

Original article:

EFFECTS OF TRICHOSTATIN A ON DRUG UPTAKE TRANSPORTERS IN PRIMARY RAT HEPATOCYTE CULTURES

Eva Ramboer*, Vera Rogiers, Tamara Vanhaecke[°], Mathieu Vinken[°]

In Vitro Toxicology and Dermato-cosmetology research group, Center for Pharmaceutical Research, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel

[°] equally contributing last authors

* Corresponding author: Eva Ramboer; In Vitro Toxicology and Dermato-cosmetology research group, Center for Pharmaceutical Research, Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel, (VUB), Laarbeeklaan 103, B-1090 Brussels, Belgium.

E-mail: eramboer@vub.ac.be, +32.2.477.45.87

<http://dx.doi.org/10.17179/excli2015-220>

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>).

ABSTRACT

The present study was set up to investigate the effects of Trichostatin A (TSA), a prototypical epigenetic modifier, on the expression and activity of hepatic drug uptake transporters in primary cultured rat hepatocytes. To this end, the expression of the sinusoidal transporters sodium-dependent taurocholate cotransporting polypeptide (Ntcp) and organic anion transporting polypeptide 4 (Oatp4) was monitored by real-time quantitative reverse transcriptase polymerase chain reaction analysis and immunoblotting. The activity of the uptake transporters was analyzed using radiolabeled substrates and chemical inhibitors. Downregulation of the expression and activity of Oatp4 and Ntcp was observed as a function of the cultivation time and could not be counteracted by TSA. In conclusion, the epigenetic modifier TSA does not seem to exert a positive effect on the expression and activity of the investigated uptake transporters in primary rat hepatocyte cultures.

Keywords: epigenetics, Trichostatin A, drug transporters, primary hepatocyte culture

Abbreviations: ABC, ATP binding cassette; B2m, beta-2 microglobulin; BSA, bovine serum albumin; DNA, complementary deoxyribonucleic acid; DNMT, DNA methyltransferase; DMSO, dimethylsulfoxide; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; Hnf-1 α , hepatocyte nuclear factor-1 α ; Hmbs, hydroxymethylbilane synthase; (m)RNA, (messenger)ribonucleic acid; Ntcp, sodium-dependent taurocholate cotransporting polypeptide; Oatp4, organic anion transporting polypeptide 4; PBS, phosphate buffered saline; RT-qPCR, real-time quantitative reverse transcriptase polymerase chain reaction; SLC, solute carrier; TBS-T, Tris-buffered saline solution; TSA, Trichostatin A; Ubc, ubiquitin C.

INTRODUCTION

Over the years, numerous animal studies have been carried out to determine the pharmacotoxicological properties of new chemical entities. However, their use has been strongly criticized and with the introduction of the 3Rs concept of Russell and Burch in

1959, more attention has been paid to the establishment of alternative *in vitro* models (EMA, 2011, 1997; Flecknell, 2002; May et al., 2009). Among the variety of liver-based *in vitro* models available today, cultures of primary hepatocytes are generally considered as the gold standard. Indeed, in these cells, the entire spectrum of biotransformation en-

zymes and drug transporters involved in hepatic drug clearance is expressed at an *in vivo*-like level, rendering this *in vitro* setting an excellent tool to evaluate drug metabolism and to predict drug-induced liver toxicity (Hewitt et al., 2007; Ramboer et al., 2013; Ulvestad et al., 2011; Vinken et al., 2012). However, primary hepatocyte cultures cope with dedifferentiation, which also negatively affects drug transporter expression (Jigorel et al., 2005; Luttringer et al., 2002; Rippin et al., 2001). Accordingly, considerable focus has been put on the development of strategies to counteract this deteriorative process, in particular by mimicking the *in vivo* hepatic micro-environment (Hewitt et al., 2007; Papeleu et al., 2002). Cultivating rat hepatocytes between 2 layers of gelled collagen (*i.e.* sandwich configuration) has been reported to lead to a better retention of drug transporter expression (Liu et al., 1998) and support the reestablishment of the canalicular network and proper localization of canalicular transporters, making this *in vitro* model often the reference system to study hepatobiliary transport (Ramboer et al., 2013). However, in this *in vitro* model the expression and functionality of the drug uptake transporters are usually still considerably decreased in function of culture time (Jacobsen et al., 2011; Jørgensen et al., 2007; Kotani et al., 2011; Tchapanian et al., 2011). Since these conventional strategies do not tackle the actual causes of dedifferentiation, new approaches that affect dedifferentiation at the most upstream level of its regulation could thereby more effectively impact this process. In this respect, epigenetic modification of hepatocellular gene expression by histone deacetylase (HDAC) inhibitors, with Trichostatin A (TSA) as a prototype, can strongly favour the differentiated phenotype in primary rat hepatocyte cultures, including boosting of functional expression of phase I biotransformation enzymes (Henkens et al., 2007). Yet, no information regarding the effect of HDAC inhibition on drug transporter expression and functionality in primary hepatocyte cultures is available. Indeed, re-

ports on the epigenetic regulation of drug transporter proteins are limited and mainly restricted to cancer research (Klaassen et al., 2011). Nevertheless, it has been repeatedly demonstrated that in mice, epigenetic mechanisms, namely DNA methylation and/ or histone acetylation, are involved in liver-specific expression of drug transporters (Douet et al., 2007; Imai et al., 2009, 2013). The latter are specialized transmembrane proteins that belong either to the solute carrier (SLC) superfamily or to the ATP binding cassette (ABC) superfamily (Li et al., 2009; Russel, 2010; Shugarts and Benet, 2009; Thompson, 2011; Zhang et al., 2006). SLC superfamily members, such as the organic anion transporting polypeptide 4 (Oatp4) and the sodium taurocholate cotransporting polypeptide (Ntcp), as well as their ABC counterparts, are frequently involved in drug-drug interactions and drug-induced toxicities (Giacomini et al., 2010; Kato et al., 2002; Thompson, 2011; Zhang et al., 2006). Hence, drug transporter research is nowadays routinely implemented in safety and efficacy assessment during the drug development process. This research area, at least in part, typically relies on *in vitro* experimentation (Ramboer et al., 2013). The current study was set up to investigate whether epigenetic modification also affects the expression and functionality of drug uptake transporters *in vitro* and the TSA-optimized primary hepatocyte cultures could represent a user-friendly alternative for the labour-intensive “sandwich” cultures for transporter research.

MATERIALS AND METHODS

Chemicals and reagents

TSA was supplied by Errant Gene Therapeutics (United States of America). The radiolabeled compounds ([³H] taurocholic acid (specific activity 5 Ci/mmol), [³H] estrone-3-sulfate (specific activity 45.6 Ci/mmol), scintillation vials and scintillation fluid (Ultima Gold MV) were purchased from Perkin Elmer (Belgium). All other chemicals were commercially available products of analyti-

cal grade and were obtained from Sigma (Belgium), unless specified otherwise. Probenecid and TSA were dissolved in dimethylsulfoxide (DMSO), with final DMSO concentrations not exceeding 1 % v/v.

Hepatocyte isolation and cultivation

Procedures for the housing of rats as well as for the isolation and cultivation of hepatocytes were approved by the local Ethical Committee of the Vrije Universiteit Brussel (Belgium). Male outbred Sprague-Dawley rats, weighing 250-275 g (Charles River Laboratories, France) were kept under controlled environmental conditions with free access to food and water. Hepatocytes were isolated by use of a 2-step collagenase method (Papeleu et al., 2006). Viable ($\geq 85\%$) hepatocytes were seeded on a plastic surface at a density of 0.57×10^5 cells/cm² (6-well plates - 9.6 cm²) or 0.82×10^5 cells/cm² (6 cm dish - 19.5 cm²) in William's medium E (Invitrogen, Belgium) supplemented with 7 ng/ml glucagon, 292 mg/ml L-glutamine, antibiotics (7.33 I.E./ml sodium benzyl penicillin, 50 µg/ml kanamycin monosulphate, 10 µg/ml sodium ampicillin, 50 µg/ml streptomycin sulphate) and 10 % v/v fetal bovine serum (Gibco, Belgium). Cell culture plates were placed in an incubator (37 °C, 5 % CO₂) and after 4 hours, cell culture media were removed and replaced by serum-free medium supplemented with 25 µg/ml hydrocortisone sodium hemisuccinate and 0.5 µg/ml insulin. All hepatocyte cultures were maintained in an incubator (37 °C, 5 % CO₂) and cell culture media were replaced daily. The cultivation conditions used were as follows: monolayer cultures either exposed to (i) 25 µM TSA or (ii) 0.083 % v/v DMSO (solvent control), or (iii) untreated monolayer cultures.

Samples were taken 4 hours after plating (T4) and on day 4 (D4) of the cultivation period.

Real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) analysis

Cells were harvested by scraping, washed with ice-cold phosphate buffered saline (PBS) and pelleted. Total cellular ribonucleic acid (RNA) extraction, complementary deoxyribonucleic acid (cDNA) production and cDNA purification were carried out as outlined elsewhere (De Kock et al., 2012). The RT-qPCR reaction mix and RT-qPCR conditions, using the StepOnePlus system (Applied Biosystems, Belgium), were established according to the manufacturer's instructions (Applied Biosystems, Belgium). Gene mixes were purchased from Applied Biosystems (Table 1). Selection of reliable housekeeping genes for normalization of the RT-qPCR data was done using qbasePLUS software (Biogazelle, Belgium), whereby beta-2 microglobulin (B2m), ubiquitin C (Ubc), hydroxymethylbilane synthase (Hmbs) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were selected as the most stable housekeeping genes (results not shown). The results were processed according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and relative messenger RNA (mRNA) expression levels of each drug transporter were expressed as fold changes normalized against the geometric means of all 4 housekeeping gene mRNAs and scaled against the mRNA expression level of T4 untreated monolayer cultures, arbitrarily set at 100 %.

Table 1: Gene expression assays used for qRT-PCR

Gene	Assay ID	Accession number
Ntcp (<i>Slc10a1</i>)	Rn00566894_m1	NM_017047.1
Oatp4 (<i>Slco1b2</i>)	Rn01492634_m1	NM_031650.2
Gapdh	Rn01775763_g1	NM_017008.3
Ubc	Rn01789812_g1	NM_017314.1
Hmbs	Rn00565886_m1	NM_013168.2
B2m	Rn00560865_m1	NM_012512.2

Western blot analysis

Cells were harvested by scraping, washed with ice-cold PBS and pelleted. Total cellular protein extraction was performed as described elsewhere (Vinken et al., 2011) and quantification was carried out according to the Bradford procedure using bovine serum albumin (BSA) as a standard (Bradford, 1976). Proteins (50 µg) were heated, fractionated on sodium dodecyl sulphate polyacrylamide (7.5 % or 10 % w/v) and blotted afterwards onto nitrocellulose membranes (Amersham, United Kingdom) (Ntcp) or polyvinylidene difluoride membranes (Bio-Rad, Germany) (Oatp4). For Ntcp the subsequent steps were performed using the Super-signal Western Blot Enhancer kit (Pierce, Belgium). Membranes were blocked with 5 % w/v non-fatty milk in Tris-buffered saline solution (20 mM tris(hydroxymethyl) amino, 135 mM NaCl) containing 0.1 % v/v Tween 20 (TBS-T). Membranes were incubated overnight at 4 °C with primary antibodies directed against a specific drug transporter (Table 2), followed by incubation for 1 hour at room temperature with a horseradish peroxidase-conjugated secondary antibody (Dako, Denmark). Excess antibody was removed by washing the membranes several times with TBS-T. Detection of the proteins was carried out by means of an enhanced chemiluminescence Western blotting system (Pierce, Belgium). For semi-quantification of the results, blots were further incubated with a primary antibody against hepatocyte nuclear factor-1 α (Hnf-1 α) (Table 2), which was previously identified as a robust housekeeping protein in primary rat hepatocyte cultures (Henkens et al., 2007). Blots were scanned and densitometric analyses were performed by using the Quantity One software (Bio-Rad, Germany). Transporter signals were normalized against the corresponding Hnf-1 α signals and were expressed as percentage of the normalized transporter signals in T4 untreated monolayer cultures, arbitrarily set at 100 %.

Sinusoidal uptake assay

Cell culture medium was removed and cells were washed twice and incubated for 10 minutes with transporter assay buffer at 37 °C [5.3 mM KCl, 1.1 mM KH₂PO₄, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 11 mM D-glucose, 10 mM HEPES, and 136 mM N-methyl glucamine (sodium-free buffer) or 136 mM NaCl (sodium-containing buffer)] (Jigorel et al., 2005). To study the activity of Ntcp and Oatp4, cells were incubated with transporter uptake buffer at 37 °C supplemented with the radiolabeled substrates (1 µCi/ml) [3H] taurocholic acid (0.2 µM) (Ntcp) and [3H] estrone-3-sulfate (0.02 µM) (Oatp4) either in the presence or absence of their inhibitors, namely sodium ions (Ntcp) and probenecid (1 mM) (Oatp4), respectively. Uptake was stopped by addition of ice-cold PBS containing 0.2 % w/v BSA (Poirier et al., 2008). After a final washing step with ice-cold PBS, cells were lysed with mammalian protein extraction reagent (Pierce, Belgium) and the intracellular accumulation of the radiolabeled substrate was measured using liquid scintillation counting (Tri-Carb, Perkin Elmer, Belgium). The total protein content from each well was determined by means of the Bradford method (Bradford, 1976) with BSA as a standard and was used to normalize uptake activity. Passive transport was determined by performing experiments at 4 °C using cold substrate solutions (Poirier et al., 2008). The difference in intracellular radioactive accumulation in the absence and presence of the respective inhibitors was considered to represent the transporter activity (Jigorel et al., 2005).

Statistical analysis

Data are expressed as mean \pm standard deviation of at least 3 independent experiments. Results were evaluated using a one-tailed or 2-tailed paired Student's *t*-test with level of significance $p \leq 0.05$. In order to determine changes in function of the cultivation time, the expression and activity of

Table 2: Primary antibodies used for Western blot analysis

Antigen	Supplier	Reference	Species	Type	Dilution
Ntcp	Santa Cruz	sc-107032	Goat	Polyclonal	1/1500
Oatp4	Santa Cruz	sc-134461	Rabbit	Polyclonal	1/2500
Hnf-1 α	Santa Cruz	sc-10791	Rabbit	Polyclonal	1/1000

transporters in T4 and D4 untreated monolayer cultures were analyzed. The effect related to the addition of DMSO or TSA to the culture medium was studied at each timepoint by comparing the results obtained in solvens control with untreated monolayers or TSA-treated hepatocytes with solvens control, respectively.

RESULTS

The effect of TSA on the mRNA expression of drug uptake transporters in primary rat hepatocyte cultures

Previous research showed that in primary rat hepatocyte cultures 25 μ M TSA positively affects the maintenance of cytochrome P450-mediated biotransformation capacity and the longevity of the cells (Henkens et al., 2007). Because of these results, the same *in vitro* system was used to investigate whether this higher functionality is also associated with effects on drug uptake transporters. As such freshly isolated rat hepatocytes were cultured in a conventional monolayer configuration either in the absence or presence of 25 μ M TSA for 4 days. Since DMSO (*i.e.* the TSA solvent) is known to favour the maintenance of the differentiated phenotype of primary hepatocytes (Isom et al., 1985), a number of cultures were separately treated with 0.083 % v/v DMSO, being the concentration used as TSA solvent. RT-qPCR analysis was performed on T4 and D4 hepatocyte samples using B2m, Gapdh, Hmbs and Ubc as a combination of housekeeping genes for normalization (Figure 1). The mRNA levels of the uptake transporters Ntcp ($p=0.037$) and Oatp4 ($p=0.015$) significantly dropped as a function of the cultivation time in untreated hepatocytes. Furthermore, no clear effect of TSA or DMSO could be seen at

both timepoints for the investigated transporters.

The effect of TSA on the protein expression of drug uptake transporters in primary rat hepatocyte cultures

In order to investigate whether the findings at the mRNA level are translated into similar protein modifications, semi-quantitative immunoblot analyses were performed on whole cell lysates, using Hnf-1 α as a housekeeping protein (Figures 2a and b). The significant downregulation in Ntcp ($p=0.023$) and Oatp4 ($p=0.031$) expression as a function of the cultivation time, observed by RT-qPCR analysis, was confirmed by immunoblot analysis and no significant effects of TSA or DMSO could be seen at both timepoints for the investigated transporters.

The effect of TSA on the activity of drug uptake transporters in primary rat hepatocyte cultures

To provide a complete picture of potential effects of TSA on uptake transporters in primary hepatocyte cultures, a number of activity assays were performed. In this respect, intracellular accumulation of radiolabelled substrates of Oatp4 ([³H] estrone-3-sulfate) and Ntcp ([³H] taurocholic acid) was assessed, both in the presence and absence of drug inhibitors, probenidic and sodium chloride, respectively. Results are presented as uptake of 1 μ Ci/ml [³H] taurocholic acid and [³H] estrone-3-sulfate after 90 seconds incubation. The uptake of both substrates at that specific concentration was shown to be linear over this time range (supplementary Figures 1 and 2).

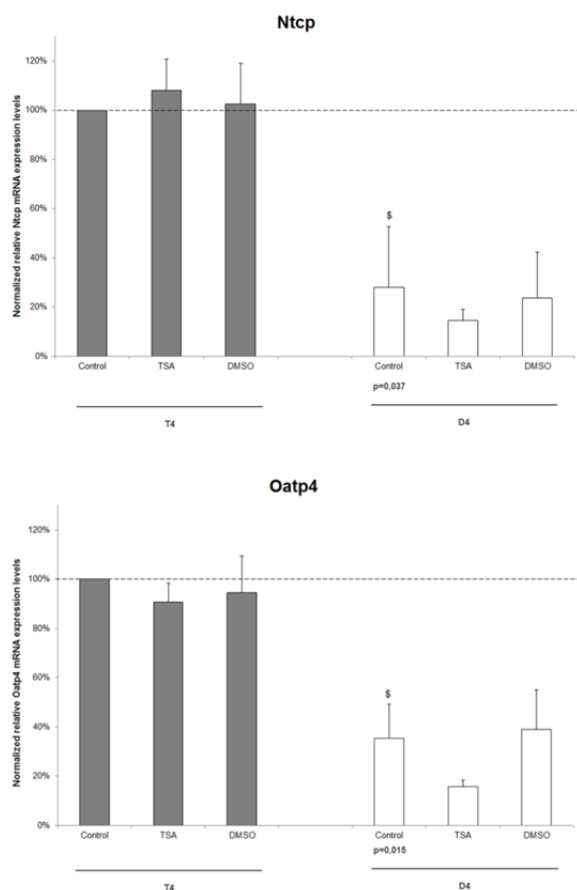


Figure 1: The effect of TSA on the mRNA expression level of drug uptake transporters in primary rat hepatocyte cultures. Freshly isolated rat hepatocytes were cultivated in 6 cm diameter dishes (19.5 cm²) as described in “2.2 Hepatocyte isolation and cultivation”. Hepatocyte samples were taken 4 hours after cell plating (T4) and at day 4 of the cultivation period (D4). Samples were subjected to RT-qPCR analysis, using B2m, Gapdh, Hmbs, Ubc as a combination of house-keeping genes for normalization. Data are expressed as mean \pm standard deviation of 3 independent experiments and normalized to the mRNA level of T4 untreated hepatocytes (arbitrarily set at 100 %, indicated with a dotted line). Statistical analyses were performed using a 2-tailed paired Student’s *t*-test with *p* value \leq 0.05 considered to be significantly different. ^s*p* \leq 0.05 when D4 untreated hepatocytes are compared with T4 counterparts. Samples collected 4 hours after cell plating are indicated with a gray bar and 4-day old cultured hepatocytes with a white bar. (D4, day 4 cultures; DMSO, dimethylsulfoxide; Ntcp, sodium taurocholate cotransporting polypeptide; Oatp4, organic anion transporting polypeptide 4; T4, 4 hours cultures; TSA, Trichostatin A)

The intracellular accumulation of the radioactive substrates underwent a clear down-regulation in the presence of the respective inhibitors, indicating the proper functioning of Ntcp and Oatp4 in the 3 cultivation condi-

tions at both timepoints (Figure 3a). However, the smaller inhibitory effect observed in D4 cultures compared to T4 cultures suggests reduced transporter functionality at this timepoint. Indeed, Ntcp and Oatp4 activity, represented by the difference in accumulation in the absence and presence of the corresponding inhibitor, was significantly decreased as a function of cultivation time. No effects related to the use of DMSO were seen at both timepoints. Only in T4 cultures, lower Oatp4 functionality (*p*=0.029) was observed in TSA-treated versus DMSO-treated hepatocytes (Figure 3b).

DISCUSSION

The current study was set up to investigate whether TSA, a HDAC inhibitor earlier shown in our laboratory to maintain cytochrome P450-mediated biotransformation competence in primary cultured rat hepatocytes (Henkens et al., 2007), also affects drug transporter-mediated phase 0 metabolism. Focus was hereby put on the sinusoidal uptake transporters Ntcp and Oatp4. When seeded in a conventional monolayer configuration, canalicular drug transporter mRNA levels in primary cultured rat hepatocytes are usually better maintained in comparison with their basolateral counterparts (Jigorel et al., 2005; Luttringer et al., 2002; Rippin et al., 2001). In analogy with previous reports, downregulated mRNA expressions were observed for Ntcp and Oatp4 as a function of the cultivation time. Regarding TSA, no significant differences could be noticed in transporter mRNA quantities of TSA-treated and DMSO-treated monolayer cultures. Consequently, TSA does not seem to affect hepatic drug transporter gene expression, at least not in this type of *in vitro* system.

Protein expression of drug transporters in conventional monolayer cultures has in general not been extensively investigated. In this study, the detected translational modulations comply with the observed variations in mRNA expression levels. As previously described (Liu et al., 1998; Rippin et al., 2001),

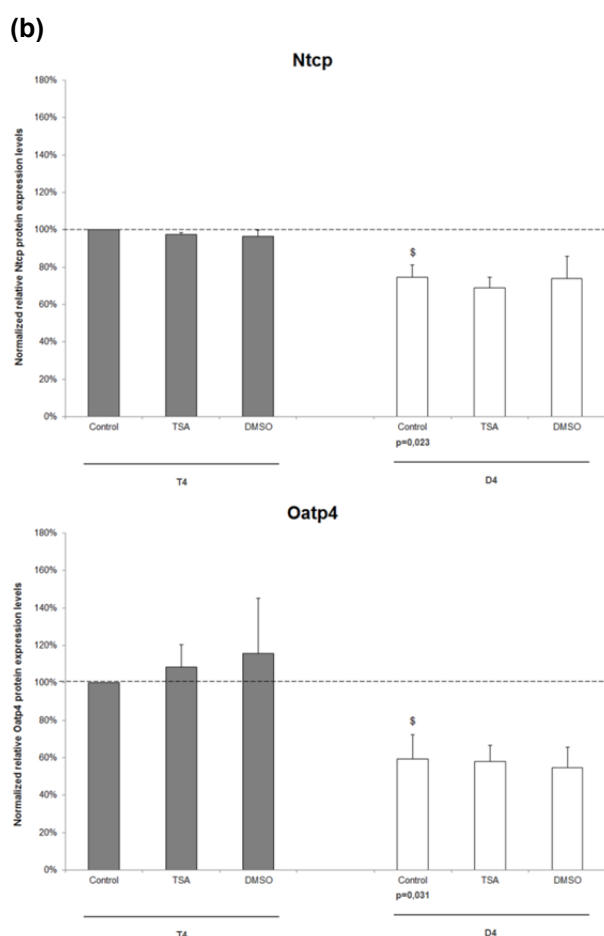
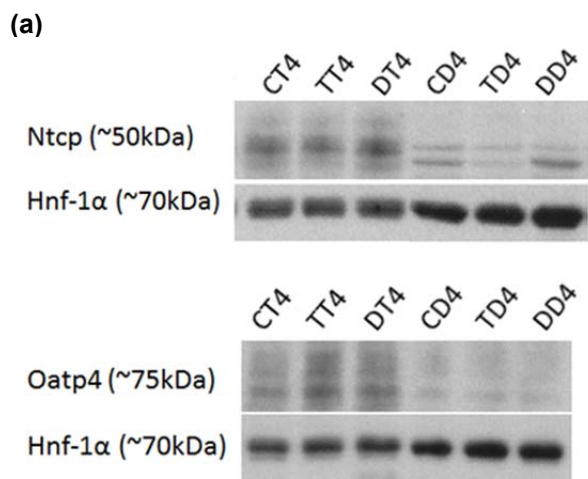


Figure 2: The effect of TSA on the protein expression levels of drug uptake transporters in primary rat hepatocyte cultures. Freshly isolated rat hepatocytes were cultivated in 6 cm diameter dishes (19.5 cm²) as described in “2.2 Hepatocyte isolation and cultivation”. **(a)** Samples were taken 4 hours after cell plating (T4) and at day 4 of the cultivation period (D4) and subjected to Western blot analysis. Results of 3 independent experiments are shown. **(b)** Densitometric analysis of the expression of drug transporters. Transporter signals were normalized against the corresponding Hnf-1 α signals and were expressed as a percentage of the normalized transporter signals in T4 untreated hepatocytes

(arbitrarily set at 100 %, indicated with a dotted line). The results are shown as mean \pm standard deviation of 3 independent experiments. Statistical analyses were performed using a 2-tailed paired Student's *t*-test with *p* values \leq 0.05 considered to be significantly different. ^{\$}*p* \leq 0.05 when D4 untreated hepatocytes are compared with T4 counterparts. Samples collected 4 hours after cell plating are indicated with a gray bar and 4-day old cultured hepatocytes with a white bar. (CD4, untreated hepatocytes D4; CT4, untreated hepatocytes T4; D4, day 4 cultures; DD4, DMSO-treated hepatocytes D4; DMSO, dimethylsulfoxide; DT4, DMSO-treated hepatocytes T4; Ntcp, sodium taurocholate cotransporting polypeptide; Oatp4, organic anion transporting polypeptide 4; T4, 4 hours cultures; TD4, TSA-treated hepatocytes D4; TSA, Trichostatin A; TT4, TSA-treated hepatocytes T4)

Ntcp protein expression levels significantly decrease as a function of the cultivation time. A similar regulation was observed for Oatp4. To the best of our knowledge, no other reports on Oatp4 protein expression in conventional monolayer cultures have yet been published. In line with other studies (Jigorel et al., 2005; Liu et al., 1998; Rippin et al., 2001), the activities of Ntcp and Oatp4 strongly declined with cultivation time. In general, DMSO had no effect on the expression and activity of the drug uptake transporters studied. Nevertheless, positive effects of DMSO on hepatocellular Ntcp and Oatp drug transporters activity in primary hepatocyte cultures have already been described by others (Jigorel et al., 2005). It should be mentioned that in the current study, DMSO was used as the solvent for TSA and was kept as low as possible (0.083 % v/v). In the study of Isom and colleagues DMSO was present in much higher concentrations (2 % v/v) in order to reach an optimal maintenance of the hepatocyte differentiated status (Isom et al., 1985).

In conclusion, the epigenetic modifier TSA does not seem to exert an effect on the expression and activity of drug uptake transporters in primary rat hepatocyte cultures although it clearly affected the metabolizing capacity (Henkens et al., 2007). Since it has been shown that DNA methylation is involved in the liver-specific expression of the investigated drug transporters (Imai et al.,

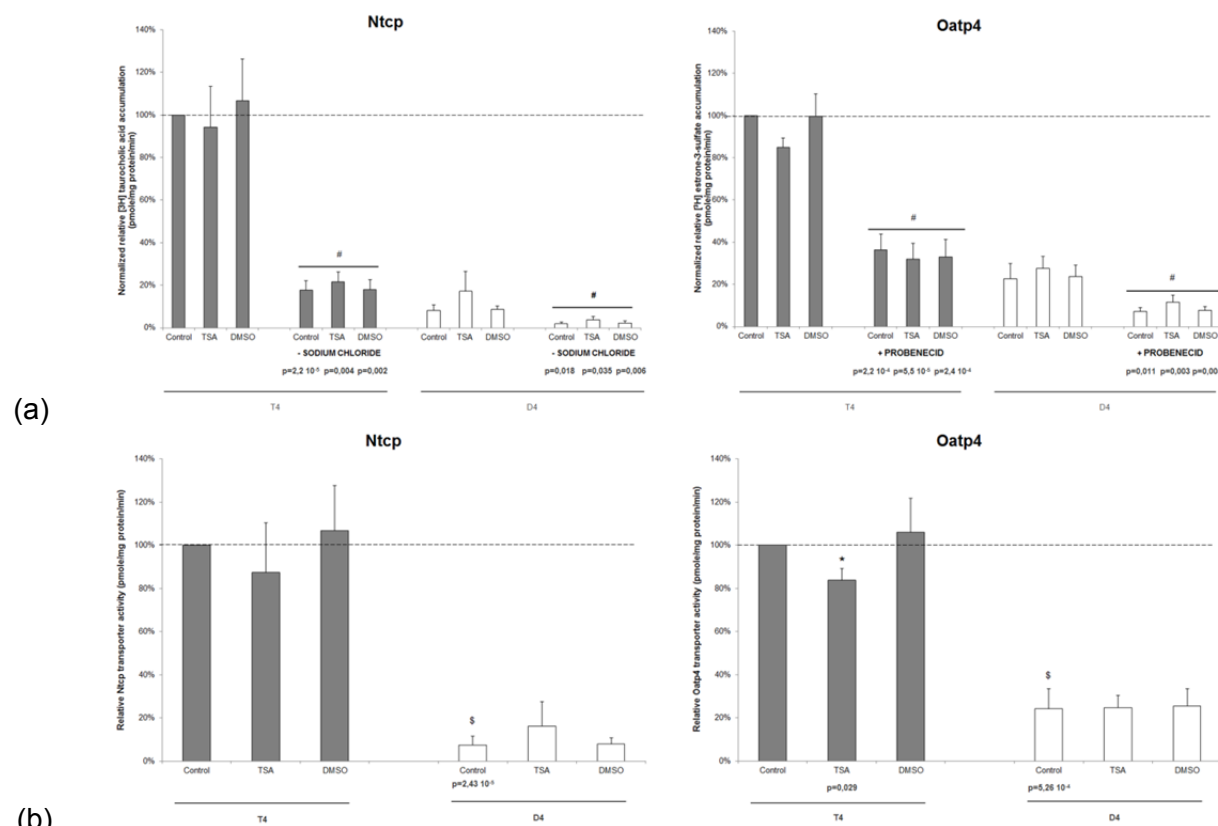


Figure 3: The effect of TSA on drug uptake transporter activity in primary rat hepatocyte cultures. Freshly isolated rat hepatocytes were cultivated in 6-well plates (9.6 cm²) as described in “2.2 Hepatocyte isolation and cultivation”. Hepatocyte cultures were incubated for 90 seconds with 1 μCi/ml radioactive substrate [³H] taurocholic acid (0.2 μM) (Ntcp) and [³H] estrone-3-sulfate (0.02 μM) (Oatp4) either in the presence or absence of their inhibitors, namely sodium ions (136 mM) (Ntcp) and probenecid (1 mM) (Oatp4), respectively. **(a)** Representation of intracellular accumulation in different experimental settings. The results were expressed as mean ± standard deviation of 4 independent experiments and normalized for the intracellular accumulation measured in the untreated monolayer cultures in the absence of the respective inhibitor at T4 of cultivation (arbitrarily set at 100 %, indicated with a dotted line). Statistical analyses were performed using a one-tailed paired Student’s t-test with p values ≤ 0.05 considered to be significantly different. #p<0.05, when compared to substrate accumulation in the absence of an inhibitor **(b)** Transporter activity, equivalent to substrate uptake in the absence of the respective inhibitor minus the uptake in the presence of the inhibitor, is determined at T4 and D4 of cultivation for Ntcp and Oatp4. These results were expressed as mean ± standard deviation of 4 independent experiments with the transporter activity of untreated monolayers 4 hours after cell plating arbitrarily set at 100 %. Statistical analyses were performed using a 2-tailed paired Student’s t-test with p values ≤ 0.05 considered to be significantly different. \$p<0.05, when D4 transporter activity is compared with functionality in T4 untreated monolayer cultures; *p<0.05, when TSA treated hepatocytes are compared with solvents control cultures. Samples collected 4 hours after cell plating are indicated with a gray bar and 4-day old cultured hepatocytes with a white bar. ([³H], tritium; D4, day 4 cultures; DMSO, dimethylsulfoxide; Ntcp, sodium taurocholate cotransporting polypeptide; Oatp4, organic anion transporting polypeptide 4; T4, 4 hours cultures; TSA, Trichostatin A)

2009; Imai et al., 2013), it could be interesting to study the effect of DNA methyltransferase (DNMT) as alternative epigenetic modulatory mechanism to improve drug transporter expression and activity *in vitro*. Furthermore, using a combination of epigenetic modulators, namely HDACi and DNMT, could also be explored as it has already been demonstrated to have a synergetic positive effect on the albumin secretory capacity of primary rat hepatocyte cultures (Fraczek et al., 2012).

Acknowledgements

This work was financially supported by the grants of the Fund for Scientific Research-Flanders-Belgium (FWO-Vlaanderen) and the Vrije Universiteit Brussel (OZR-VUB). The authors are grateful to Ms. Tinke Vanhalewyn and Mr. Paul Claes for their dedicated technical assistance.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-54.

De Kock J, Najar M, Bolleyn J, Al Battah F, Rodrigues RM, Buyl K, et al. Mesoderm-derived stem cells: the link between the transcriptome and their differentiation potential. *Stem Cells Dev.* 2012;21:3309-23.

Douet V, Heller MB, Le Saux O. DNA methylation and Sp1 binding determine the tissue-specific transcriptional activity of the mouse *Abcc6* promoter. *Biochem Biophys Res Commun.* 2007;354:66-71.

EMA. Position adopted by the CPMP on 19 February 1997 on replacement of animal studies by *in vitro* methods (CPMP/SWP/728/95). 1997.

EMA. Concept paper on the need for revision of the position on replacement of animal studies by *in vitro* methods (CPMP/SWP/728/95). 2011.

Flecknell P. Replacement, reduction and refinement. *ALTEX.* 2002;19:73-8.

Fraczek JE, Vinken M, Tourwé D, Vanhaecke T, Rogiers V. Synergetic effects of DNA demethylation and histone deacetylase inhibition in primary rat hepatocytes. *Invest New Drugs.* 2012;30:1715-24.

Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, et al. Membrane transporters in drug development. *Nat Rev Drug Discov.* 2010;9:215-36.

Henkens T, Papeleu P, Elaut G, Vinken M, Rogiers V, Vanhaecke T. Trichostatin A, a critical factor in maintaining the functional differentiation of primary cultured rat hepatocytes. *Toxicol Appl Pharmacol.* 2007;218:64-71.

Hewitt NJ, Lechón MJ, Houston JB, Hallifax D, Brown HS, Maurel P, et al. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev.* 2007;39:159-234.

Imai S, Kikuchi R, Kusuhara H, Yagi S, Shiota K, Sugiyama Y. Analysis of DNA methylation and histone modification profiles of liver-specific transporters. *Mol Pharmacol.* 2009;75:568-76.

Imai S, Kikuchi R, Kusuhara H, Sugiyama Y. DNA methylation and histone modification profiles of mouse organic anion transporting polypeptides. *Drug Metab Dispos.* 2013;41:72-8.

Isom HC, Secott T, Georgoff I, Woodworth C, Mummaw J. Maintenance of differentiated rat hepatocytes in primary culture. *Proc Natl Acad Sci USA.* 1985;82:3252-6.

Jacobsen JK, Jensen B, Skonberg C, Hansen SH, Badolo L. Time-course activities of Oct1, Mrp3, and cytochrome P450s in cultures of cryopreserved rat hepatocytes. *Eur J Pharm Sci.* 2011;44:427-36.

Jigorel E, Le Vee M, Boursier-Neyret C, Bertrand M, Fardel O. Functional expression of sinusoidal drug transporters in primary human and rat hepatocytes. *Drug Metab Dispos.* 2005;33:1418-22.

Jørgensen L, Van Beek J, Lund S, Schousboe A, Badolo L. Evidence of Oatp and Mdr1 in cryopreserved rat hepatocytes. *Eur J Pharm Sci.* 2007;30:181-9.

Kato Y, Suzuki H, Sugiyama Y. Toxicological implications of hepatobiliary transporters. *Toxicology.* 2002;181-182:287-90.

- Klaassen CD, Lu H, Cui JY. Epigenetic regulation of drug processing genes. *Toxicol Mech Methods*. 2011; 21:312-24.
- Kotani N, Maeda K, Watanabe T, Hiramatsu M, Gong LK, Bi YA, et al. Culture period-dependent changes in the uptake of transporter substrates in sandwich-cultured rat and human hepatocytes. *Drug Metab Dispos*. 2011;39:1503-10.
- Li P, Wang GJ, Robertson TA, Roberts MS. Liver transporters in hepatic drug disposition: an update. *Curr Drug Metab*. 2009;10:482-98.
- Liu X, Brouwer KL, Gan LS, Brouwer KR, Stieger B, Meier PJ, et al. Partial maintenance of taurocholate uptake by adult rat hepatocytes cultured in a collagen sandwich configuration. *Pharm Res*. 1998;15:1533-9.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25: 402-8.
- Luttringer O, Theil FP, Lavé T, Wernli-Kuratli K, Guentert TW, de Saizieu A. Influence of isolation procedure, extracellular matrix and dexamethasone on the regulation of membrane transporters gene expression in rat hepatocytes. *Biochem Pharmacol*. 2002;64:1637-50.
- May JE, Xu J, Morse HR, Avent ND, Donaldson C. Toxicity testing: the search for an in vitro alternative to animal testing. *Br J Biomed Sci*. 2009;66:160-5.
- Papeleu P, Elaut G, Rogiers V, Vanhaecke T. Cell cultures as in vitro models for biotransformation studies. In: Pandalia SG (ed). *Recent research developments in drug metabolism and disposition* (pp 199-234). Kerala, India: Transworld Research Network, 2002.
- Papeleu P, Vanhaecke T, Henkens T, Elaut G, Vinken M, Snykers S, et al. Isolation of rat hepatocytes. *Methods Mol Biol*. 2006;320:229-37.
- Poirier A, Lavé T, Portmann R, Brun ME, Senner F, Kansy M, et al. Design, data analysis, and simulation of in vitro drug transport kinetic experiments using a mechanistic in vitro model. *Drug Metab Dispos*. 2008;36:2434-44.
- Ramboer E, Vanhaecke T, Rogiers V, Vinken M. Primary hepatocyte cultures as prominent in vitro tools to study hepatic drug transporters. *Drug Metab Rev*. 2013;45:196-217.
- Rippin SJ, Hagenbuch B, Meier PJ, Stieger B. Cholestatic expression pattern of sinusoidal and canalicular organic anion transport systems in primary cultured rat hepatocytes. *Hepatology*. 2001;33:776-82.
- Russel FGM. Transporters: importance in drug absorption, distribution, and removal. In: Pang KS (ed). *Enzyme- and transporter-based drug-drug interactions, progress and future challenges* (pp 27-49). New York: Springer, 2010.
- Russell WMS, Burch RL. *The principles of human experimental technique*. London: Methuen and Co., 1959.
- Shugarts S, Benet LZ. The role of transporters in the pharmacokinetics of orally administered drugs. *Pharm Res*. 2009;26:2039-54.
- Tchapanian EH, Houghton JS, Uyeda C, Grillo MP, Jin L. Effect of culture time on the basal expression levels of drug transporters in sandwich-cultured primary rat hepatocytes. *Drug Metab Dispos*. 2011; 39:2387-94.
- Thompson TN. The clinical significance of drug transporters in drug disposition and drug interactions. In: Howard PB (ed). *Pharmacokinetics in drug development* (pp 285-313). New York: Springer, 2011.
- Ulvestad M, Björquist P, Molden E, Asberg A, Andersson TB. OATP1B1/1B3 activity in plated primary human hepatocytes over time in culture. *Biochem Pharmacol*. 2011;82:1219-26.
- Vinken M, Decrock E, Doktorova T, Ramboer E, De Vuyst E, Vanhaecke T, et al. Characterization of spontaneous cell death in monolayer cultures of primary hepatocytes. *Arch Toxicol*. 2011;85:1589-96.
- Vinken M, Vanhaecke T, Rogiers V. Primary hepatocyte cultures as in vitro tools for toxicity testing: quo vadis? *Toxicol In Vitro*. 2012;26:541-4.
- Zhang L, Strong JM, Qiu W, Lesko LJ, Huang SM. Scientific perspectives on drug transporters and their role in drug interactions. *Mol Pharm*. 2006;3:62-9.