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Review article

Membrane transporter data to support kinetically-informed chemical risk assessment using non-animal methods: Scientific and regulatory perspectives

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ABSTRACT

Humans are continuously exposed to low levels of thousands of industrial chemicals, most of which are poorly characterised in terms of their potential toxicity. The new paradigm in chemical risk assessment (CRA) aims to rely on animal-free testing, with kinetics being a key determinant of toxicity when moving from traditional animal studies to integrated *in vitro-in silico* approaches. In a kinetically informed CRA, membrane transporters, which have been intensively studied during drug development, are an essential piece of information. However, how existing knowledge on transporters gained in the drug field can be applied to CRA is not yet fully understood. This review outlines the opportunities, challenges and existing tools for investigating chemical-transporter interactions in kinetically informed CRA without animal studies. Various environmental chemicals acting as substrates, inhibitors or modulators of transporter activity or expression have been shown to impact TK, just as drugs do. However, because pollutant concentrations are often lower in humans than drugs and because exposure levels and internal chemical doses are not usually known in contrast to drugs, new approaches are required to translate transporter data and reasoning from the drug sector to CRA. Here, the generation of *in vitro* chemical-transporter interaction data and the development of transporter databases and classification systems trained on chemical datasets (and not only drugs) are proposed. Furthermore, improving the use of human biomonitoring data to evaluate the *in vitro-in silico* transporter-related predicted values and developing means to assess uncertainties could also lead to increase confidence of scientists and regulators in animal-free CRA. Finally, a systematic characterisation of the transportome (quantitative monitoring of transporter abundance, activity and maintenance over time) would reinforce confidence in the use of experimental transporter/barrier systems as well as in established cell-based toxicological assays currently used for CRA.

1. Introduction

Humans are continuously exposed to low levels of thousands of industrial chemicals such as pesticides, metals, food contaminants and cosmetic ingredients. However, little is known about the possible impacts of these substances on human health, even though epidemiological studies indicate that certain environmental chemicals can exert deleterious effects in humans. The aim of chemical risk assessment (CRA) is to provide an understanding of the nature, magnitude and probability of a chemical to adversely affect humans, animals or the environment. CRA takes into account both hazard and exposure and informs regulatory risk management decisions in a range of different industrial sectors, such as chemicals, pesticides, pharmaceuticals, cosmetics, and food and feed. Traditionally, animal testing has provided the gold standard for assessing CRA accepted by regulatory authorities. However, policies in the EU and US are shifting away from animal studies. A clear demonstration of this shift is the ban on animal testing for cosmetic ingredients and products in the European Union since March 2013 (Cosmetics Directive EC:1223/2009) and in California since September 2018. Another illustration is the EU Directive on the

Table 1

Illustrations of environmental chemicals from various classes interacting with different human drug transporters.

Class of pollutants	Chemicals	Transporter	Nature of the interaction	Reference (PMID)
Plasticizer	Bisphenol A	OCT1, MATE1, OATP1B1, OAT3	Inhibition of activity	(Bruyere et al., 2017)
		P-gp/MDR1, BCRP	Regulation of mRNA and protein expression	(Sieppi et al., 2017; Speidel et al., 2018)
	Diethylhexyl phthalate	P-gp/MDR1	Regulation of activity	(Angelini et al., 2011)
Surfactant	Perfluorooctane sulfonate (PFOS)	OATP1B1, OATP1B3, OATP2B1	Substrate	(Zhao et al., 2017)
	Perfluorooctanoate (PFOA)	OAT4	Substrate	(Kummu et al., 2015; Yang et al., 2010)
Organophosphorus pesticide	Fenamiphos, phosmet	OCT1, OCT2, MATE1	Inhibition of activity	(Agarwala et al., 2004; Chedik et al., 2018b)
	Phosalone, diazinon	P-gp/MDR1	Inhibition of activity	
	Chlorpyrifos	P-gp/MDR1		
Organochlorine pesticide	Chlordane, heptachlor	OCT1, MRP2, BCRP	Inhibition of activity	(Bucher et al., 2014)
		MRP2	Induction of mRNA expression	
	Dieldrin, 4,4′-DDT	P-gp/MDR1	Inhibition of activity	(Nicklisch et al., 2016)
	Endosulfan	P-gp/MDR1	Substrate	(Bain and LeBlanc, 1996)
	Methoxychlor	MRP1	Substrate	(Tribull et al., 2003)
Fungicide	Propiconazole	P-gp/MDR1	Inhibition of activity	(Mazur et al., 2015)
Herbicide	2,4-dichlorophenoxyacetic acid (2,4-D)	OAT1, OAT3	Substrate	(Nozaki et al., 2007)
	Paraquat	OCT2, MATE1	Substrate	(Chen et al., 2007)
		P-gp/MDR1	Substrate	(Wen et al., 2014)
Cyanotoxin	Microcystin-LR	OATP1B1, OATP1B3	Substrate	(Fischer et al., 2005)
Mycotoxin	Aflatoxin B_1	OAT1, OAT3, OCT1, OCT2	Substrate	(Tachampa et al., 2008)
	Phalloidin	OATP1B1, OATP1B3	Substrate	(Meier-Abt et al., 2004)
	Ochratoxin A	OAT1, OAT3	Substrate	(Takeuchi et al., 2001)
Marine biotoxin	Okadoic acid	P-gp/MDR1	Substrate	(Ehlers et al., 2014)
Heterocyclic aromatic amine	PhIP	BCRP	Substrate	(Pavek et al., 2005)
	Trp-P-1, Trp-P-2	OCT1, OCT2	Inhibition of activity	(Sayyed et al., 2019)
Polycyclic aromatic hydrocarbons	Benzo(a)pyrene	BCRP	Induction of mRNA and protein expression	(Seidel et al., 2005)
Heavy metal	Arsenic	MRP2	Substrate and induction of protein expression	(Roggenbeck et al., 2015)
	Antimony	MRP1	Substrate	(Vernhet et al., 1999)

The different chemical -transporter interactions are described in details in point 2.1.

Protection of Animals used for Scientific Purposes, reinforcing the principle of the Three Rs (Replacement, Reduction and Refinement of animal procedures; Directive 2010/63/EU). In the US, the Federal program "Toxicology in the 21st century" (Tox21) aims to evaluate the utility of *in vitro* assays and *in silico* models as alternative approaches to toxicity testing (Thomas, 2018). This new paradigm in CRA, taken together with observed inter-species differences, financial and ethical concerns, created a need to develop reliable and cost-effective alternative (non-animal) methods to assess chemical safety. The two facets of CRA include evaluation of the toxicokinetics (TK)* of a compound, relating external exposure to internal target-site dose, and its toxicodynamics (TD), relating the target-site dose to and observable toxicity response (dose-response relationship). TK data provide essential information on the absorption, distribution, metabolism and excretion (ADME) processes of a substance within the body, allowing quantitative relationships to be established between the external chemical dose and the toxicity response (Coecke et al., 2013; Tsaioun et al., 2016). Therefore, when moving from traditional animal studies to integrative approaches based on *in vitro* and *in silico* methods, information on TK is a key element in CRA (Bessems et al., 2015; Coecke et al., 2013; ECHA, 2014; EFSA, 2014; FDA, 2017a, 2018).

*Page note: In this paper, the term TK is used to refer to the kinetics of toxicants specifically, even though it remains indistinguishable from the concept of pharmacokinetics applied to therapeutic drugs.

Initially discovered in the 1980s as causing multidrug resistance in chemotherapy by actively pumping anticancer drugs out of tumour cells (Juliano and Ling, 1976), membrane transporters were later also found to be localised in healthy tissues affecting the disposition of a variety of drugs (Klaassen and Aleksunes, 2010). Going beyond the cancer field, transporters were then intensively studied during drug development as they play key roles in ADME processes affecting drug pharmacokinetics

and mediating adverse drug-drug interactions (DDI). In 2010, an International Transporter Consortium (ITC) comprising industrial, regulatory and academic scientists was formed to identify clinically relevant transporters and suitable *in vitro* and *in vivo* methods as well as appropriate computational models to better characterise transporter-drug interactions (Brouwer et al., 2013; Giacomini et al., 2010; Zamek-Gliszczynski et al., 2013).

Drug transporters belong to two super-families: ATP-binding cassette (ABC) transporters, acting as efflux pumps through ATPase-dependent primary active transport, and solute carrier (SLC) transporters, acting mostly as drug uptake transporters through facilitated diffusion or secondary active transport. Among all transporters, seven transporters were initially emphasised for their clinical significance by the ITC: P-glycoprotein (P-gp), also called multidrug resistance 1 protein (MDR1/encoded by *ABCB1* gene), breast cancer resistance protein (BCRP/*ABCG2*), organic anion transporting polypeptide (OATP1B1 and 1B3/*SLCO1B1 and 1B3*), organic anion transporter (OAT1 and 3/ *SLC22A6 and A8*) and organic cation transporter (OCT2/*SLC22A2*) (Giacomini et al., 2010). Subsequently, the ITC further updated the list to highlight additional transporters of emerging importance, such as bile salt export pump (BSEP/*ABCB11*), multidrug resistance-associated proteins (MRPs/*ABCCs*), multidrug and toxin extrusions (MATE1 and 2K/*SLC47A1 and 2*), OCT1 (*SLC22A1*), and OATP2B1 (*SLCO2B1*) (Hillgren et al., 2013; Zamek-Gliszczynski et al., 2018). Based on the ITC recommendations, the European Medicines Agency (EMA), the U.S. Food and Drug Administration (FDA) and the Japanese Ministry of Health, Labour and Welfare (MHLW) recommended conducting transporter studies for any new drug. All three agencies released final (EMA, 2012), draft (FDA, 2012) or tentative (MHLW, 2014) regulatory guidelines. Revised guidelines were released in 2017 by the FDA including new *in vitro* guidance and encouraging the conduct of

transporter studies at earlier stages of drug development (FDA, 2017a). The field of transporters is growing at a rapid pace, as reflected by the impressive increase in the number of transporter experiments per FDA-approved drug, increasing from 6 in 2013 to 22 in 2016 (Yu et al., 2018).

Owing to the historical interest of the pharmaceutical sector, a significant amount of knowledge and data exists for the so-called drug transporters. More recently, besides drugs, transporters have been shown to interact with various types of environmental chemicals, such as pesticides, industrial chemicals, mycotoxins, food process-derived chemicals (*e.g.* burned meat-derived heterocyclic aromatic amines) and heavy metals (Table 1) (Chedik et al., 2018a; Epel et al., 2008; Fardel et al., 2012; Leslie et al., 2005; Van Herwaarden and Schinkel, 2006; Wilks and Tsatsakis, 2014).

While the role of metabolising enzymes in kinetically informed CRA is well documented, the role of transporters is only starting to be recognised as a major kinetic determinant and thus as an essential piece of information in animal-free CRA (Bessems et al., 2014, 2015; Paini et al., 2017a; Paini et al., 2017b; Paini et al., 2019). However, how the existing knowledge on drug transporters could be applied to CRA is not yet fully understood. In a first attempt to capture the current state-of-play and challenges in the application of *in vitro* and *in silico* methods to study transporters for CRA purposes, we created a survey that was disseminated among transporter experts (Clerbaux et al., 2018). A key finding of the survey was that transporters are being investigated primarily during drug development, but also for CRA purposes of food and feed contaminants, industrial chemicals, cosmetics, nanomaterials and in the context of environmental toxicology, by applying both *in vitro* and *in silico* tools. Furthermore, the respondents identified various challenges related to the interpretation and use of transporter data from non-animal methods. Overall, it was considered that a mechanistically-anchored *in vitro-in silico* approach, validated against available human data, would increase confidence in the use of transporter data within an animal-free CRA. Based on the survey results and on recently published data on chemical-transporter interactions at various biological membrane barriers, we aim here to review the *in vivo* relevance and current applications of chemical-transporter interactions for human CRA. This review makes recommendations on the applicability of the extensive existing knowledge and tools available from the pharmaceutical sector to study drug transporters in support of CRA using alternative methods. The challenges and needs specific to the toxicological community are compared with those shared with the pharmaceutical sector.

2. Chemical-transporter interactions: *in vivo* relevance and potential impacts on human TK

2.1. Chemical-transporter interactions: potential impacts on human TK

Membrane-embedded transport proteins represent the functional part of the biological barriers of the body as they mediate the cellular uptake and efflux of compounds. Besides the well-studied drug transporters, the literature on additional transporters of clinical and toxicological relevance is continuously emerging. In this review, the focus is on transporters expressed at external and internal biological barriers relevant for human toxicology (Fig. 1) and with which environmental chemicals have been shown, or are presumed, to interact (Table 1). These include transporters expressed in the gut, liver, kidney and brain, which are considered pivotal for determining the kinetics of xenobiotics and already intensively studied for drugs (Giacomini et al., 2010). Regarding external barriers, transporter data are becoming increasingly available also for lungs at mRNA and protein expression (Bosquillon, 2010; Sakamoto et al., 2013) and for skin at the mRNA levels (Alriquet et al., 2015; Fujita et al., 2017; Fujiwara et al., 2014; Giacone et al., 2018; Hashimoto et al., 2017; Ito et al., 2008; Osman-Ponchet et al., 2017, 2014), potentially opening new avenues to explore the impacts of chemical-transporter interactions on pulmonary and dermal absorption.

Finally, transporters expressed at key barriers implicated in reproduction and development, such as the placenta, testis and mammary glands, albeit representing some transient physiological situations, are considered in this review as they play critical role in reproductive and development toxicity and the health consequences may be serious for the foetus, infant or pregnant woman.
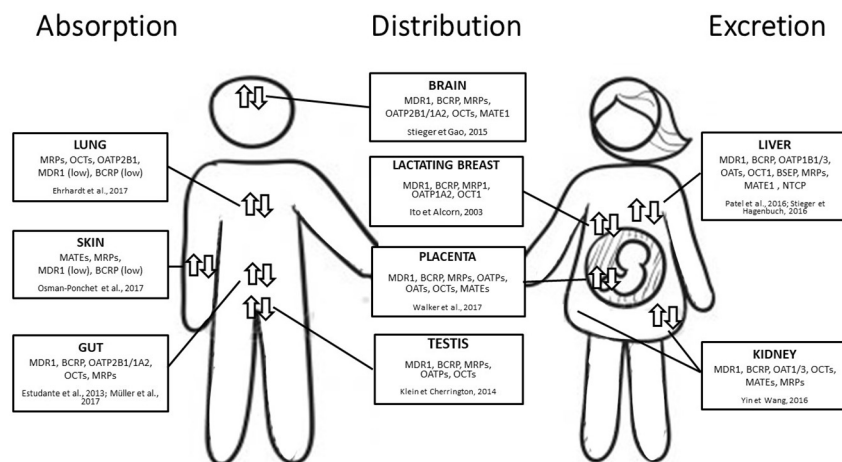
At these key biological external and internal barriers, transporters affect ADME processes, and thereby TK in general, either because chemicals are substrates of transporters or because they modulate transporter activity or expression. By controlling chemical access to various tissues, thereby modulating chemical concentration within the body, transporters influence TK. In addition, when inside target tissues, chemical may induce toxic response, therefore by allowing access to these tissues, transporters impact TD as well. This dual role of transporters is discussed here in terms of the ADME processes they interfere with.

2.1.1. Chemicals as substrates of transporters

At the level of external barriers, if a chemical is a transporter substrate, its **absorption** can be favoured or restricted. The impact of transporters on intestinal absorption is intensively documented for drugs (Müller et al., 2016) and has been demonstrated for some pesticides like paraquat and different pyrethroids (Silva et al., 2015; Zastre et al., 2013). Influx transporters were shown to be involved in their intestinal uptake whereas efflux transporters, located on the apical side of enterocytes, may limit oral ingestion of pesticides, by pumping them back into the gut lumen leading to their elimination in feces (Silva et al., 2015; Zastre et al., 2013). In lungs, whether transporters are involved in pulmonary uptake or in protective efflux of volatile environmental contaminants still need to be demonstrated (Leslie et al., 2005). In the skin, in contrast to the gut, the efflux transporters P-gp and MRP1 seem to play an absorptive role, transporting substrates from the surface to the dermis (Giacone et al., 2018; Hashimoto et al., 2013; Ito et al., 2008; Osman-Ponchet et al., 2017), in which case one may expect an increased pollutant concentration in the epidermis with a potentially increasing toxic effect in the skin. SLC transporters are also expressed at mRNA levels in the skin but their function is not yet clear (Alriquet et al., 2015). Thus transporters are present in the skin and could in theory modulate dermal absorption of chemicals that come into contact with the skin, such as drugs, cosmetics or pollutants.

Regarding **distribution** to tissues inside the body, efflux transporters are known to restrict drug access at the major blood-tissue barriers (Fig. 1) and therefore reduce therapeutic efficacy, notably in the central nervous system (Löscher and Potschka, 2005). In contrast, when effluxing environmental contaminants, they play a protective role by preventing their permeation and accumulation in sensitive tissues, such as brain, testis, or foetus (Klein and Cherrington, 2014; Myllynen et al., 2008; Oosterhuis et al., 2008). An exception to this protective role is in the lactating mammary glands in humans and farm animals, where BCRP and MDR1 handle the secretion of pollutants into milk, thereby exposing breast-fed infants and dairy consumers to harmful chemicals, such as certain carcinogen heterocyclic aromatic amines (Jonker et al., 2005; Van Herwaarden and Schinkel, 2006).

Furthermore, substrates of transporters in **excretory** organs, such as liver or kidney, are eliminated from the body into the bile or urine respectively. For example, OCT2, MATE1 and MDR1 transporters participate in the urinary elimination of the pesticide paraquat and thereby protect against its subsequent toxic renal accumulation (Chen et al., 2007; Wen et al., 2014). Furthermore, in the kidney, some chemicals are reabsorbed from the primitive urine back into the body *via* transporters. As an example, uptake of perfluorooctanoate (PFOA) *via* OAT4 at the apical side of proximal tubular cells has been shown to mediate renal reabsorption, which contributes significantly to the long half-life of this fluorochemical observed in humans (Yang et al., 2010). Of note, interactions between a network of transporters in a given tissue, such as uptake at the basolateral membrane and efflux at the apical side, may

Fig. 1. Transporters expressed at the external and internal biological barriers of the body where they can impact the absorption, distribution and excretion of a compound. Up-arrow: uptake; down-arrow: efflux.

result in a coordinated vectoral direction of transport for absorption, distribution and excretion.

Finally, transporters can work in concert with **metabolic** enzymes, notably in the liver to an extent that the uptake process has been called phase 0 and the efflux, phase III in relation to metabolic phase I and II (Döring and Petzinger, 2014). This transport-metabolism interplay is well-established for drugs (Shi and Li, 2014) and may need to be considered for chemicals as well. Investigating chemical metabolites as substrates of transporters might also be particularly important in cases where transporters are involved in handling metabolites instead or in addition to parent compounds (Chedik et al., 2018a; Lanning, 1996).

2.1.2. Chemicals that modulate or inhibit transporter activity

Various drugs have been withdrawn from the market following DDI due to transporter inhibition (Huang and Woodcock, 2010). Inhibition of transporters can occur through direct binding to transporters or *via* (non)-competitive mechanisms, as already described for drugs. Similarly, environmental pollutants could inhibit activity of the major drug transporters discussed here, but so far this has only been demonstrated *in vitro*. It is for example the case for various pesticides, which block MDR1 activity (Bain and LeBlanc, 1996), but also interfere with BCRP, OCT1 and OCT2 functions (Chedik et al., 2018a). In the same way, bisphenol A has been shown to block activity of different SLC transporters (Bruyere et al., 2017), whereas various marine persistent organic pollutants inhibit that of P-gp (Nicklisch et al., 2016). Similar to DDI, inhibition of transporter activity may result in pollutant-drug interactions, with the pollutant acting as the perpetrator. As an example, the *in vitro* assessed inhibition of OATP1B1 and -1B3 activity by various pollutants could lead to increased systemic exposure of their drug substrates, especially of the widely prescribed statins, potentially causing myopathy (Le Vee et al., 2015). In the skin, interactions between pollutants and cosmetic ingredients, such as sunscreen, and topical drugs may be hypothesised as well (Giacone et al., 2018).

Besides drugs, inhibition of transporter activity by pollutants may also affect the disposition of endogenous substrates of transporters, notably hormones or bile acids, which may contribute to some of the toxic effects of pollutants. Diesel exhaust particle extracts, for example, markedly inhibit OATP activity *in vitro*. This has been proposed to contribute to the endocrine disruption caused by those particles due to altered transport of endogenous steroid hormones (Le Vee et al., 2015).

Finally, post-translational modifications also affect the level of transporter activity (Czuba et al., 2018; Xu and You, 2017). Further investigations would be needed to assess the role of pollutants on post-translational modifications contributing to functional expression of transporters.

2.1.3. Chemicals that modulate transporter expression

Finally, drugs can alter transporter expression levels by inducing or repressing transcriptional activation in a tissue-specific manner. For example, the anti-tuberculosis drug rifampicin alters the mRNA expression of many drug transporters in skin, liver or renal cells, and severe transporter-mediated rifampicin-induced DDI have been reported *in vivo* (Benson et al., 2016; Jigorel et al., 2006; Osman-Ponchet et al., 2014). Similarly, chemicals, such as dioxin or organochlorine pesticides, have been shown to modulate transporter mRNA expression *in vitro* (Jigorel et al., 2006; Aleksunes et al., 2012; Bucher et al., 2014; Fardel et al., 2012). This kind of transporter modulation probably occurs by activating nuclear receptors, such as the aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Amacher, 2016; Lemaire et al., 2004).

Importantly, endogenous signaling molecules, such as hormones or bile acids, can modulate transporter expression levels, thereby impacting chemical TK. This is exemplified by the sex difference observed in serum half-life of the fluorochemical PFOA due to hormonally-regulated functional expression of OAT transporters involved in renal clearance (Worley and Fisher, 2015). While transporter induction or repression can impact kinetics, variations in transporter functional expression due to genetic polymorphisms play a key role in the inter-individual variability of pharmacokinetics and chemical TK (Engström et al., 2016; Maeda and Sugiyama, 2008).
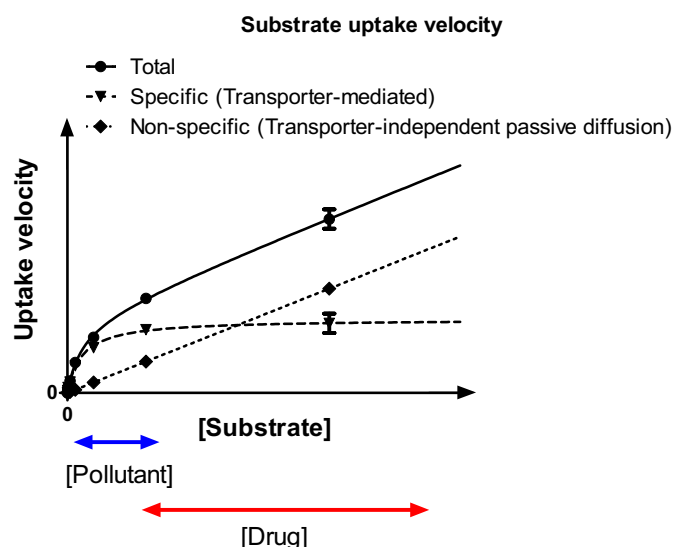
2.2. Chemical-transporter interactions: in vivo toxicological relevance in humans

Compared with the drug area, there is little, if any, direct evidence about the *in vivo* toxicological relevance of chemical-transporter interactions for humans. A key consideration when judging the relevance of chemical-transporter interactions is the relatively low levels of pollutants reached *in vivo*. Concentrations of pollutants in human blood are mostly in the pM or nM range (Chedik et al., 2018a), which is much lower than concentrations of administered drugs. This is the case for humans that are chronically-exposed to low occupational or environmental chemicals rather than high accidental or intentional (poisoning) exposure scenarios.

2.2.1. Chemicals as substrates of transporters

The fact that pollutant concentrations are usually low in environmentally-exposed humans suggests that when a pollutant is a high-affinity substrate for a defined transporter, the transporter is likely to be implicated in its passage across plasma membranes (Fig. 2). For uptake of substrate chemicals, the part of transporter-independent

Fig. 2. The relative impact of transporter-unrelated passive diffusion compared to transporter-mediated uptake across the membrane depends on the substrate concentration. At low substrate concentrations (usually the case for environmental pollutants), the relative contribution of uptake transporter-mediated velocity to total uptake is maximal. By contrast, at high substrate concentrations (as in drugs), the transporter-independent passive diffusion may be more significant.

passive diffusion may be minor compared to transporter-mediated uptake (Fig. 2).

This also has implications for efflux transporters that are not saturated, due to the low chemical concentrations, and may thus limit chemical transfer by continuously expelling it. This could happen for example in the gut, at the blood-brain or placental-fetal barrier where efflux transporters may limit access of chemicals to the gut, brain or foetus, respectively. The lipophilic or hydrophilic nature of the chemical should also be considered as this will contribute to its major transport pathway.

2.2.2. Chemicals inhibit transporter activity

Similarly, for inhibition of transporter activity, pollutant concentrations required *in vitro* have been seen in the 1–100 μM range, thus much higher than realistic *in vivo* blood concentrations (Chedik et al., 2018a). Furthermore, P-gp and BCRP, being generally co-expressed at the same sites and having broad and partly overlapping substrate specificity, are believed to act synergistically to potentiate the barrier effect (Agarwal et al., 2011; Burger et al., 2004; DeVries et al., 2007; Polli et al., 2009). Whether pollutants act *in vivo* to inhibit this P-gp/BCRP synergy remains to be assessed. In the pharmaceutical sector, ITC experts concluded that the levels of marketed drugs are not sufficient at the human blood-brain barrier to inhibit the efflux transporters to a clinically relevant extent (Kalvass et al., 2013). It follows then that *in vivo* transporter inhibition by pollutants is unlikely to occur due to low chemical concentrations. This likely precludes chemical-drug, chemical-chemical or chemical-endogenous substrate interactions in humans. However, this assertion can be challenged. First, the tissue concentration also has to be considered since it might be much higher than in the plasma (as cell volume is much smaller) and thus can lead to inhibition of carrier-mediated transport dependent of the intracellular concentration. This is particularly relevant for efflux transporters whose substrate binding sites are within the cell (membrane), whereas the binding sites of uptake transporters are facing blood. However, intracellular concentrations are often poorly characterised, even for drugs. Secondly, similar to the occurrence of polypharmacy in the drug area, humans are rarely exposed to a single contaminant but rather to mixtures of pollutants. Inhibition of transporter activity by pollutants

may impact other pollutant kinetics, inducing pollutant-pollutant interactions and those inhibitory effects towards transporters may have synergistic effects (Chedik et al., 2018b). And it is worth to note that chemical concentrations targeting transporters are likely much higher than those interacting with their targets, which may impact TD.

2.2.3. Chemicals modulate transporter expression

Consistent with the previous discussion, the *in vivo* concentration of a single chemical is most likely lower than the concentration required to modulate transporter expression levels, making this type of interaction less likely to occur *in vivo*. However, once again, the combined effects of multiple chemicals on transporter expressions could be of relevance, especially if the interaction leads to synergistic effects.

Overall, chemicals as substrates, inhibitors or modulators of transporter activity or expression may impact ADME processes at the different key biological external and internal barriers, just as drugs do. However, some chemical specificities have to be taken into account: (i) the concentrations of pollutants in human blood are generally much lower than administrated drugs, rendering it less likely that the chemical-transporter interactions are of relevance for human TK, except maybe in the case of mixtures; (ii) the given dose of a drug is known, which is usually not the case for environmental chemicals; and (iii) CRA scientists lack human *in vivo* evidence. Such disparities make it challenging to directly translate transporter-mediated interactions data and reasoning from the pharmaceutical sector to environmental toxicology. CRA-specific approaches will be required to evaluate the *in vivo* relevance of chemical-transporter interactions in humans.

3. Current applications of transporter kinetic data to support CRA

Even if the *in vivo* relevance of chemical-transporter interactions remains to be clearly demonstrated, transporter kinetic data can still be applied for CRA purposes, such as improving *in vitro* to *in vivo* extrapolation (IVIVE), better characterising *in vitro* toxicological assays, or prioritising further assessments (Clerbaux et al., 2018).

In the context of IVIVE, the inclusion of transporter kinetic data has already been shown to correct *in vitro* to *in vivo* discrepancies due to transporter differences related to sex, species, life-stage, diseases, diet and transporter polymorphisms (Mallick, 2017; Ménochet et al., 2012; Worley and Fisher, 2015; Yoon et al., 2014; Zhang et al., 2017; Zhang and Unadkat, 2017). Importantly, IVIVE inconsistencies could also be due to transporter artefacts associated with the *in vitro* systems used to evaluate the cytotoxic or metabolic effects of chemicals (Fischer et al., 2017; Zaldivar Comenges et al., 2017). *In vitro* metabolic results, for example, are dependent on the real intracellular concentration since metabolism occurs inside the cells. Such intracellular concentration is modulated not only by passive but also active transport across the membranes. If the *in vitro* system used does not thoroughly recapitulate the *in vivo* transportome profile, the nominal concentration added to the system will not correspond to the real intracellular concentration. In such cases, it will be difficult to predict correctly the dose-response relationship in this chosen assay. Polarity, expression and maintenance of transporter activity, representing the transportome profile, have to be monitored carefully and compared to *in vivo* situations in order to extrapolate data obtained from *in vitro* cell culture into *in vivo* metabolic or toxicity information (Godoy et al., 2013).

As an example, the difficulties in maintaining a differentiated hepatocyte phenotype over time in culture result in strongly reduced expression of some of the transporters (Godoy et al., 2013). Also while passive diffusion is comparable between rat and human hepatocytes, interspecies differences have been observed for active uptake, thereby confounding direct scaling of clearance rates obtained in rat hepatocytes to humans (Ménochet et al., 2012). Furthermore, frequent IVIVE underpredictions of *in vivo* hepatic clearance based on *in vitro* metabolic studies are due to the fact that active uptake actually represents the rate-limiting step of metabolism which is not taken into account (De

Bruyn et al., 2016; Lundquist et al., 2014; Parker and Houston, 2008; Soars et al., 2007).

Similarly, in various cell-based systems used in nephrotoxicity evaluation, such as human kidney proximal tubule epithelial cells (HK − 2), the mRNA expression of uptake transporters (OAT1, OAT3 and OCT2) was not detected and the mRNA expression of the apical efflux transporters (MDR1, MRPs) was low relative to normal *in vivo* human tissue levels, calling into question the predictive value obtained for transporter related toxicities (Jenkinson et al., 2012; Tiong et al., 2014). For example, OCT2 mediates the uptake of compounds into tubular cells, thereby inducing their nephrotoxicity. Reduced uptake functionality in HK-2 cells will give underpredicted nephrotoxicity data for chemicals that are OCT2 substrates (Nieskens et al., 2018).

Globally, transporter mRNA/protein levels and activities are affected by culture conditions in cell-based systems (Godoy et al., 2013; Tiong et al., 2014). Thus, systematically characterising the transportome profile of the *in vitro* systems used for toxicity testing is a prerequisite to assure their relevance for CRA purposes.

Finally, humans are continuously exposed to thousands of chemicals, but only a small portion of them have undergone significant toxicological evaluation, leading to the need to screen and prioritise the remaining unevaluated chemicals. Using transporter data could help to develop screening and prioritisation strategies (Guseman et al., 2016).

4. Existing tools and future needs to investigate chemical-transporter interaction without animal studies

Several methods and tools have been developed over the years to study transporters. These have been principally developed by pharmaceutical scientists, but could benefit the toxicological community. However, several challenges in the interpretation and use of transporter data still exist. Some are common to pharmaceutical and toxicological safety assessment, while others are specific to CRA. The latter are further discussed here.

4.1. Experimental tools

4.1.1. In vivo: human data and animal studies

(Pre)-clinical, epidemiological and imaging studies in humans with transporter genetic variants have highlighted the role played by transporters in the *in vivo* disposition of drugs (Giacomini et al., 2010). For pharmaceutical compounds, kinetics may ultimately be fully characterised during human clinical trials, while this is clearly not the case for environmental contaminants. Performing TK assays for pollutants on humans is not conceivable due to ethical considerations. Therefore the amount of human *in vivo* TK data for pollutants is still very limited (Fardel et al., 2012). Some associations however between chemicals, diseases and transporter polymorphisms may support the *in vivo* role played by transporters for pollutants, such as MDR1 polymorphism associated with Parkinson's disease and exposure to pesticides, or with colorectal cancer and ingestion of chemical carcinogens found in meat (Andersen et al., 2009; Narayan et al., 2015). In the quest for human TK data to support CRA, biomonitoring studies can provide chemical concentrations in blood, urine, breast milk or sweat offering essential data that can be used to validate predictions generated by non-animal kinetic studies. Furthermore, besides exogeneous substrates such as drugs and pollutants, transporters have physiological substrates. Referred to as biomarkers, the evaluation of the urine or plasma concentration of endogenous substrates is emerging as a potentially powerful tool to assess the *in vivo* functionality of transporters in humans (Chu et al., 2018; Yee et al., 2016). As examples, pyridoxic acid and homovanilic acid may serve as plasma biomarkers of OAT1 and/or OAT3 activities while coproporphyrin I and III may constitute suitable *in vivo* biomarkers to gauge OATP1B1 and/or 1B3 activity (Shen et al., 2018, Shen et al., 2017). However, despite this progress, the biomarker list needs to be further expanded for most of transporters, and the

existing biomarkers need further characterisation and validation (Rodrigues et al., 2018).

In addition to human data, the use of various knock-out, mutated or humanised mice have illustrated the *in vivo* role of transporters in the disposition of drugs (Durmus et al., 2016; Jiang et al., 2011; Klaassen and Lu, 2008) and environmental contaminants such as mycotoxins, insecticides, mercury or food carcinogen (Lu et al., 2008; Torres et al., 2011; Vlaming et al., 2014). Non-invasive imaging as used in the pharmaceutical industry (Ricketts et al., 2011) can reduce the number of animals and can allow refinement of animal experimentation when exploring chemical TK. However, animal studies are banned in the cosmetics sector in the EU (EU Cosmetics Regulation) and are starting to be phased out in the US. Furthermore, animal studies are time- and resource-consuming and could pose a challenge to scientists in evaluating species differences when extrapolating the findings to humans.

4.1.2. In vitro: expression and cellular systems – barrier models

The plethora of *in vitro* assays currently available to study transporters (Volpe, 2016) and their applications and limitations have been extensively reviewed (Brouwer et al., 2013; Giacomini et al., 2010; Riley et al., 2016; Zamek-Gliszczynski et al., 2013). Briefly, two general types of *in vitro* systems are used to study active transport kinetics: (i) expression systems overexpressing a defined transporter, spanning from membrane-based vesicles, oocytes, to transporter-transfected or -transduced immortalised cell lines (*e.g.* CHO, HEK, HeLa or MDCKII) and (ii) cellular systems presenting overall transportome profile, including primary cells (*e.g.*, hepatocytes) and relevant cell lines (*e.g.*, Caco-2, HepaRG).

Expression systems can be used to estimate kinetic parameters for the overexpressed transporter. By contrast, cellular systems can be optimised to estimate kinetic parameters specific to uptake, metabolism or efflux, as well as the interplay of multiple processes (Zamek-Gliszczynski et al., 2013). The cellular systems currently available as external biological barrier models (lung, skin, gut), in which transporters are directly implicated in the barrier properties, have been reviewed in (Gordon et al., 2015). In the case of the placental barrier, the utility of a limited number of *in vitro* human cell lines have been reviewed in the context of studying the role of placental transporters on fetal exposure (Vähäkangas and Myllynen, 2009).

Even if simplified *in vitro* systems are routinely used to study transporters, important concerns are still raised regarding the use of these conventional approaches (Clerbaux et al., 2018; Mallick, 2017). In common with the pharmaceutical field, improvements of current methodology for transporter interaction studies are needed, while considering the economic, reproducibility and high-throughput capacity aspects. The sharing and standardisation of protocols should be the next priority to ensure high-quality data that are consistent across laboratories. This may require interlaboratory validation studies involving also the drug sector, such as the 'P-gp IC50 Initiative' established to assess interlaboratory variability in the determination of P-gp inhibitory potency (Bentz et al., 2013). The various experimental parameters impacting transporter mRNA/protein and functional expression should be clearly defined when developing, validating or using a given transporter *in vitro* method (Clerbaux et al., 2018; Riley et al., 2016). Thus, similar to the need for transportome characterisation in toxicological test systems (as detailed in point 3), experts emphasised the need to quantitatively characterise transporter abundance and activity in experimental transporter systems and barrier models. This should ensure (i) consistency across laboratories and (ii) reliable IVIVE in comparison to their *in vivo* counterparts (Clerbaux et al., 2018; Gordon et al., 2015). To this end, *in vitro* systems could be characterised against a set of uptake and efflux markers (Ménochet et al., 2012) or *via* a LC-MS/MS proteomic-based strategy (Kumar et al., 2015; Sakamoto et al., 2013). By knowing the differences between *in vitro* and *in vivo*, the performance of the cellular model could be improved by treating cells with corresponding known inducers or inhibitors, or using the

knowledge as a correction factor within *in silico* predictive models (Clerbaux et al., 2018; Turco et al., 2011).

In addition, systemic regulators of transporter expression could be lost *in vitro* resulting in poor predictive value. As an example, *in silico* models considering hormonally-regulated renal transporter expression were used to correct *in vitro* data to match the sex-difference of PFOA plasma levels observed *in vivo* in rats (Worley and Fisher, 2015). However, in both the toxicology and pharmacology fields, regulation of transporter expression is understudied due to a lack of adequate experimental systems (Clerbaux et al., 2018). Furthermore, information of transporter expression regulation is not presently required for drugs, according to the FDA and EMA recommendations. However, information on transporter induction or repression is of particular importance for CRA purposes, as no human *in vivo* data are available, in contrast to the plethora of clinical data available in pharmacology. Studying transporter expression regulation could also be particularly relevant for chronic exposure to environmental chemicals.

Finally, investigators should be clear on their research question so as to select the right method and experimental system. A good understanding of the limitations of the system is essential to ensure that data are analysed and interpreted in an appropriate fashion (Riley et al., 2016).

Regarding CRA more specifically, the amount of existing *in vitro* data assessing transporter-chemical interactions is low. High-throughput *in vitro* screening experiments are needed to generate information on chemical-transporter interactions (Chedik et al., 2018a; Fardel et al., 2012). When considering large series of chemicals, it is probably easier to search for inhibitors first and then investigate positive compounds as potential substrates. The rationale is that (i) substrates can often inhibit the transport of other compounds by competitive mechanisms and (ii) the search for inhibitors can be performed with fluorescent probes in high-throughput assays whereas identifying transporter substrates requires a dedicated analytical method for each chemical, which may be expensive and time-consuming. However, inhibitors are not necessarily substrates as the capacity to inhibit a transporter also depends on the nature of the substrate (Hacker et al., 2015). Direct measurement of the transport of a chemical, if possible, therefore remains the gold standard approach for evaluating chemical-transporter interactions.

4.1.3. In silico: computational models

In combination with experimental studies, several *in silico* tools have been developed to evaluate transporter-substrate interactions or to integrate active transport data at a systemic level. Quantitative Structure-Activity Relationship (QSAR), pharmacophoric and docking models are used for virtual profiling of chemical-transporter interactions as substrates or inhibitors (Danielson et al., 2018; Pajeva and Christoph Globisch, 2009; Sedykh et al., 2013; Welch et al., 2015; You et al., 2015). Physiologically-based kinetic (PBK) modelling enables the quantitative description of the ADME processes (Kim et al., 2017) and the relative importance of transporters in driving the *in vivo* TK of chemicals, as already shown for PFAO or polychlorobiphenyl (Lohitnavy et al., 2008; Worley and Fisher, 2015). Recently, FDA scientists reported the use of PBK models as a tool to evaluate the contribution of major transporters to drug ADME processes, and summarised the PBK models provided in regulatory submissions by drug developers to illustrate the transporter-related questions that arise in light of regulatory assessments (Pan et al., 2016). A similar exercise with the available data would benefit the toxicological community. Furthermore, PBK models are increasingly being used as an effective tool for designing toxicology experiments and for conducting extrapolations essential for RA (Paini et al., 2017b; Paini et al., 2019). Commercial PBK modelling software tools, such as Simcyp, PKSim and GastroPlus among others, allow predictions of drug disposition in virtual patient populations. QSAR-based software tools, including free ones such as SwissADME (Daina et al., 2017), allow the evaluation of

passage across biological barriers and/or interactions with transporters, mainly P-gp. SwissADME was used to predict the intestinal absorption and transport across the blood-brain barrier of a large set of pesticides (n = 338) (PMID 28665355).

Finally, Deep Learning could also dramatically influence predictive toxicology, encompassing TK and ADME processes predictions, in the near future (Ekins, 2016; Mayr et al., 2016). Similar to the successes in the other fields, such as vision recognition, Deep Learning outperformed many other *in silico* approaches, considerably improving the predictive performance of computational methods in toxicology (Mayr et al., 2016).

While the computational field is evolving rapidly, several challenges still exist in the interpretation and use of *in silico* transporter data for assessing the safety of both drugs and chemicals. These challenges include mechanistic understanding of transporter biology or interaction, robustness of the *in silico* models, broad substrate specificity of transporters, accuracy or size of the dataset, lack of metabolite libraries or divergent membrane topologies (Clerbaux et al., 2018; Giacomini et al., 2010). Again, quantitative knowledge of transporters at protein and functional levels in cell lines and tissues, and for multiple species, should be systematically and carefully reported. This knowledge can be applied as a scaling factor in PBK models, such as for oral drug absorption (Harwood et al., 2012). To enhance the overall predictive performance of transporter-based PBK models, it is necessary to have a mechanistic understanding of transporter biology for proper representation in the PBK models in addition to knowledge of transporter specificity for the chemical of interest (Pan et al., 2016; Clerbaux et al., 2018).

Lastly, of particular relevance for CRA, the uncertainties in computational biokinetic models have to be systematically characterised and documented in order for risk assessors and regulators to evaluate their confidence in the models (Clerbaux et al., 2018). This is especially important in cases where no or few clinical data are available for model calibration/validation.

4.2. Decision-support tools

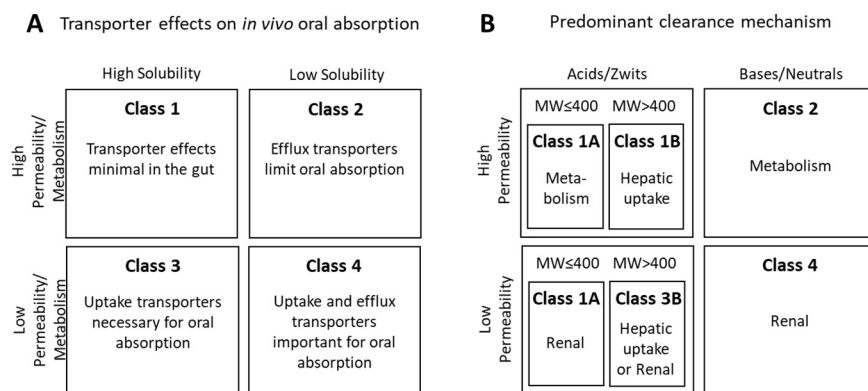#### 4.2.1. Guidelines and guidance documents

Guidance documents on the conduct of transporter studies have been released by drug regulatory agencies (EMA, 2012; FDA, 2012, 2017a; MHLW, 2014). They recommend investigating the involvement of transporters in the absorption, distribution and excretion processes of new compounds and their interplay with metabolic enzymes as well as their implication in DDI. Not all transporters have to be studied in all cases. To support drug developers with the choice and interpretation of relevant transporter studies, flow diagrams and decision trees represent a central part of the regulatory recommendations. Similar decision trees adapted for chemicals could represent a valuable roadmap to orient industrials on relevant transporter investigations.

Furthermore, despite the usefulness of transporter kinetic data and of TK in general, there are few legal requirements in the EU and US chemicals legislation for the generation of TK data, and not at all for transporter studies. However, the use of TK data, including transporter kinetic parameters, is widely recommended to support the assessment of systemic toxicity of chemicals using alternative approaches to animal testing (Bessems et al., 2014; Casati et al., 2013; Corvi et al., 2013; ECHA, 2014; EFSA, 2014; Prieto et al., 2014).

4.2.2. Classification systems

Similar to decision trees, classification systems represent invaluable tools to avoid costly and inefficient testing in early drug discovery. The biopharmaceutics classification system (BCS), which classifies drugs in four classes according to their permeability and solubility, was first established to predict *in vivo* oral absorption from *in vitro* measurements (Amidon et al., 1995). Based on this, the FDA supports waivers of bioequivalence studies for highly permeable and highly soluble BCS

A Transporter effects on *in vivo* oral absorption

	High Solubility	Low Solubility
High Permeability/ Metabolism	**Class 1** Transporter effects minimal in the gut	**Class 2** Efflux transporters limit oral absorption
Low Permeability/ Metabolism	**Class 3** Uptake transporters necessary for oral absorption	**Class 4** Uptake and efflux transporters important for oral absorption

B Predominant clearance mechanism

	Acids/Zwits		Bases/Neutrals
High Permeability	MW≤400 **Class 1A** Meta-bolism	MW>400 **Class 1B** Hepatic uptake	**Class 2** Metabolism
Low Permeability	MW≤400 **Class 1A** Renal	MW>400 **Class 3B** Hepatic uptake or Renal	**Class 4** Renal

Fig. 3. Predictions of transporter effects on *in vivo* intestinal absorption using the B(DD)CS (A) and of predominant clearance mechanisms using the ECCS (B) based on some physicochemical characteristics of the compound, such as permeability, solubility, molecular weight and/or ionization state. Adapted from (Benet, 2013; Varma et al., 2015).

Class 1 drugs (FDA, 2017b). The BCS, which is the most highly cited paper in the pharmaceutical sciences, has driven the development of similar approaches. First, the observation that compounds with high intestinal permeability (Class 1 and 2) are mainly eliminated by metabolism while others (Class 3 and 4) are eliminated unchanged by biliary or renal excretion led to the development of the biopharmaceutics drug disposition classification system (BDDCS), in which the permeability parameter was replaced by the extent of metabolism (Wu and Benet, 2005).

Interestingly, this classification scheme allows predictions of uptake and efflux transporter effects on *in vivo* oral absorption (Fig. 3A). Highly permeable and highly soluble Class 1 compounds may be substrates for transporters *in vitro*; however due to their high solubility, high concentrations in the gut saturate transporters so that transporter effects on oral absorption may be minimal *in vivo*. Highly permeable Class 2 compounds may pass through the gut membranes by passive diffusion unaided by uptake transporters. However, their low solubility will limit their intestinal concentration, thereby preventing saturation of efflux transporters. Consequently, efflux transporters may limit intestinal absorption of Class 2 compounds (Wu and Benet, 2005). For the poorly permeable Class 3 compounds, uptake transporters will be necessary for absorption and efflux transporters may be important when sufficient permeation is achieved *via* an uptake transporter. Due to the low permeability and low solubility of Class 4 compounds, both uptake and efflux transporters play an important role in oral absorption (Fig. 3B) (Wu and Benet, 2005).

Reflecting the high degree of interest in these systems and expanding from predictions of oral absorption, the extended clearance classification system (ECCS) was proposed to identify the predominant clearance mechanism based on the ionization state, molecular weight and permeability generated from *in vitro* and *in silico* methods (Varma et al., 2015). ECCS Class 1B covers compounds for which transporter-mediated hepatic uptake is the rate-determining clearance process (Fig. 2B). Based on this, the Hepatic Clearance Classification System and Extended Clearance Model have been proposed focusing on hepatic clearance (Camenisch et al., 2015; Fan et al., 2014). More recently, a classification system for excipients (BCSE) has been proposed based on the ability of an excipient to interfere with intestinal metabolism and efflux mechanisms (Vasconcelos et al., 2017).

By predicting the effects of transporters on oral absorption and major routes of elimination (metabolism, hepatic or renal), similar classification systems for chemicals would clearly aid in choosing the right methodology for incorporating relevant transporter studies to support CRA without the need for animal studies. However, as already stressed, environmental pollutants will be present in relatively low intestinal concentrations compared to administered drugs. Consequently, to predict oral absorption, no chemicals would be expected to behave as BCS-BDDCS Class 1 or 3. All highly permeable chemicals would act as Class 2 ("efflux transporter limit oral absorption") even if highly soluble, as no saturation of the efflux pumps would be achieved, in

contrast to drugs. While chemicals with low permeability, even highly soluble ones such as glyphosate, would likely behave as Class 4 ("efflux and uptake transporters impact oral absorption"), if they are substrates of transporters, considering that their intestinal concentration remains low. As the concern for pollutants is generally the opposite as for drugs, *e.g.* to limit bioavailability, this would be protective. However, highly soluble and permeable pollutants could still behave as Class 1 and pass through the membranes if they are not substrates of efflux transporters. These considerations are still theoretical and a classification system built on chemical datasets, taking into consideration low intestinal concentrations, would clearly represent a useful tool to support animal-free CRA.

It is important to note that the BCS, BDDCS, ECCS and BCSE systems allow for reliable predictions of oral absorption, the major routes of elimination, and the impact of transporters at intestinal, hepatic and renal level. However, they may have limited value for predicting (transporter-mediated) dermal absorption, inhalation or penetration at the internal blood-tissue barriers relevant for reprotoxicity, such as the placenta or testis. For the brain, it has been shown that the prediction of brain disposition of orally administered drugs may be improved using BDDCS (Broccatelli et al., 2012). Similarly, the same system as BCSE could be applicable to cosmetic ingredients based on their interactions with transporters identified in the skin. However, there is need to characterise the transporter interactions of the main excipients used in cosmetic products.

4.3. Knowledge sharing tools

4.3.1. Transporter databases

Several large-scale efforts have already been implemented to systematically gather transporter data into databases (Table from Clerbaux et al., 2018, summarising existing transporter databases). However, such databases mostly constitute transporter information for drugs, while databases based on environmental chemicals, such as pesticides, metals, food contaminants or cosmetic ingredients, are lacking. Again, several experts emphasised that there is a need for publicly available databases reporting transporter protein levels and activities in cell lines *versus in vivo* tissues, across species and in normal and diseased human individuals (Clerbaux et al., 2018; Gordon et al., 2015).

4.3.2. Adverse Outcome Pathway (AOP)

A way forward to help scientists in designing relevant transporter-related CRA studies and risk assessors in evaluating them, may be the use of integrated framework like the Adverse Outcome Pathway (AOP) approach (Wilks and Tsatsakis, 2014). AOPs are designed to provide a structured mechanistic representation of critical toxicological effects that span over different layers of biological organisation (Vinken, 2013). They are a modular-linear representation of a sequence of events, consisting of a molecular initiating event (MIE), a series of intermediate steps, called key events (KE) linked by key event

relationships (KER), and an adverse outcome (AO) (Villeneuve et al., 2014). OECD guidelines have been published to support the development of AOPs (OECD, 2018). During the development and evaluation stages, AOPs are available in the AOP wiki (https://aopwiki.org/aops) with different status. AOPs can serve different purposes, including the establishment of computational prediction models for regulatory toxicology (Wittwehr et al., 2017), such as QSARs to describe membrane transport interactions, or to help in better inclusion of transporter data into CRA.

Several AOPs are related to membrane transporters. As examples, the AOP 27 in a late stage of evaluation, describes cholestasis triggered by drug-mediated inhibition of the BSEP transporter, whereas the AOP 138 still under evaluation, is related to the OAT1 transporter inhibition leading to renal failure and mortality. Another drug transporter-mediated adverse outcome that would worth to be developed into AOP for human CRA purposes is the potential inhibition by endocrine disruptor chemicals, such as bisphenol A, of transporter(s) mediating uptake or efflux of hormones (FitzGerald and Wilks, 2014).

5. Scientific and regulatory perspectives

The extensive knowledge and data on drug transporters gained in the pharmaceutical sector could be adapted, improved and used in the chemical safety field, especially to gain increased confidence within an animal-free strategy. Compared to the pharmaceutical sector, however, CRA scientists are confronted with a lack of available human data, relatively low chemical concentrations upon environmental exposure, unknown exposure levels and potential synergistic mixture effects. All these factors reinforce the importance of developing reliable CRA-adapted methods, incorporating appropriate chemical-transporter interactions, to gain confidence in CRA based on *in vitro* and *in silico* methods.

Current integrative *in vitro-in silico* approaches that are kinetically and mechanistically informed, starting with *in vitro* and QSAR methods to generate input parameters for PBK modelling, including estimates of transporter interactions. Model predictions need to be validated with available and relevant *in vivo* data. In this review, we highlighted some areas for further improvements in CRA, including the need to: (i) characterise toxicological (cytotoxic or metabolic) *in vitro* methods in terms of their transportome profile; (ii) develop and perform high-throughput transporter *in vitro* assays to generate data on chemical-transporter interactions; (iii) develop transporter databases and classification systems trained on *in vitro* chemical-based datasets; (iv) make better use of human biomonitoring data to evaluate the *in vitro-in silico* predicted values; and (v) develop means to quantitatively assess uncertainties.

Furthermore, although a vast amount of knowledge has been gained during recent decades regarding transporters in many tissues of the body, there is still limited data in other toxicologically relevant organs. For example, at the blood-eye barrier, there is a lack of direct information on the role of transporters in humans. Some data indicate that transporters could be involved in the ocular disposition of compounds (Lee and Pelis, 2016; Nakano et al., 2014; Stieger and Gao, 2015; Tomi and Hosoya, 2010). However, more studies are needed to unravel the role of transporters in eyes, especially relevant for topically applied compounds such as cosmetics. Finally, despite their importance in toxicology, very little is known regarding the functional relevance of drug transporters in cardiovascular organs such as the heart, or in slowly perfused fat tissues. These tissues are hypothesised to accumulate some xenobiotics which may be substrates for tissue-specific drug transporters (Couture et al., 2006; Grube et al., 2006; Moreno-Navarrete et al., 2011). Additionally, transporter-independent diffusion of lipophilic chemicals may contribute to their tissue accumulation, notably in fat tissues (La Merrill et al., 2013).

Finally, transporters are quite well conserved through evolution as they play key roles in ADME processes and are protective in keeping xenobiotics out of tissues and organisms. This means that the impact of chemical-transporter interactions on environmental species could be similar to humans and in some cases even threaten the sustainability of a population (Epel et al., 2008; Hawthorne and Dively, 2011; Luckenbach et al., 2014). Further studies on environmental species, such as bees or aquatic organisms, could provide *in vivo* relevance for chemical-transporter interactions. Similarly, transporter kinetic data could represent key determinants of environmental toxicology.

In conclusion, this review outlines some of the opportunities and challenges in considering chemical-transporter interactions in support of kinetically informed CRA without the need for animal studies. In doing so, we hope that it initiates dialogue between the various stakeholders world-wide to collaboratively draft relevant decision trees and guidance documents for chemical-transporter interaction to promote resource-effective toxicity testing while increasing the confidence of scientists and regulators in animal-free CRA.

Disclaimer

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