat > 2000 mg/kg bw

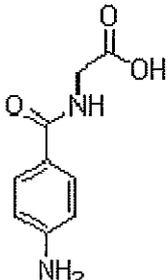
3.2.1.2 Preparation of the Test Item Solutions

A 2 mM stock solution in experimental buffer (HBSS, supplemented with 20 mM HEPES, pH 7.4) was prepared for unlabeled E and DME. Each solution was aliquoted and stored at 4°C. Additional working solutions of E and DME were freshly prepared as necessary in experimental buffer (HBSS, supplemented with 20 mM HEPES, pH 7.4).

3.2.3 Chemical Structure and Characterization of Substrates and Inhibitors

3.2.3.1 Substrate: p-Aminohippuric Acid

p-Aminohippuric acid (PAH) was purchased by Sigma-Aldrich and was used as a substrate for hOAT1 (Lot no. 0001433124).

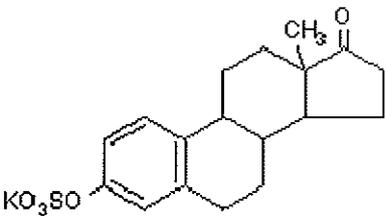
Structural formula: 	Chemical name:	N-(4-Aminobenzoyl)glycine
	Synonym:	p-Aminohippuric acid
	Molecular formula:	$\text{NH}_2\text{C}_6\text{H}_4\text{C}(\text{O})\text{NHCH}_2\text{COOH}$
	Solubility	Clear colorless to yellow solution at 50 mg/mL in H_2O
	Molecular weight:	194.19 g/mol

^3H -p-Aminohippuric acid (aminohippuric acid, P-[GLYCYL-2- ^3H]) was purchased by PerkinElmer (Lot no.3632431).

Specific activity:	1-5 Ci (37.0-185GBq)/mmol
Purity:	>97%
Storage temperature:	2-8°C

3.2.3.2 Substrate: Estrone 3-sulfate

Estrone 3-sulfate (ES) was purchased by Sigma-Aldrich and was used as a substrate for hOAT3 and hOAT4 (Lot no. 067K4039).

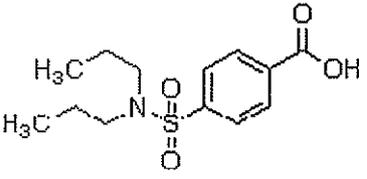
Structural formula: 	Chemical name:	1,3,5(10)-Estratrien-17-one 3-sulfate
	Synonym:	Estrone 3-sulfate potassium salt
	Molecular formula:	C ₁₈ H ₂₁ O ₅ SK
	Molecular weight:	388.52 g/mol

[³H]Estrone sulfate, ammonium salt, [6,7-³H(N)] was purchased by PerkinElmer (Lot no.3632437).

Specific activity:	40-60 Ci/mmol (1480-2220 GBq/mmol)
Purity:	>97%
Storage temperature:	-20°C

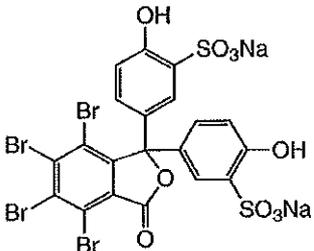
3.2.3.3 Inhibitor: Probenecid

Probenecid (*p*-(Dipropylsulfamoyl) benzoic acid) was purchased by Sigma-Aldrich (Lot no. 118K0893) and was used as an inhibitor for hOAT1 and hOAT3.

Structural formula: 	Chemical name:	<i>p</i> (Dipropylsulfamoyl)benzoic acid
	Synonym:	Probenecid
	Molecular formula:	C ₁₃ H ₁₉ NO ₄ S
	Molecular weight:	285.36 g/mol

3.2.3.4 Inhibitor: Sulfobromophthalein

Sulfobromophthalein disodium salt hydrate (BSP) was purchased by Carl Roth GmbH&Co KG (Lot no. 499150456) and was used as an inhibitor for hOAT4.

Structural formula: 	Chemical name:	Sulfobromophthalein disodium salt hydrate
	Synonym:	
	Molecular formula:	C ₂₀ H ₈ Br ₄ O ₁₀ S ₂ Na ₂
	Molecular weight:	838 g/mol

3.2.3.6 Preparation of Stock and Working Solutions

The stock solutions of ES (MW: 388.52 g/mol) and BSP (MW: 838 g/mol) were prepared in water in a final concentration of 50 mM. The stock solution of PAH (MW: 194.19 g/mol) was prepared in water in a final concentration of 100 mM. Each solution was aliquoted and stored at -20°C. Additional working solutions of ES, BSP, and PAH were freshly prepared as necessary in water.

The stock solution of probenecid (MW: 285.36 g/mol) was prepared in dimethyl sulfoxide (DMSO for molecular biology) in a final concentration of 100 mM. Additional working solutions of probenecid, were freshly prepared as necessary in DMSO.

The final incubation solutions were prepared in HBSS (supplemented with 20 mM HEPES, pH 7.4) by serial dilutions such that the DMSO content was equal to 1% (v/v). All vector-transfected control cells were also treated with 1% DMSO.

The [³H]PAH and [³H]ES working solutions were prepared in experimental buffer.

3.3 Thawing Procedure and Proliferation Conditions

All cells were handled under a laminar flow. For disinfection purpose, all critical instruments for handling of cells were purified with 70% ethanol in advance.

Cryogenic vials containing about 3 x 10⁶ hOAT-transfected or vector-transfected HEK cells were thawed in a water bath at 37°C until a small rest of ice was present. The cell suspension was immediately and completely transferred into a cell culture dish 100 mm containing 10 mL of 37°C-

temperated PAA-medium (PAA, Quantum 286 for epithelial cells with L-glutamine, 1% penicillin/streptomycin). The hOAT3-transfected cells were cultured in PAA-medium additionally containing 175 µg/mL hygromycine B. After 24 h the medium was replaced by new medium.

OAT-transfected and vector-transfected HEK cells were grown on 100 mm or 150 mm cell culture dishes at 37°C in a humidified 5% CO₂ atmosphere.

All HEK cell lines (hOAT1, hOAT3, hOAT4 and vector-HEK-cells) were tested for mycoplasma - before starting transport experiments using Mycoplasma Detection Kit for conventional PCR.

3.4 Passaging of HEK Cell Lines

After removal of the medium, the cells were washed with 5-10 mL of PBS. The adherent cells were exposed to 1-2 mL trypsin-EDTA solution for 5 min at room temperature. To the cell culture plate 3-4 mL medium was added and the suspension was transferred into a falcon tube and centrifuged at 124 x g for 3 min. The cell pellet was resuspended in about 1-3 mL medium.

Confluent grown cell cultures were split twice a week 1:4 to 1:10. For counting in a Neubauer-counting chamber, cells were diluted 1:20 in medium. A fraction of harvested cells was used for transport experiments.

3.5 Preparation of HEK Cells for Uptake Assays

For uptake assays, 24-well plates were pretreated with poly-D-lysine hydrobromide solution (2 mg/mL). Therefore, each well was coated with 0.5 mL poly-D-lysine solution and incubated for at least 15-30 min. After complete removal of the solution the plates were dried for 30-60 min. Before use in transport experiments freshly harvested cells were seeded into 24 well plates (2 x 10⁵ cells in 0.5 mL medium per well) and cultured for 3 days.

3.6 OAT Uptake Assay

Growth medium was aspirated and each well was rinsed twice with 0.5 mL PBS buffer and then preincubated in 0.5 mL incubation buffer HBSS (supplemented with 20 mM HEPES, pH 7.4) for 20 min at 37°C. The incubation buffer was removed and 200 µL incubation buffer containing the radiolabeled and non-radiolabeled substances was added to each well and incubated at 37°C for designated time intervals. After incubation, the uptake was terminated by aspirating the reaction mixture and washing the cells three times with 1 mL ice-cold PBS buffer. Cells were solubilized with

0.5 mL of 1N NaOH over night. [³H] content was measured after addition of 2.5 mL scintillation solvent in a LS 6000 Beckmann scintillation counter.

3.6.1 Uptake Transport Experiments in hOAT1-transfected HEK Cells

3.6.1.1 Inhibition Study

The PAH incubation buffer contained [³H]PAH and unlabeled PAH in a final concentration of 10 μM and 100 μM, respectively. To characterize the inhibitory potential of “E” and “DME” each test item was added in two different concentrations to 10 and 100 μM [³H]PAH, respectively. Inhibition of hOAT1-mediated PAH uptake by 100 μM probenecid was performed as control experiment. In detail the following concentrations were used:

Substrate	I. Substrate Concentration (μM)	II. Substrate Concentration (μM)	Inhibitor	Inhibitor Concentration (μM)
PAH	10	100	-	-
	10	100	E	20
	10	100	DME	200
	10	100	Probenecid	100

After 3 min the incubation was terminated. All experiments were conducted at least on 2 separate days. On each day, all experiments were performed as triplicates.

3.6.2 Uptake Transport Experiments in hOAT3-transfected HEK Cells

3.6.2.1 Inhibition Study

The ES incubation buffer contained [³H]ES and unlabeled ES in a final concentration of 1 μM and 10 μM, respectively. To characterize the inhibitory potential of “E” and “DME” each test item was added in two different concentrations to 1 μM and 10 μM [³H]ES, respectively. Inhibition of hOAT3-mediated ES uptake by 100 μM probenecid was performed as control experiment. In detail the following concentrations were used:

Substrate	I. Substrate Concentration (μM)	II. Substrate Concentration (μM)	Inhibitor	Inhibitor Concentration (μM)
ES	1	10	-	-
	1	10	E	20
	1	10	DME	200
	1	10	Probenecid	100

The incubation was terminated after 1 min. All experiments were conducted at least on 2 separate days. On each day, all experiments were performed as triplicates.

3.6.3 Uptake Transport Experiments in hOAT4-transfected HEK Cells

3.6.3.1 Inhibition Study

The ES incubation buffer contained [³H]ES and unlabeled ES in a final concentration of 1 μM and 10 μM, respectively. To characterize the inhibitory potential of “E” and “DME” each test item was added in two different concentrations to 1 μM and 10 μM [³H]ES, respectively. Inhibition of hOAT4-mediated ES uptake by 10 μM BSP was performed as control experiment. In detail the following concentrations were used:

Substrate	I. Substrate Concentration (μM)	II. Substrate Concentration (μM)	Inhibitor	Inhibitor Concentration (μM)
ES	1	10	-	-
	1	10	E	20
	1	10	DME	200
	1	10	BSP	10

The incubation was terminated after 1 min. All experiments were conducted at least on 2 separate days. On each day, all experiments were performed as triplicates.

3.7 Determination of the Protein Amount

Reagents and equipment for protein determination were purchased from following suppliers:

Coomassie Serva Blue G	Serva, Heidelberg, Germany
Ethanol 99.6%	Carl Roth, Karlsruhe, Germany
Phosphoric acid 85%	AppliChem, Darmstadt, Germany
5x lysis buffer	Promega, Madison, WI, USA
BSA, albumin fraction V	Carl Roth, Karlsruhe, Germany
Photometer Mithras LB 940	Berthold Technologies, Bad Wildbad; Germany

Cellular protein amount was determined in parallel from 6 wells from a different 24-well reference plate per cell line and experimental day using a method described by Bradford 1976 [8].

For 250 mL Bradford-reagent (4x) were used:	70 mg Serva Blue G
	50 mL ethanol (96%)
	100 mL phosphoric acid (85%)

Cell monolayers in 24 well plates were washed 3 x with 500 μ L PBS buffer and incubated for lysis 30-60 min in 100 μ L 1 x lysis buffer (5 x lysis buffer; Promega; diluted 1:5 in PBS buffer) per well. Cell lysate was filled up with PBS buffer to 1 mL per well and mixed vigorously. Plates were stored at -20°C .

Protein determination was performed in 96 well plates (Sarstedt; flat bottom) in duplicate. BSA (stock solution: 1 mg/mL) was used as standard for a calibration curve with the following concentrations (0, 50, 75, 100, 150, 200, 250, 300 $\mu\text{g}/\text{mL}$)

20 μ L of BSA-standards or 20 μ L sample was mixed with 200 μ L Bradford-reagent (diluted 1:4 in PBS buffer from stock reagent) per well. After 10-20 min incubation at room temperature, absorption was measured at 590 nm (Microplate Reader, Mithras LB 940, Berthold).

3.8 Data Analysis and Results

The absolute amount (pmol) of the substrate uptake was calculated within the given time and related to the determined protein values. Initially, the specific activity was calculated according the labeling ($\mu\text{Ci}/\text{mmol}$) and the concentration of the substrate. Additionally, the radioactivity (dpm) of aliquots of each substrate solution was determined. From this, the specific radioactivity, so called “*standard*”, was calculated according the following formula:

$$SA = \frac{St_{dpm}}{[S] * V_s * 10^6}$$

SA: specific radioactivity (dpm/pmol)

St_{dpm} : mean radioactivity of standards (dpm)

[S]: substrate concentration ($\mu\text{mol}/\text{L}$)

V_s : volume of the aliquot substrate solution (L)

The specific radioactivity from each condition was used to determine the uptake values U of every sample, which was calculated according the following formula:

$$U = \frac{RA_{sample}}{SA * P}$$

U: uptake (pmol/mg protein)

SA: specific radioactivity (dpm/pmol)

RA_{sample} : radioactivity of one well of the 24 well plate (dpm)

P: protein amount (mean of six wells from the 24 well reference plate) (mg protein)

Initial uptake rate v for each well was calculated according to the following formula:

$$v = \frac{U}{t}$$

v: uptake rate (pmol/mg protein/min)

U: uptake (pmol/mg protein)

t: incubation interval (min)

OAT-transporter mediated uptake rate (net-uptake) was obtained by subtracting the uptake rate in vector-transfected HEK cells from the uptake rate in OAT-HEK cells as described below:

$$v^{OAT} = v^{OAT-HEK} - v^{vector-HEK}$$

v^{OAT} : initial uptake rate (OAT mediated) (pmol/mg protein/min)

v : initial uptake rate (pmol/mg protein/min)

The percentage of uptake inhibition was calculated from the net-uptake from control experiments in the absence of added inhibitor (100%). Means \pm average deviation (av. dev.) were calculated from two independent experiments on 2 separate days.

4. Results

4.1 Inhibition of hOAT1 mediated [³H]PAH uptake by E and DME

To determine, if E and DME are hOAT1 inhibitors, the inhibitory potential towards the uptake of a probe substrate was analyzed.

For these inhibition studies PAH was used as a specific hOAT1 probe substrate at concentrations of 10 μ M and 100 μ M [9, 10]. The inhibitory effect of E and DME on the hOAT1 mediated [³H]PAH uptake was evaluated at two concentrations (20 and 200 μ M).

As shown in Figure 1 and Table 1, the test item E shows only at a higher concentration of 200 μ M a significant inhibition of the hOAT1-mediated [³H]PAH uptake. The uptake of [³H]PAH was reduced by 35% (10 μ M PAH) and by 36% (100 μ M). Two tested concentrations of DME (20 μ M and 200 μ M) had no inhibitory effect on the [³H]PAH net-uptake (10 μ M and 100 μ M) in hOAT1-transfected HEK cells. Probenecid was used as a specific positive control inhibitor on the hOAT1 mediated [³H]PAH uptake. In this two experiments 100 μ M of probenecid showed a significant inhibition of 10 μ M [³H]PAH (89%) and of 100 μ M [³H]PAH (82%), respectively.

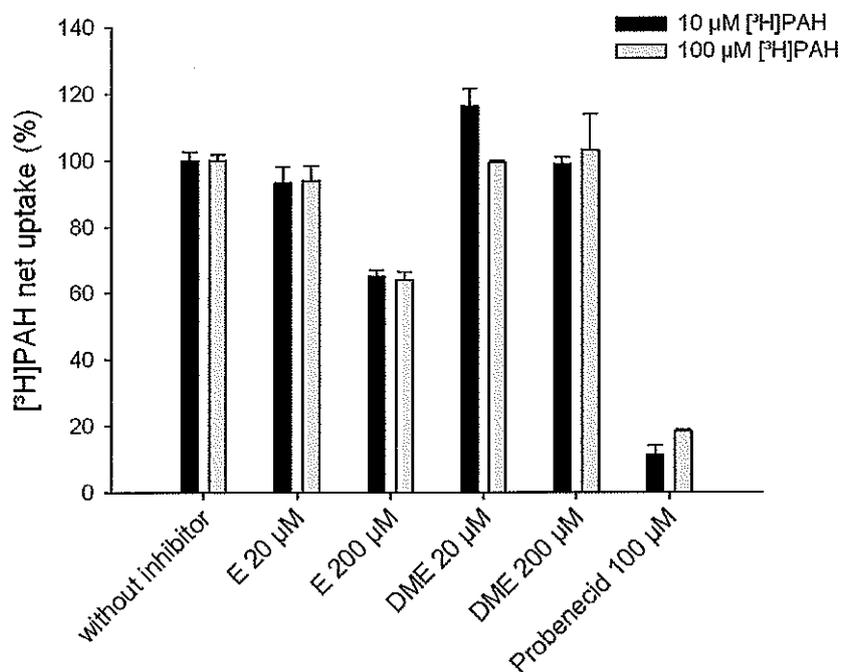


Figure 1 Inhibitory effect of E and DME (20 and 200 μ M) towards the [³H]PAH (10 μ M and 100 μ M) net-uptake in hOAT1-transfected HEK cells. Shown are percentage means (net-uptake) of 2 experiments \pm average deviation.

4.2 Inhibition of hOAT3-mediated [³H]ES uptake by E and DME

To determine, if E and DME are hOAT3 inhibitors, the inhibitory potential towards the uptake of a probe substrate was analyzed.

For inhibition studies [³H]ES was used as a specific hOAT3 substrate at concentrations of 1 μ M and 10 μ M [9, 10]. The inhibitory effect of E and DME towards the hOAT3 mediated [³H]ES uptake was evaluated at two concentrations (20 μ M and 200 μ M).

As shown in Figure 2 and Table 1, the test item E showed a significant concentration dependent inhibition of the hOAT3-mediated ES uptake. The inhibitory potential of E on the ES uptake (1 μ M) was $34 \pm 1.8\%$ (20 μ M) and $76 \pm 0.5\%$ (200 μ M), respectively. At a higher ES concentration of 10 μ M ES the inhibitory effect of E was lower with $16 \pm 1.9\%$ (20 μ M) and $59 \pm 3\%$ (200 μ M), respectively. DME showed a slight inhibition of the [³H]ES net-uptake in hOAT3-transfected HEK cells. At a tested concentration of 1 μ M [³H]ES the inhibitory effect of 20 μ M and 200 μ M was $10 \pm 7.5\%$ and $24 \pm 4\%$, respectively. Probenecid was used as a specific positive control inhibitor on the hOAT3 mediated [³H]ES uptake. In this experiments 100 μ M of probenecid showed a significant inhibition of 1 μ M [³H]ES (94%) and 10 μ M [³H]ES (87%), respectively.

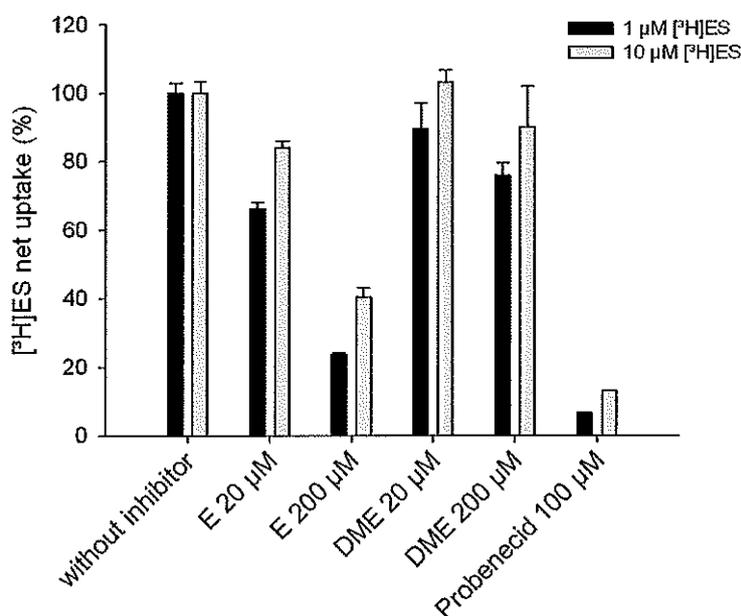


Figure 2 Inhibitory effect of E and DME (20 and 200 μ M) towards the [³H]ES (1 μ M and 10 μ M) net-uptake in hOAT3-transfected HEK cells. Shown are percentage means (net-uptake) of 2 experiments \pm average deviation.

4.3 Inhibition of hOAT4 mediated [³H]ES uptake by E and DME

To determine, if E and DME are hOAT4 inhibitors, the inhibitory potential towards the uptake of a probe substrate was analyzed.

For inhibition studies [³H]ES was used as a specific hOAT3 substrate at concentrations of 1 μM and 10 μM [9, 10]. The inhibitory effect of E and DME towards the hOAT4 mediated [³H]ES uptake was evaluated at two concentrations (20 μM and 200 μM).

As shown in Figure 3 and Table 1, the test item E showed a significant concentration dependent inhibition of the hOAT4-mediated ES uptake. The inhibitory potential of E on the ES uptake (1 μM) was 27±2.0% (20 μM) and 73±1.0 % (200 μM), respectively. At a higher ES concentration of 10 μM the inhibitory effect of E was slightly reduced with 15±0.4% (20 μM) and 59±2.0% (200 μM), respectively. DME showed also a concentration dependent inhibition of the [³H]ES net-uptake in hOAT3-transfected HEK cells. With the concentration of 1μM [³H]ES the inhibitory effect of the highest tested concentration of DME (200 μM) was 62±0.2%. With the concentration of 10μM [³H]ES the inhibitory effect of the highest tested DME concentration (200 μM) was slightly lower with 52±3%. BSP was used as a specific positive control inhibitor on the hOAT4 mediated [³H]ES uptake. In this experiments 10 μM of BSP showed a significant inhibition of 1 μM [³H]ES (95%) and 10 μM [³H]ES (92%), respectively.

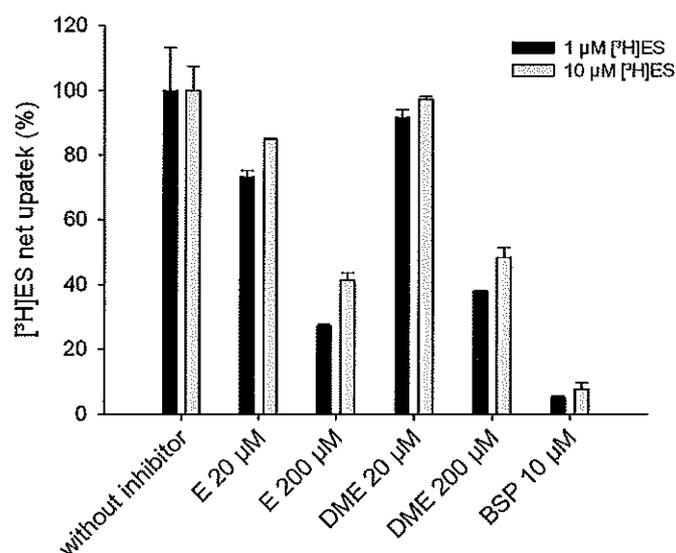


Figure 3 Inhibitory effect of E and DME (20 and 200 μM) towards the [³H]ES (1 μM and 10 μM) net-uptake in hOAT4-transfected HEK cells. Shown are percentage means (net-uptake) of 2 experiments ± average deviation.

5. Discussion and Conclusion

The tested compounds E and DME are the main rat metabolites of BYI08330 (spirotetramat), an insecticide developed at BayerCropScience. From toxicological studies in rats there is evidence that BYI08330-enol (“E”) and BYI08330-desmethyl-enol (“DME”) may interact with OATs.

In the ADME study in rats, plasma concentrations of approximately 10 to 550 μM of E and DME will be reached after low to moderate dose levels of spirotetramat (2 to 100 mg/kg bw). Therefore, 20 μM and 200 μM were used in the orientating OAT-interaction studies with the enol and the desmethyl-enol.

Transfected cell lines with rat organic anion transporter 1 and 3 are not available to date and OAT4 is not detected in rodents; therefore, human OATs stably transfected in HEK-cell were used in this orientating study to characterize whether E and DME are interacting with hOAT1-mediated p-aminohippuric acid (PAH) uptake and with hOAT3- as well as hOAT4-mediated estrone sulfate (ES) uptake, respectively.

The radio labeled p-aminohippurate (PAH) is the prototypical test anion for OAT1 used in this study. Cellular localization and mode of operation of OAT1 and OAT3 are the same. The substrate specificity of OAT1 and OAT3 overlap but are not identical. In general, OAT3 handles bulkier and more lipophilic organic anions than OAT1 does. The usual test substrate for OAT3 is estrone-3-sulfate (ES), because the uptake is easily detectable (low background in non-expressing cells) and the affinity of OAT3 is very high; therefore, ES was used in this studies as a probe substrate [11].

Two concentrations of PAH and ES at the K_m and $1/10 K_m$ value, determined by PortaCellTec biosciences, were used to characterize the inhibitory potential of E and DME. Probenecid is a well known competitive inhibitor of hOAT1 and hOAT3 and was used in all experiments as a positive control [12, 13].

hOAT1-mediated [^3H]PAH uptake was only significantly inhibited by 200 μM E by a maximum value of $36\pm 2\%$ (100 μM PAH). The second test substance DME showed no interaction with the OAT1-mediated PAH uptake.

hOAT3-mediated [^3H]ES uptake was significant inhibited by both concentrations of E by a maximum value of $34\pm 2\%$ (20 μM) and $76\pm 1\%$ (200 μM). The test substance DME had a much lower inhibitory effect on the OAT3-mediated ES-uptake (1 μM) with values up to a maximum $10\pm 8\%$ (20 μM) and $24\pm 4\%$ (200 μM).

hOAT4- mediated [³H]ES uptake was significantly reduced by both test substances. The inhibitory effect was concentration dependent with a maximum value at the lower ES concentration (1 μM) of 73±1 (200 μM E) and 62±0.3 % (200 μM DME), respectively.

In conclusion, in this orientating study the human OAT transporters were identified to play an important role in the distribution and elimination of these two metabolites E and DME.

Substrates affinity and inhibitory potential of human, mouse and rat OATs are highly overlapping as summarized in the review by VanWert et al. 2010 [14] Nevertheless, kinetic data of human, mouse and rat OATs, especially for OAT1 and OAT3, demonstrated some variations for several drugs. This could be due to different expression systems, experimental setups and other experimental parameters (e.g. substrate concentration, uptake time).

This orientating inhibition study performed in human OAT HEK-cells could very well represent the interaction of the BYI08330-enol and the BYI08330-demethyl-enol with OATs in mammalian species and should help to understand the elimination kinetics of E and DME via kidney.

Because the enol metabolite of spirotetramat is partly conjugated by glucuronic acid in mice and humans, other important transporters like OATPs, mainly expressed in the liver, and the efflux multidrug transporters (e.g. MDR1, MRP2, MRP4) could also be involved in the elimination of this metabolite.

6. References

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7. Appendix

Table 1 Tabulated Summary

Study No.	PCT_009-11
Test System	hOAT1, hOAT3 and hOAT4-transfected and vector HEK cells
Test Item	BYI08330-enol (E) and BYI08330-desmethyl-enol (DME)
Analytical Method	Measurement of radio labeled probe substrate uptake (at K_m and $1/10 K_m$) in cells measured by liquid scintillation counting in presence and absence of E and DME and one positive control inhibitor

Experimental Results:

		Inhibitory effect of test compound added (%)				
Transporter	[³ H] Substrate	E (20 μM)	E (200 μM)	DME (20 μM)	DME (200 μM)	Control inhibitor
		mean±av.dev.	mean±av.dev.	mean±av.dev.	mean±av.dev.	mean±av.dev.
hOAT1	10 μM [³ H]PAH	7 ± 5	35 ± 2	-16 ± 6	1 ± 2	89 ± 3
	100 μM [³ H]PAH	6 ± 5	36 ± 2	1 ± 1	-3 ± 11	81 ± 0.3
hOAT3	1 μM [³ H]ES	34 ± 2	76 ± 1	10 ± 8	24 ± 4	94 ± 0.2
	10 μM [³ H]ES	16 ± 2	59 ± 3	-3 ± 4	10 ± 12	87 ± 0.01
hOAT4	1 μM [³ H]ES	27 ± 2	73 ± 1	9 ± 3	62 ± 0.3	95 ± 0.4
	10 μM [³ H]ES	15 ± 0.4	59 ± 2	3 ± 1	52 ± 3	92 ± 2

Control inhibitors: hOAT1 and hOAT3 experiments: probenecid (100 μM); for hOAT4 experiments: sulfobromophthalein (10 μM)

Results are given as mean +/- average deviation inhibitory effect from two independent experiments

hOAT: human organic anion transporter

PAH: p-aminohippuric acid

ES: estrone 3-sulfate

BSP: sulfobromophthalein



Table 2 Inhibition of [³H]PAH (10 μM) uptake (5 min incubation) by E and DME (20 μM and 200 μM) and by probenecid (100 μM) in hOAT1-HEK and vector-HEK-Cells.

Inhibition of substrate uptake (PAH) by inhibitor (E and DME) in OAT1-HEK and vector-HEK cells

Assay No.	Substrate solution (S _{0,exp})		Specific activity (SA)	Inhibitor/ Test item	Conc.	vector-HEK				OAT1-HEK				Net-Uptake v (pmol/mg/min)			Net uptake (%)			Inhibitory effect (%)					
						RA _{sample}	Uptake v (pmol/mg/min)			RA _{sample}	Uptake v (pmol/mg/min)			Per experiment	Mean	Av. Dev.	Per experiment	Mean	Av. Dev.	Per experiment	Mean	Av. Dev.			
						Test Item (pL)	(dpm/ well)	Per well	Mean	S.D.	(dpm/ well)	Per well	Mean	S.D.	Per experiment	Mean	Av. Dev.	Per experiment	Mean	Av. Dev.	Per experiment	Mean	Av. Dev.		
PCT-009-11 1	25665	23657	473	-	0	1923	5	5	0,3	18768	47	47	0	42	44	1	103	3	-3	0	3				
	20427					1754	4			18509	47											18468	47		
	24379	1730				4	17915			51	17771											50	17313	49	
	22867	1722				5	16606			45	17688											48	17074	46	
	23298	21781				1653	5			17771	50											16579	45	17074	46
	19179					1814	6			16579	45														
2	22841	22223	444	-	20	1888	5	4	0,1	16606	45	45	1,6	42	41	1	98	4,9	7	4,9					
	23230					1633	4			17768	48										17074	46			
	20497	*					17074			46	17074										46				
	22780	1758				5	16511			45	16511										45				
	23245	22804				1718	5			16588	45										16588	45			
	22387					1768	5			16579	45														
1	23186	21994	440	-	200	1849	4	4	0,1	11354	31	31	0,4	27	28	2	63	1,8	37	35	2				
	19607					1882	4			11630	32											11630	32		
	23189	1700				5	11618			32	11618											32			
	20246	1597				5	12795			36	12795											36			
	22864	21907				1691	5			12447	35											12447	35		
	22810					1615	5			11879	33														
2	22854	22176	444	-	20	1788	5	5	0,1	19604	53	56	4,6	52	51	1	122	5,5	-22	-16	5,5				
	23314					1837	5			22913	62											20180	54		
	20361	1804				5	20180			54	20180											54			
	23316	2211				7	19922			55	19922											55			
	22777	22228				2011	6			20230	56											20230	56		
	20592					1752	5			19857	55														
1	22338	22360	447	-	200	1968	5	5	0,3	17827	48	48	0,7	43	43	0	101	2	-1	1	2				
	22453					2006	5			17754	47											18223	49		
	22290	1802				5	18223			49	18223											49			
	20908	1893				6	17635			49	17635											49			
	23343	22191				2052	6			17462	49											17462	49		
	22423					2012	6			18175	51														
2	20914	21097	422	-	100	2033	6	6	0,1	3934	11	12	0,7	6	5	1	14	2,9	86	89	2,9				
	19704					2058	6			4025	11											4393	12		
	22674	1982				6	4393			12	4393											12			
	18226	1944				6	3302			10	3302											10			
	22122	20766				2077	7			3515	10											3515	10		
	21950					2261	7			3744	11														

S.D. Standard deviation

Av. Dev. Average Deviation

Substrate PAH

Substrate Conc. (μM) 10

Volume aliquot substrate solution (L) 0,000005

Incubation time (min) 3

Protein (P) determination (mg/well):

(vector-HEK cells in assay 1) 0,280 (OAT1-HEK cells in assay 1) 0,279

(vector-HEK cells in assay 2) 0,259 (OAT1-HEK cells in assay 2) 0,270

* data points were excluded of the calculation

Table 3 Inhibition of [³H]PAH (100 μM) uptake (5 min incubation) by E and DME (20 μM and 200 μM) and by probenecid (100 μM) in hOAT1-HEK and vector-HEK-Cells.

Inhibition of substrate uptake (PAH) by inhibitor (E and DME) in OAT1-HEK and vector-HEK cells

Assay No.	Substrate solution (S _{100μM})		Specific activity (SA) (dpm/μmol)	Inhibitor Test (nm)	Conc	vector-HEK				OAT1-HEK				Net-Uptake v (pmol/mg/min)			Net uptake (%)			Inhibitory effect (%)		
						RA _{sample}	Uptake v (pmol/mg/min)			RA _{sample}	Uptake v (pmol/mg/min)			Per experiment	Mean	Av. Dev.	Per experiment	Mean	Av. Dev.	Per experiment	Mean	Av. Dev.
						Test Item (μM)	(dpm/well)	Per well	Mean	S.D.	(dpm/well)	Per well	Mean									
PCT-009-11	1	20848	21787	44	0	1848	50			11754	322			267	262	5	98	100	2	0	2	
		21133				1898	52	51	1,1	11720	321	317	8									
		23379				1822	50			11259	309											
		18105				1562	50			9100	288											
		17621				1562	50	50	0,7	9574	313	308	8									
		14843				1518	49			8408	308											
	4	21004	22767	46	20	1843	47			11241	285			239	246	7	89	94	4,5	11	6	4,5
		23511				1920	49	48	1,5	10745	282	288	8									
		23786				1808	46			10721	281											
		17814								10689	320											
		18287				2145	69	66	4,3	10770	323	318	4									
		18194				1868	63			10502	315											
1	22530	21197	42	200	1884	51			7954	224			164	168	3	62	64	2	38	38	2	
	22151				1832	50	50	0,7	7307	206	214	9										
	18809				1850	50			7572	213												
	16795				1572	52			8966	228												
	16840				1673	55	53	1,7	8847	224	224	3										
	17058				1614	53			6771	221												
1	23289	23255	47	20	2199	56			12268	315			263	260	3	99	99	0,7	1	1	0,7	
	23076				2215	57	55	2,7	12690	325	318	6										
	23391				2026	52			12207	314												
	17109				1800	58			9724	320												
	15038				1987	64	62	3,2	9685	319	319	1										
	18190				1952	63			8673	318												
1	22874	22001	44	200	2142	58			11369	309			246	269	23	92	103	11	8	-3	11	
	22620				2241	61	59	1,5	11237	305	305	4										
	20410				2143	58			11106	302												
	17183				2194	72			11482	383												
	16240				2242	74	73	0,9	10015	334	365	27										
	16186				2191	72			11337	378												
1	23492	22215	44	100	2236	60			4124	111			49	48	0	18	19	0,3	82	81	0,3	
	22205				2419	65	63	2,5	4146	111	111	0										
	20947				2347	63			4125	111												
	17879				2207	67			3738	115												
	17221				2058	62	67	5,2	3684	113	115	3										
	18813				2402	72			3870	119												

S.D. Standard deviation

Av. Dev. Average Deviation

Substrate PAH
 Substrate Conc. (μM) 100
 Volume aliquot substrate solution (L) 0,000005
 Incubation time (min) 3
 Protein (P) determination (mg/well):
 (vector-HEK cells in assay 1) 0,280 (OAT1-HEK cells in assay 1) 0,279
 (vector-HEK cells in assay 2) 0,307 (OAT1-HEK cells in assay 2) 0,302

* data points were excluded from the calculation

Table 7 Inhibition of [³H]ES (10 μM) uptake (1 min incubation) by E and DME (20 μM and 200 μM) and by probenecid (100 μM) in hOAT4-HEK and vector-HEK-Cells.

Inhibition of substrate uptake (ES) by inhibitor (E and DME) in OAT4-HEK and vector-HEK cells

Assay No.	Substrate solution (St _{q-m})		Specific activity (SA)	Inhibitor/ Test item	Conc	vector-HEK				OAT4-HEK				Net-Uptake v (pmol/mg/min)			Net uptake (%)			Inhibitory effect (%)			
						RA _{app}	Uptake v (pmol/mg/min)			RA _{app}	Uptake v (pmol/mg/min)			Per experiment	Mean	Av. Dev.	Per experiment	Mean	Av. Dev.	Per experiment	Mean	Av. Dev.	
						(dpm/well)	Per well	Mean	S.D.	(dpm/well)	Per well	Mean	S.D.										
PCT-009-11	(dpm/5 μL)	Mean (dpm/5μL)	(dpm/μmol)		Test item (μM)	(dpm/well)	Per well	Mean	S.D.	(dpm/well)	Per well	Mean	S.D.	Per experiment	Mean	Av. Dev.	Per experiment	Mean	Av. Dev.	Per experiment	Mean	Av. Dev.	
	19616	19821	396		0	632	7			20950	199												
	20642	19205				622	6	6	0,1	20822	188	197	2	191			108				-8		
	19205					616	6			20445	195				206	15		101	7		-1	7	
	12315	12850	257		0	224	4			12243	204												
2	13267	12850	257		0	212	3	4	0,4	14649	245	225	20	222			93				7		
	12967					259	4			13583	227												
1	17620	17718	354		20	512	8			16098	171												
	17705					554	9	9	0,6	16246	173	170	4	161			84				16		
	17828					690	9			15515	165				175	14		85	0,4		15	0,4	
	13629					234	4			11343	192												
	12643	12699	254		20	246	4	4	0,2	11687	197	193	4	189			85				15		
	11825					263	4			11240	190												
1	12708	12921	258		200	436	6			5742	84												
	12871					438	6	6	0,4	5466	80	80	3	74			39				61		
	13183					394	6			5297	77				85	11		41	2		59	2	
	12451					214	4			5504	97												
	12563	12169	243		200	228	4	4	0,3	5994	106	101	5	97			44				56		
	11494					200	4			5824	99												
1	13187	13110	262		20	366	6			13067	188												
	13202					390	6	6	0,4	13724	188	190	7	184			96				4		
	12942					412	6			12798	184				201	17		97	0,9		3	0,9	
	12479					221	4			13157	223												
	12254	12819	252		20	201	3	4	0,3	13185	224	221	5	217			98				2		
	13125					238	4			12651	215												
1	13830	13824	276		200	384	5			6749	92												
	13295					407	6	6	0,4	6626	90	92	1	86			45				55		
	14241					419	6			6807	93				100	14		48	3		52	3	
	11629					245	5			6207	117												
	10853	11390	228		200	257	5	5	0,1	6521	123	119	4	114			51				49		
	11689					254	5			6150	116												
1	11382	11558	231		10	529	9			1156	19												
	11538					482	9	9	0,6	1152	19	19	1	11			6				94		
	11773					457	8			1246	20				16	5		8	2,0		82	2,0	
	10929					237	4			1216	23												
	12149	11253	225		10	258	5	5	0,4	1514	29	26	3	21			10				90		
	10681					282	5			1377	26												

S.D. Standard deviation

Av. Dev. Average Deviation

Substrate Estrone sulfate (ES)

Substrate Conc. (μM) 10

Volume aliquot substrate solution (L) 0,000005

Incubation time (min) 1

Protein (P) determination (mg/well):

(vector-HEK cells in assay 1) 0,245 (OAT4-HEK cells in assay 1) 0,265
 (vector-HEK cells in assay 2) 0,237 (OAT4-HEK cells in assay 2) 0,233