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Abstract

Cigarette smoke condensate (CSC) has previously been shown to impair activity and expression of hepatic drug transporters. In the present study, we provided evidence that CSC also hinders activity of organic anion transporters (OATs), notably expressed at the kidney level. CSC thus *cis*-inhibited OAT substrate uptake in OAT1- and OAT3-transfected HEK293 cells, in a concentration-dependent manner (IC_{50}=72.1 µg/mL for OAT1 inhibition and IC_{50}=27.3 µg/mL for OAT3 inhibition). By contrast, OAT4 as well as the renal organic cation transporter (OCT) 2 were less sensitive to the inhibitory effect of CSC (IC_{50}=351.5 µg/mL and IC_{50}=226.2 µg/mL, for inhibition of OAT4 and OCT2, respectively). OAT3 activity was further demonstrated to be blocked by some single chemicals present in cigarette smoke such as the heterocyclic amines AαC (IC_{50}=11.3 µM) and PhIP (IC_{50}=1.9 µM), whereas other major cigarette smoke components used at 100 µM, like nicotine, the nitrosamine NNK and the polycyclic aromatic hydrocarbons benzo(a)pyrene and phenanthrene, were without effect. AαC and PhIP however failed to *trans*-stimulate activity of OAT3, suggesting that they were not substrates for this transporter. Taken together, these data establish OAT1 and OAT3 transporters as targets of cigarette smoke chemicals, which may contribute to smoking-associated pharmacokinetics alterations.

Key-words: Drug transporter; cigarette smoke; pharmacokinetics; organic anion transporter; heterocyclic amines.

1. Introduction

Cigarette smoke is a complex mixture of more than 5,000 chemicals, including nicotine, polycyclic aromatic hydrocarbons, heterocyclic amines, nitrosamines, aminobiphenyls and metals like cadmium, lead and arsenic (Talhout et al., 2011). It is highly toxic for human health, notably causing cancers, cardiovascular diseases, endocrine disruption and stroke (Ezzati et al., 2005; Sasco et al., 2004; Windham et al., 2005). These well-established and pleomorphic deleterious effects of smoking have been linked to the activation of various cellular and molecular pathways (Sobus and Warren, 2014), including ones related to drug detoxifying proteins. Indeed, cigarette smoke-contained chemicals like polycyclic aromatic hydrocarbons induce expression of the drug metabolizing enzymes cytochrome P-450 (CYP) 1A1 and CYP1B1 in various tissues, through mobilizing the aryl hydrocarbon receptor signaling cascade (Kitamura and Kasai, 2007). CYP1A1/1B1 can in turn bio-activate polycyclic aromatic hydrocarbons into reactive metabolites, that cause DNA adducts and mutation, which ultimately may result in cancers, notably lung cancers (Moorthy et al., 2015; Nebert et al., 2004). Up-regulation of hepatic CYPs, but also of other drug metabolizing enzymes like glutathione S-transferases, by cigarette smoke additionally results in increased metabolism of various drugs, and thereby contributes to impaired pharmacokinetics in smokers (Miller, 1989; Sohn et al., 2015).

In addition to drug metabolizing enzymes, drug transporters, which belong to either the solute carrier (SLC) or the ATP-binding cassette (ABC) transporter superfamilies, are now recognized as major contributors to drug absorption, disposition and elimination (Giacomini et al., 2010). These transporters, mainly expressed in organs/tissues implicated in drug disposition and elimination such as gut, blood-brain barrier, liver and kidney, also constitute targets for various cigarette smoke chemicals. Indeed, some polycyclic aromatic hydrocarbons and polycyclic aromatic hydrocarbon metabolites have been shown to be handled, and/or to

regulate, the ABC transporters breast cancer resistance protein (BCRP/*ABCG2*) and multidrug resistance protein (MRP) 4 (*ABCC4*) (Ebert et al., 2005; Gelhaus et al., 2012). The heterocyclic amine 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine (PhIP) as well as the heavy metal cadmium interact with BCRP activity (Kummu et al., 2012; van Herwaarden et al., 2003). Cadmium is moreover transported by the SLC transporters organic cation transporter (OCT) 1 (*SLC22A1*), OCT2 (*SLC22A2*), multidrug and toxin extrusion protein (MATE) 1 (*SLC47A1*) and MATE2-K (*SLC47A2*) (Soodvilai et al., 2011; Yang et al., 2017). Another heavy metal present in cigarette smoke, *i.e.*, arsenic, is handled by the ABC transporters MRP1 (*ABCC1*) and MRP2 (*ABCC2*) (Leslie, 2012) and regulates their expression (Takeshita et al., 2003; Vernhet et al., 2001).

Interestingly, cigarette smoke condensate (CSC) and cigarette smoke extract as whole mixtures impair activity and/or expression of various ABC and/or SLC transporters (Pan et al., 2009; Sayyed et al., 2016; Takano et al., 2016; van der Deen et al., 2007). CSC notably blocks activity of hepatic transporters handling anionic drugs such as the canalicular ABC transporter MRP2 and the sinusoidal SLC transporters organic anion transporting polypeptide (OATP) 1B1 (*SLCO1B1*) and OATP1B3 (*SLCO1B3*) (Sayyed et al., 2016). Functional activity of MRP1, which handles drugs conjugated to anionic ligands (Cole and Deeley, 2006), has also been shown to be altered in lung epithelial cells exposed to cigarette smoke extract (van der Deen et al., 2007). Whether CSC may additionally block other SLC drug transporters handling anionic drugs, such as organic anion transporter (OAT) 1 (*SLC22A6*) and OAT3 (*SLC22A8*), remains however unknown. This issue is likely important to consider because OAT1 and OAT3, notably located at the basolateral pole of proximal tubule cells and acting as dicarboxylate exchangers, play a major role in tubular secretion of drugs, and by this way, in their renal elimination and pharmacokinetics (Burckhardt, 2012). OAT1 and OAT3 are consequently identified as drug transporters that have to be studied during the

pharmaceutical development of new molecular entities according to a regulatory point of view, notably because inhibition of these transporters can be the cause of drug-drug interactions (DDI) (Giacomini et al., 2010; Maeda and Sugiyama, 2013). The present study was therefore designed to analyze the effects of CSC and various cigarette smoke-contained chemicals on activity of these OATs.

2. Materials and Methods

2.1 Chemicals

CSC was supplied by Murty Pharmaceuticals (Lexington, KY, USA). It was prepared by smoking University of Kentucky's 3R4F standard research cigarettes on a Federal Trade Commission smoke machine (Nagaraj et al., 2006). The amount of smoke particulates, collected on a glass fiber filter, was determined by weight increase of the filter. It corresponds to a mean of 9.5 mg total particular matter/cigarette (Eldridge et al., 2015). CSC was finally prepared by dissolving the collected smoke particulates in dimethyl sulfoxide (DMSO) to yield a 40 mg/mL solution. Probenecid, amitriptyline, verapamil, 6-carboxyfluorescein, nicotine, benzo(a)pyrene, phenanthrene, 4-aminobiphenyl, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), glutarate, sodium arsenate and lead chloride were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France), whereas cadmium chloride was from Merck Millipore (Fontenay-sous-Bois, France). The heterocyclic amines PhIP, 2-amino-9H-pyrido**[**2**,**3-b**]**indole (AαC), 2-amino-3-methyl-9H-pyrido[2**,**3-b]indole (MeAαC) and 3-amino-1-methyl-[5H]-pyrido[4.3-b]indole (Trp-P-2) were from Santa Cruz Biotechnology (Dallas, TX, USA). [6,7-^{3}H(N)]-estrone-3-sulfate (E3S) (specific activity 51.8 Ci/mmol), p-[glycyl-2-^{3}H]-aminohippuric acid (PAH) (specific activity 3.0 Ci/mmol), and [1-^{14}C] tetra-ethylammonium (TEA) (specific activity 3.5 mCi/mmol) were from Perkin-Elmer (Courtaboeuf, France).

2.2 Cell culture

HEK293 cells overexpressing OAT1 (HEK-OAT1 cells), OAT3 (HEK-OAT3 cells), OCT2 (HEK-OCT2 cells) or MATE2-K (HEK-MATE2-K cells), kindly provided by Technologie Servier (Orléans, France) and characterized in previous studies (Bruyere et al., 2017; Chedik et al., 2017), were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Saint Aubin, France) supplemented with 10% (vol/vol) fetal calf serum, 10 IU/mL penicillin, 10 µg/mL streptomycin, 1% nonessential amino acids, and 1 µg/mL insulin. HEK293 cells overexpressing OAT4 (HEK-OAT4 cells) were prepared by transduction of HEK293 cells by lentiviral pLV-EF1-hOAT4-hPGK-GFP vector, as previously described (Jouan et al., 2014). Construction of the lentiviral vector, production of lentivirus supernatants, transduction of HEK293 cells and cloning and initial characterization of HEK-OAT4 cells were performed by Vectalys (Labège, France). HEK-OAT4 cells were next routinely cultured in DMEM medium as described above.

2.3 Transporter activity assays

The effects of CSC and cigarette smoke-contained chemicals on SLC transporter activities were analyzed through determining intracellular accumulation of reference substrates using a well-defined transport medium, as previously reported (Chedik et al., 2017; Sayyed et al., 2016). The transport assay medium consisted of 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM D-glucose, 10 mM HEPES, and 136 mM NaCl; pH was adjusted to 7.4 value, except for the pH-sensitive MATE2-K transport assay for which pH was set at 8.4 (Chedik et al., 2017). Cells were first washed with phosphate-buffered saline and then incubated at 37 °C for 5 min with transport assay buffer containing reference substrates, in the presence or absence of reference transporter inhibitors, CSC or cigarette

smoke chemicals. The used substrates were: 33.3 nM [^{3}H]-PAH (for OAT1 activity), 3.86 nM [^{3}H]-E3S (for OAT3 or OAT4 activities), 10 µM 6-carboxyfluorescein (for OAT1 or OAT3 activities) and 28.6 nM [^{14}C]-TEA (for OCT2 or MATE2-K activities). The used reference inhibitors were: 10 mM probenecid (for OAT1 and OAT3 activities), 2 mM probenecid (for OAT4 activity), 200 µM amitriptyline (for OCT2 activity) and 200 µM verapamil (for MATE2-K activity). Cells were next washed twice with ice-cold phosphate-buffered saline (PBS) and finally lysed in distilled water. Intracellular accumulation of radiolabeled substrates was next measured by scintillation counting of cell lysates with a Beckman LS6500 (Beckman Coulter Inc, Fullerton, CA, USA). Intracellular accumulation of 6-carboxyfluorescein was determined by spectrofluorimetry using a SpectraMax Gemini SX spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA); excitation and emission wavelengths were 492 and 517 nm, respectively. Values of substrate accumulation were then normalized to total protein content, determined by the Bradford method (Bradford, 1976). Data were finally expressed as percentage of substrate accumulation found in control cells not exposed to reference inhibitor, CSC or cigarette smoke chemicals. Percentage of reduction of substrate accumulation was defined as 100% (accumulation in control cells) minus percentage of substrate accumulation in the presence of CSC or cigarette smoke chemical, whereas percentage of stimulation of substrate accumulation corresponded to percentage of substrate accumulation in the presence of CSC or cigarette smoke chemical minus 100 % (accumulation in control cells). Data were also alternatively expressed as percentage of transporter activity found in control cells, arbitrarily set at 100%, according to the following equation:

$$\%\ \text{SLC transporter activity} = \frac{(\text{Accumulation}_{\text{CSC/chemical}} - \text{Accumulation}_{\text{Reference inhibitor}}) \times 100}{\text{Accumulation}_{\text{Control}} - \text{Accumulation}_{\text{Reference inhibitor}}} \quad \text{(A)}$$

where $\text{Accumulation}_{\text{CSC/chemical}}$ corresponds to substrate accumulation in the presence of CSC or cigarette smoke chemical, $\text{Accumulation}_{\text{Control}}$ corresponds to substrate accumulation in

control cells and $Accumulation_{Reference\ inhibitor}$ corresponds to substrate accumulation in the presence of a reference transporter inhibitor.

Half maximal inhibitory concentrations (IC_{50}) of CSC or some cigarette smoke-contained chemicals toward transporter activities were determined from nonlinear regression of concentration-response data based on the four parameter logistic function. They were calculated using GraphPad Prism software (GraphPad Software, La Jolla, CA) using the following equation:

$$A = \frac{100}{1 + 10^{(([I] - LogIC50)x\ Hill\ slope)}} \quad (B)$$

where A is the percentage of transporter activity for a given concentration of CSC or cigarette smoke-contained chemical determined as described in equation (A), [I] is the CSC or cigarette smoke-contained chemical concentration in the medium, and Hill slope is a coefficient describing the steepness of the curve.

2.4 Trans-stimulation assays

Trans-stimulation assays were performed in HEK-OAT1 and HEK-OAT3 cells using the OAT1/OAT3 substrate dicarboxylate glutarate as a reference trans-stimulating agent (Apiwattanakul et al., 1999; Sweet et al., 2003). Briefly, HEK-OAT1 and HEK-OAT3 cells were first incubated with 1 mM glutarate, 80 or 320 µg/mL CSC or 100 µM cigarette smoke chemical for 15 min at 37°C. After washing with PBS, cells were next re-incubated with the OAT1/OAT3 substrate 6-carboxyfluorescein for 5 min at 37°C in the transport assay medium. Intracellular accumulation of the fluorescent dye was finally determined as reported above. Data were expressed as percentages of 6-carboxyfluorescein accumulation found in control cells not exposed to glutarate, CSC or cigarette smoke chemicals.

2.5 Chemical combination assays

The combined effects of the heterocyclic amines PhIP and AαC on OAT3 activity were evaluated by median drug effect analysis, as previously described (Chou, 2006). Briefly, the inhibitory effects of combinations of PhIP and AαC towards OAT3-mediated transport were studied as a fixed constant ratio (2:11), chosen according to the IC_{50} value of each chemical, using 2-fold serial dilutions with several concentration points below and above IC_{50} values of each heterocyclic amine used as a single chemical (Chou, 2010). Data from combination and single chemical effects towards OAT3 activity were then processed using the CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA), allowing thus to determine combination index (CI) values. The CI is a numerical index that is calculated using the following equation (Zhao et al., 2004):

$$CI = \frac{C_{PhIP/Mixture}}{C_{PhIP}} + \frac{C_{A\alpha C/Mixture}}{C_{A\alpha C}} \qquad (C)$$

where $C_{PhIP/Mixture}$ and $C_{A\alpha C/Mixture}$ are the concentrations of PhIP and AαC applied in combination to achieve x % of OAT3 activity inhibition, whereas C_{PhIP} and $C_{A\alpha C}$ represent the concentrations of individual agents to accomplish the same efficacy. Combinations were considered as synergistic when $CI<0.8$, additive when $0.8 \leq CI \leq 1.2$ and antagonistic when $CI>1.2$ (Bijnsdorp et al., 2011).

2.6 Statistical analysis

Quantitative data related to transporter activity inhibition were usually expressed as means ± SEM from at least three independent experiments. They were statistically analyzed through analysis of variance (ANOVA) followed by Dunnett's post-hoc test using GraphPad Prism software. The criterion of significance was $p < 0.05$.

3. Results

3.1 Inhibition of OAT activities by CSC

The effects of two concentrations of CSC, *i.e.*, 80 and 320 µg/mL, which correspond to non-cytotoxic concentrations similar to those retained in previous studies (Cohen et al., 2009; Sayyed et al., 2016), were first analyzed in OAT-transfected HEK293 cells. As shown in Fig. 1, both 80 and 320 µg/mL CSC significantly decreased PAH and E3S accumulation in HEK-OAT1 and HEK-OAT3 cells, respectively. CSC used at 320 µg/mL notably reduced PAH uptake by 80.0 $\pm$ 2.3 % and that of E3S by 79.4 $\pm$ 2.1 %. These inhibitory effects of CSC on OAT1 and OAT3 activity were close to those of the reference OAT inhibitor probenecid (Fig. 1). They were concentration-dependent, with CSC IC_{50} values lower for OAT3 (27.3 $\pm$ 1.7 µg/mL) than for OAT1 (72.1 $\pm$ 1.7 µg/mL) (Fig. 2). As shown in Fig. 1, CSC used at 320 µg/mL also decreased uptake of E3S in HEK293 cells transfected by OAT4, a renal apical organic/anion exchanger involved in reabsorption of some anionic drugs in proximal tubules (Burckhardt and Burckhardt, 2011; Ekaratanawong et al., 2004). The lower concentration of 80 µg/mL CSC was however inactive on OAT4-mediated transport of E3S (Fig. 1) and the IC_{50} value of CSC towards OAT4 activity was found to be rather high (351.5 $\pm$ 1.6 µg/mL) (Fig. 2).

We then considered whether *in vitro* inhibition of OATs by CSC may be relevant in terms of DDI. For this, we applied the criteria retained by the US Food and Drug Administration (FDA) for plasma inhibitor concentration, according to which a potential DDI with respect to a drug inhibiting a transporter can be predicted from *in vitro* data when the maximum total plasma (bound plus unbound) concentration (C_{max}) of the investigated drug at steady state ([I]) divided by its *in vitro* inhibitory potency (IC_{50}) is greater than or equal to 0.1 ($[I]/IC_{50} \geq 0.1$) (Giacomini et al., 2010; Maeda and Sugiyama, 2013). The maximal total plasma concentration reached by CSC in plasma of smokers, *i.e.*, I_{CSC}, is unfortunately not reported in the literature according to the best of our knowledge. In this context, it is however noteworthy that smoking induces *in vivo* expression of cytochromes P-450 1A1, 1A2 and 1B1

expression in human tissues, notably in the liver (Chang et al., 2003). Because such inductions of these drug metabolizing enzymes requires CSC concentrations in the 15-45 µg/mL range in cultured cells (Sayyed et al., 2016), it can therefore be hypothesized that such a concentration range is reached *in vivo* for smokers. The higher value of this range of concentrations, *i.e.*, 45 µg/mL, may be retained as I_{CSC} value, with the caution that CSC contains more than 5,000 chemicals that may have, for each of them, specific pharmacokinetics profile. Applying this I_{CSC} for DDI risk assessment indicated that the ratio I_{CSC}/IC_{50} is $\geq$0.1 for each OAT (Table 1). Potential DDI due to cigarette smoke may concern therefore OAT1, OAT3 and OAT4.

In addition to OAT activities, those of the SLC transporters OCT2 (found at the basolateral pole of proximal tubular cells) and MATE2-K (expressed at the apical pole of proximal tubular cells), implicated in renal tubular secretion of cationic drugs (Motohashi and Inui, 2013), were impacted by CSC. CSC used at 320 µg/mL thus decreased TEA uptake in HEK-OCT2 and HEK-MATE2-K cells (Fig. S1A). MATE2-K activity was however more sensitive to inhibition by CSC (IC_{50}=61.4 $\pm$ 2.3 µg/mL) than OCT2 activity (IC_{50}=226.2 $\pm$ 3.0 µg/mL) (Fig. S1B).

3.2 Inhibition of OAT1 and OAT3 activities by cigarette smoke-contained chemicals

The effects of some reference cigarette smoke-contained chemicals, including nicotine, 4-aminobiphenyl, the polycyclic aromatic hydrocarbons benzo(a)pyrene and phenanthrene, the nitrosamine NNK, the heterocyclic amines AαC, MeAαC, Trp-P-2 and PhIP and the metals cadmium, lead and arsenic (van Leeuwen et al., 2005), on OAT1- and OAT3-mediated uptake of the reference OAT substrate 6-carboxyfluorescein were next analyzed. As shown in Fig. 3A, for chemicals used at 100 µM, only the heterocyclic amine PhIP was found to significantly reduce accumulation of the dye in HEK-OAT1 cells; this decrease was marked (by 92.1 $\pm$ 0.8 %) and similar to that occurring in response to the

reference OAT inhibitor probenecid. Inhibitory effect of PhIP towards OAT1 activity was next shown to be concentration-dependent (IC_{50}=3.3 ± 1.3 µM) (Fig. 3B). With respect to OAT3-mediated uptake of 6-carboxyfluorescein, it was found to be significantly reduced by the heterocyclic amines AαC, Trp-P-2 and PhIP, as well as by the reference inhibitor probenecid (Fig. 4A). The blocking effects of AαC and PhIP towards OAT3 activity were concentration-dependent (AαC IC_{50}=11.3 ± 1.3 µM and PhIP IC_{50}=1.9 ± 0.4 µM) (Fig. 4B). PhIP, used at 100 µM, which blocked both OAT1 and OAT3 activity, however failed to hinder OAT4-mediated E3S uptake in HEK-OAT4 cells (Fig. S2), thus demonstrating that its inhibitory effect towards OAT1 and OAT3 was rather specific. 100 µM Aαc also did not inhibit OAT4 activity (Fig. S2). In contrast to heterocyclic amines, metals were found to *cis*-stimulate OAT3-mediated 6-carboxyfluorescein uptake; cadmium, lead and arsenic thus enhanced dye accumulation by 67.5 ± 20.5 %, 75.2 ± 15.4 % and 53.8 ± 22.0 %, respectively (Fig. 4A). Nicotine, 4-aminobiphenyl, the nitrosoamine NNK, the polycyclic aromatic hydrocarbons benzo(a)pyrene and phenanthrene and the heterocyclic amine MeAαC failed to significantly impair OAT3 activity (Fig. 4A).

Because CSC is a complex mixture of chemicals, its effect on SLC transporters, notably OAT3, may be hypothesized to reflect the combined effect of chemicals contained in CSC, and not the effect of only a single chemical. In this context, the exact nature of chemical mixture effects, *i.e.*, synergistic, additive or antagonistic, may be important to determine. To get initial information about this point, we characterized the effects of the combination PhIP/AαC towards OAT3 activity through calculating CI using the CompuSyn software. As shown in Table 2, the low concentrations of PhIP/AαC (up to 3.02 µM) were found to exert synergistic inhibition towards OAT3-mediated transport (CI<0.8), whereas higher concentrations were mostly additive (0.8≤CI≤1.2). Only the very high mixture concentration of 153.2 µM exhibited antagonistic effect (CI>1.2) (Table 2).

Finally, we investigated whether PhIP can *trans*-stimulate 6-carboxyfluorescein uptake in HEK-OAT1 and HEK-OAT3 cells, which may constitute an argument in favor of the transport of the heterocyclic amine by OAT1 and/or OAT3 (Apiwattanakul et al., 1999; Sweet et al., 2003). Pre-loading with PhIP however resulted in *trans*-inhibition, and not *trans*-stimulation, of dye uptake in both HEK-OAT1 and HEK-OAT3 cells (Fig. 5). Pre-loading with 80 or 320 µg/mL CSC also led to *trans*-inhibition of dye uptake (Fig. 5). AαC similarly *trans*-inhibited 6-carboxyfluorescein accumulation in HEK-OAT1 cells. By contrast, pre-loading with glutarate resulted in a *trans*-stimulation of 6-carboxyfluorescein accumulation in HEK-OAT1 and HEK-OAT3 cells (Fig. 5), as expected for an OAT1/3 substrate like glutarate (Apiwattanakul et al., 1999; Sweet et al., 2003).

4. Discussion

CSC and cigarette smoke extract have been recently demonstrated to impair activity and/or expression of various drug transporters in hepatic or pulmonary lung cells (Sayyed et al., 2016; Takano et al., 2016; van der Deen et al., 2007), thus supporting the idea that drug transporters may constitute general targets of cigarette smoke. The data reported in the present study fully support this hypothesis through demonstrating that CSC can inhibit activity of the anionic drug transporters OAT1, OAT3 and OAT4, mainly expressed at the kidney level. Renal transporters of cationic drugs such as OCT2 and MATE2-K were additionally affected by CSC. These renal and also other non-renal transporters whose activity is affected by CSC are summarized in Table 3. It is noteworthy that the sensitivity of these transporters to the inhibitory effects of CSC depends on the nature of transporters. Indeed, when considering IC_{50} values, some transporters such as the SLC transporters OAT3, OCT1, MATE1, OATP1B1 and OATP1B3 and the ABC transporter BCRP can be arbitrarily considered as potently inhibited by CSC (IC_{50}<50 µg/mL) (Table 3). Other transporters, like OAT1, sodium

taurocholate co-transporting polypeptide (NTCP/*SLC10A1*) and MATE2-K, are more moderately inhibited by CSC (50 µg/mL<IC_{50}<150 µg/mL). Activity of other transporters, such as the ABC transporters P-glycoprotein, encoded by the multidrug resistance gene 1 (*MDR1/ABCB1*), and MRP2, and the SLC transporter OCT2, can be considered as more weakly impacted by CSC (150 µg/mL<IC_{50}<250 µg/mL), whereas those of OAT4 and OATP2B1 were only marginally affected (IC_{50}>250 µg/mL) (Table 3). This classification of transporters according to their sensitivity to inhibition by CSC does not correspond to functional classification of transporters according to the nature of substrates. For example, both transporters handling preferentially anionic drugs (OATP1B1, OATP1B3 and OAT3) and those transporting cationic drugs (OCT1, MATE1) are potently inhibited by CSC. This diversity of transporters inhibited by CSC can be hypothesized to reflect the structural diversity of the numerous chemicals contained in cigarette smoke (Talhout et al., 2011) and potentially acting as transporter inhibitors. Interestingly, other complex mixtures of environmental chemicals such as diesel exhaust particle extract has similarly been shown to inhibit various unrelated drug transporters (Le Vee et al., 2015).

The differential sensitivity of OATs to inhibition by CSC, *i.e.*, OAT3 is potently blocked, whereas OAT1 is more moderately inhibited and OAT4 is more marginally affected, discards any general unspecific inhibitory effect of CSC towards OATs and beyond, towards drug transporters. The inhibition of OAT3 activity by CSC therefore likely implicates direct and specific interactions of CSC-contained chemicals with substrate and/or regulatory drug binding sites on the transporter, as classically reported for drug transporter inhibitors (Montanari and Ecker, 2015). In this context, a role for nicotine, polycyclic aromatic hydrocarbons, NNK and 4-aminobiphenyl is unlikely, owing to the failure of these chemicals to modulate OAT3 activity. An implication of metals such as cadmium, lead and arsenic can also be excluded, because these compounds rather *cis*-stimulated, and did not inhibit, OAT3

activity. By contrast, some heterocyclic amines may be involved, notably PhIP and AαC, which potently inhibited OAT3 activity as single agents. To validate this hypothesis, AαC and PhIP concentrations in CSC have however to be evaluated and confronted to AαC and PhIP concentrations required for inhibiting OAT3 activity. Regarding AαC, which is the most abundant mutagenic heterocyclic amine found in cigarette smoke (Konorev et al., 2015; Turesky et al., 2007), its amount is estimated to 60-250 ng/cigarette (Zhang et al., 2011). Because one cigarette approximately generates a mean of 9.5 mg CSC (Eldridge et al., 2015), the concentration of AαC normalized to CSC particulate matter is likely 6.32-26.3 ng/mg CSC. CSC IC_{50} towards OAT3 activity, *i.e.*, 27.3 µg/mL, thus theoretically contains 0.1725-0.718 ng/mL AαC, which corresponds to 0.942-3.92 nM AαC. Such AαC concentrations are far from those inhibiting OAT3, which are in the 10 µM range (Fig. 4B), thus discarding any direct contribution of AαC to OAT3 inhibition by CSC. A similar conclusion can likely be drawn for putative direct PhIP contribution to OAT3 or OAT1 activity inhibition by CSC, knowing moreover that PhIP is thought to be less abundant than AαC in cigarette smoke (Konorev et al., 2015; Manabe et al., 1991). OAT1 and OAT3 inhibition by CSC may therefore likely implicate chemicals that remain to be characterized. Alternatively, OAT1 and OAT3 inhibition may reflect the combining effects of various chemicals, which may display synergism or additivity. This hypothesis is supported by the fact that the combination of AαC/PhIP was demonstrated to exert synergistic inhibition towards OAT3 activity when considering low concentrations of the mixture. Mixture of chemicals, rather than individual ones, may have therefore to be considered when addressing effects of environmental contaminants, including putative inhibitory effects towards transporters. In agreement with this conclusion, a realistic combination of marine pollutants as well as binary mixtures of pesticides have been demonstrated to block P-glycoprotein activity (Nicklisch et al., 2016; Pivcevic and Zaja, 2006).

In addition to OAT1 and OAT3, various ABC efflux pumps interact with heterocyclic amines. Indeed, BCRP transports PhIP, and by this way, restricts human exposure to this carcinogen through decreasing its uptake from the gut lumen and mediating its hepatobiliary and intestinal elimination (van Herwaarden et al., 2003). MRP2, MRP3 and P-glycoprotein have also been shown to handle PhIP and its genotoxic metabolites (Vlaming et al., 2014). By contrast, OAT1 and OAT3 are unlikely to transport PhIP because this heterocyclic amine failed to *trans*-stimulate their activity. In the same way, AαC *trans*-inhibited OAT3 activity and is therefore probably not a substrate for this transporter. Taken together, these data indicate that OAT1 and OAT3 are unlikely to contribute to toxicokinetics of heterocyclic amines like PhIP and AαC, in contrast to the ABC efflux pumps reported above. More generally, CSC as a whole mixture *cis*-inhibited, but failed to *trans*-stimulate activity of OAT1 and OAT3, suggesting that CSC-contained chemicals are not transported by OAT1/OAT3.

The *in vivo* relevance of OAT1/OAT3 inhibition by CSC and the possible consequences in terms of cigarette smoke toxicity or drug pharmacokinetics for smokers remains to be determined. It is nevertheless noteworthy that DDI risk assessment, performed according to FDA criteria and with a putative theoretical value of 45 µg/mL for maximal plasma CSC concentration, indicated that potential DDI due to cigarette smoke may concern OAT1, OAT3 and OAT4. OAT3, which is the most sensitive OAT to inhibitory effects of CSC, is likely the first one to be considered in this context of DDI caused by smoking, which, besides, is known to impair renal functions (Cooper, 2006; Orth, 2002). Renal secretion of drugs handled by OAT3, such as, for example, the antibiotics benzylpenicillin and cephaloridine, the anticancer drug methotrexate and lipids-lowering statins (Burckhardt, 2012), may consequently be compromised in smokers. Further studies are likely required to validate or not this hypothesis, knowing moreover that many substrates of OAT3 are shared

with OAT1 (Nigam et al., 2015a), which is less sensitive to inhibition by CSC and which may therefore compensate putative renal OAT3 inhibition in smokers. Importantly, besides drugs, various endogenous hormones, nutrients and metabolites are substrates for OAT3 (Nigam et al., 2015b). Data from Oat3-knock-out mice have additionally revealed a role for the transporter in several metabolic pathways, including the tricarboxylic acid cycle, nucleotide and amino acid metabolism, prostaglandin and steroid metabolism, as well as the metabolism of dietary flavonoids (Wu et al., 2013). Transport of endogenous OAT3 substrates as well as OAT3-related physiological ways may therefore be affected by OAT3 inhibition due to smoking, which may contribute to the pleomorphic toxicity of cigarette smoke. Finally, because OAT3 is also expressed at the choroid plexus, the brain capillary endothelium, and retina (Miyajima et al., 2011; Urquhart and Kim, 2009), putative consequences of its inhibition at these extra-renal sites may also deserve attention for smokers.

In summary, CSC and some cigarette smoke-contained chemicals like the heterocyclic amine PhIP were shown to *in vitro* inhibit OATs, notably OAT3. Such an inhibition of OAT3 activity may contribute to alterations of pharmacokinetics or some adverse toxic effects caused by smoking.

Acknowledgments

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Appendix A Supplementary data

Supplementary figures.

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Legends to figures

Fig. 1. Effects of CSC on OAT1, OAT3 and OAT4 activity.

HEK-OAT1, HEK-OAT3 and HEK-OAT4 cells were incubated with reference substrates (PAH for OAT1 and E3S for OAT3 and OAT4) for 5 min, in the absence (control) or presence of the reference inhibitor probenecid or of CSC used at 80 and 320 µg/mL. After washing, intracellular accumulation of substrate was determined by scintillation counting. Data are expressed as percentages of substrate accumulation found in untreated control cells, arbitrarily set at 100 %. They are the means $\pm$ SEM of at least three independent determinations. *, $p<0.05$ when compared to control cells.

Fig. 2. Concentration-dependent effects of CSC towards OAT1, OAT3 and OAT4 activity.

HEK-OAT1, HEK-OAT3 and HEK-OAT4 cells were incubated with reference substrates for 5 min in the absence or presence of various concentrations of CSC (from 0.3 to 320 µg/mL for OAT1/OAT3 and from 0.3 to 640 µg/mL for OAT4) or of the reference OAT inhibitor probenecid. OAT activities were next calculated as described in Materials and Methods and are expressed as percentage of those found in control cells not exposed to CSC, arbitrarily set at 100 %. Data are the means $\pm$ SEM of at least three independent assays. CSC IC_{50} values are indicated on the right of each graph.

Fig. 3. Effects of various cigarette smoke-contained chemicals on OAT1 activity.

(A) HEK-OAT1 cells were incubated with the reference OAT substrate 6-carboxyfluorescein in the absence (control) or presence of the reference inhibitor probenecid or of various cigarette smoke chemicals, each used at 100 µM. The CSC chemicals tested were: nicotine, the nitrosamine NNK, the polycyclic aromatic hydrocarbons benzo(a)pyrene and

phenanthrene, 4-aminobiphenyl, the heterocyclic amines AαC, MeAαC, Trp-P-2 and PhIP and the metals cadmium, lead and arsenic. After washing, intracellular accumulation of substrate was determined by spectrofluorimetry. Data are expressed as percentages of substrate accumulation found in untreated control cells, arbitrarily set at 100 %. They are the means ± SEM of at least three independent determinations. *, $p<0.05$ when compared to control cells. (B) HEK-OAT1 cells were incubated with 6-carboxyfluorescein for 5 min in the absence or presence of various concentrations of PhIP (from 0.01 to 100 µM) or of the reference OAT inhibitor probenecid. OAT1 activities were next calculated as described in Materials and Methods and are expressed as percentages of those found in control cells not exposed to CSC, arbitrarily set at 100 %. Data are the means ± SEM of at least three independent assays. PhIP IC_{50} value is indicated on the right of the graph.

Fig. 4. Effects of various cigarette smoke-contained chemicals on OAT3 activity.

(A) HEK-OAT3 cells were incubated with the reference OAT substrate 6-carboxyfluorescein in the absence (control) or presence of the reference inhibitor probenecid or of various cigarette smoke chemicals, each used at 100 µM. The CSC chemicals tested were: nicotine, the nitrosamine NNK, the polycyclic aromatic hydrocarbons benzo(a)pyrene and phenanthrene, 4-aminobiphenyl, the heterocyclic amines AαC, MeAαC, Trp-P-2 and PhIP and the metals cadmium, lead and arsenic. After washing, intracellular accumulation of substrate was determined by spectrofluorimetry. Data are expressed as percentages of substrate accumulation found in untreated control cells, arbitrarily set at 100 %. They are the means ± SEM of at least three independent determinations. *, $p<0.05$ when compared to control cells. (B) HEK-OAT3 cells were incubated with 6-carboxyfluorescein for 5 min in the absence or presence of various concentrations of PhIP (from 0.01 to 100 µM) or AαC (from 0.1 to 300 µM) or of the reference OAT inhibitor probenecid. OAT3 activities were next calculated as

described in Materials and Methods and are expressed as percentages of those found in control cells not exposed to CSC, arbitrarily set at 100 %. Data are the means ± SEM of at least three independent assays. IC_{50} values are indicated on the right of each graph.

Fig. 5. *Trans*-inhibitory effects of CSC and heterocyclic amines towards OAT1 and OAT3 activity.

HEK-OAT1 and HEK-OAT3 cells were first incubated for 15 min at 37°c in the absence (control) or presence of 1 mM glutarate, 80 or 320 µg/mL CSC, 100 µM PhIP or 100 µM AαC (only for HEK-OAT3 cells). After washing, cells were next re-incubated with 6-carboxyfluorescein for 5 min at 37°C. Intracellular accumulation of dye was then determined by spectrofluorimetry. Data are expressed as percentages of dye accumulation in control cells, arbitrarily set at 100 %, and are the means ± SEM of three independent experiments. *, $p<0.05$ when compared to control cells.

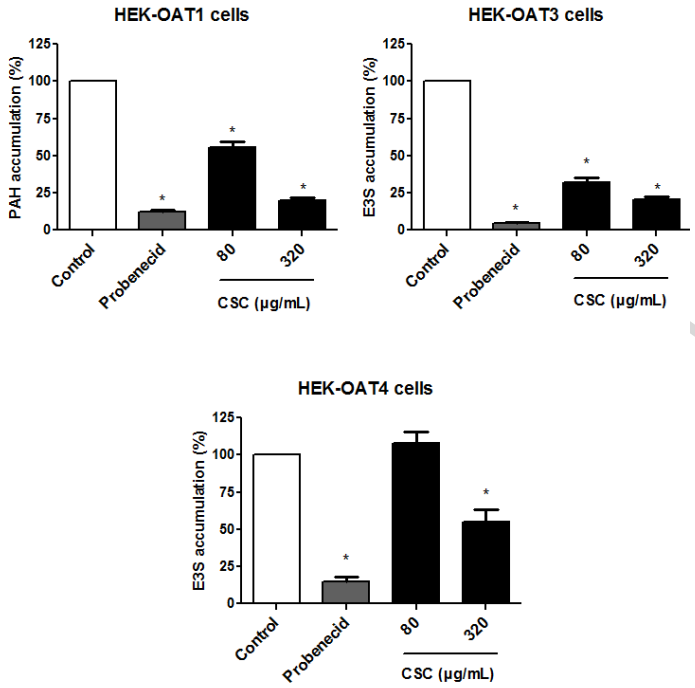

Figure 1

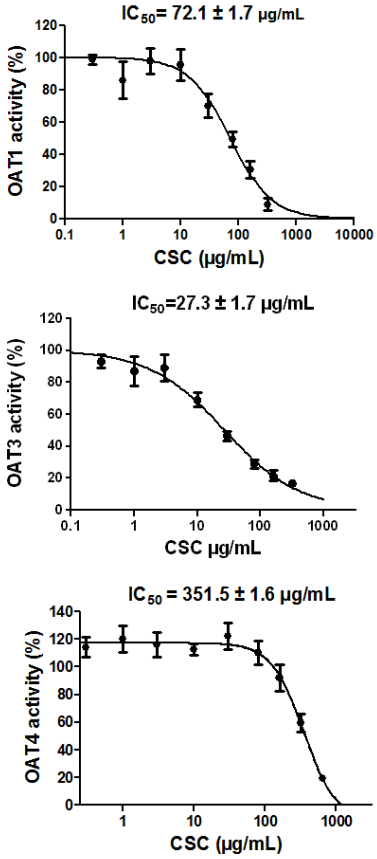

Figure 2

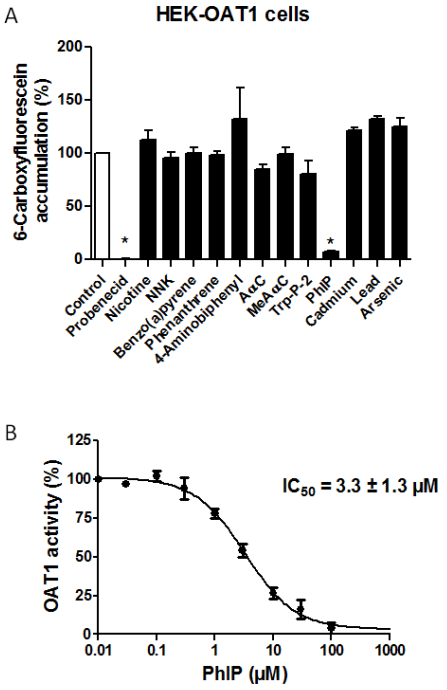

Figure 3

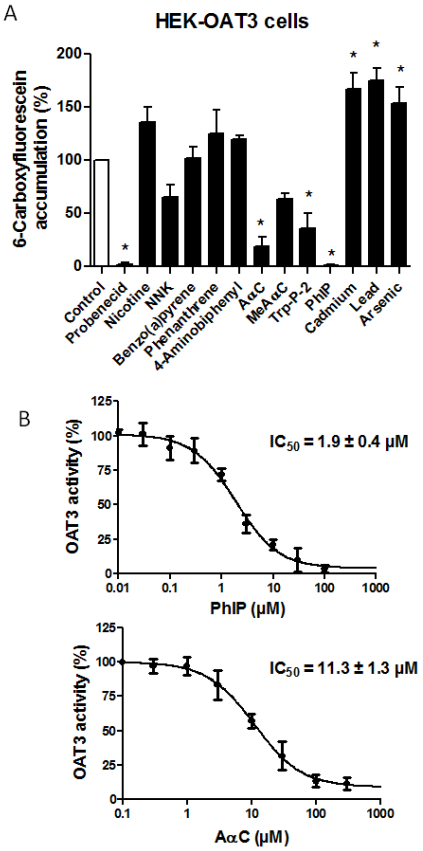

Figure 4

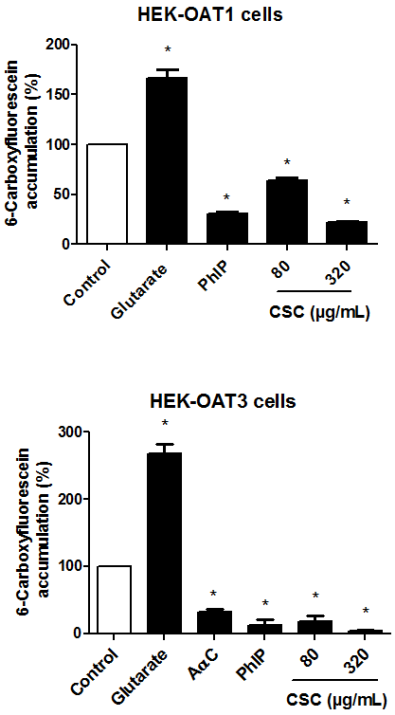

Figure 5

Table 1

Prediction of potential drug-drug interactions (DDI) for CSC-mediated inhibitions of OATs according to FDA criteria for plasma inhibitor concentration[a]

SLC transporter	CSC IC_{50} (µg/mL)	Ratio $[I_{CSC}]^b$/CSC IC_{50}	Prediction of potential DDI according to FDA criteria
OAT1	72.1	0.62	DDI
OAT3	27.3	1.65	DDI
OAT4	351.5	0.13	DDI

[a]FDA criteria related to plasma inhibitor concentration: a potential clinical DDI may be predicted when $[I]/IC_{50} \geq 0.1$ (with [I]=maximum total plasma (bound plus unbound) inhibitor concentration)
[b]I_{CSC} was estimated to 45 µg/mL

Table 2

Combination index (CI) values for OAT3 inhibition by combined treatment with PhIP and AαC.

% inhibition OAT3 activity	CI[a]	Mixture concentration[b]	Nature of effect[c]
10	0.62	0.06 µM	Synergistic
20	0.67	0.25 µM	Synergistic
30	0.71	0.67 µM	Synergistic
40	0.75	1.46 µM	Synergistic
50	0.79	3.02 µM	Synergistic
60	0.85	6.24 µM	Additive
70	0.91	13.7 µM	Additive
80	1.01	36.0 µM	Additive
90	1.22	153.2 µM	Antagonistic

[a]CI values were calculated using CompuSyn software.

[b]The ratio [PhIP]:[AαC] is 2:11.

[C]Synergistic effect: CI<0.8; additive effect: 0.8≤CI≤1.2; antagonistic effect: CI>1.2.

Table 3

Summary of drug transporter inhibition by CSC.

Drug transporter	CSC IC_{50} (µg/mL)	Nature of inhibition[a]
OATP1B1	7.2[b]	Strong
OATP1B3	7.6[b]	Strong
OATP2B1	>320[b]	Marginal
OAT1	72.1	Moderate
OAT3	27.3	Strong
OAT4	351.5	Marginal
NTCP	140[b]	Moderate
OCT1	12.5[b]	Strong
OCT2	226.2	Weak
MATE1	6.1[b]	Strong
MATE2-K	61.4	Moderate
P-gp	224.3[b]	Weak
MRP2	197.4[b]	Weak
BCRP	37.4[b]	Strong

[a]Strong inhibition: CSC IC_{50}>50 µg/mL; moderate inhibition: 50 µg/mL<CSC IC_{50}<150 µg/mL; weak inhibition: 150 µg/mL<CSC IC_{50}<250 µg/mL; weak inhibition: CSC IC_{50}>250 µg/mL.
[b]According to Sayyed et al., 2016.

Highlights

- CSC impaired activity of the renal drug transporters OATs
- OAT3 was the most potently inhibited transporter (CSC IC_{50}=27.3 µg/mL)
- CSC components like the heterocyclic amines AαC and PhIP inhibited OAT3 activity
- AαC and PhIP, as well as CSC, however failed to *trans*-stimulate OAT3 activity