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PERSPECTIVE

Disruption of small molecule transporter systems by Transporter-Interfering Chemicals (TICs)

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Small molecule transporters (SMTs) in the ABC and SLC families are important players in disposition of diverse endo- and xenobiotics. Interactions of environmental chemicals with these transporters were first postulated in the 1990s, and since validated in numerous *in vitro* and *in vivo* scenarios. Recent results on the co-crystal structure of ABCB1 with the flame-retardant BDE-100 demonstrate that a diverse range of man-made and natural toxic molecules, hereafter termed transporter-interfering chemicals (TICs), can directly bind to SMTs and interfere with their function. TIC-binding modes mimic those of substrates, inhibitors, modulators, inducers, and possibly stimulants through direct and allosteric mechanisms. Similarly, the effects could directly or indirectly agonize, antagonize or perhaps even prime the SMT system to alter transport function. Importantly, TICs are distinguished from drugs and pharmaceuticals that interact with transporters in that exposure is unintended and inherently variant. Here, we review the molecular mechanisms of environmental chemical interaction with SMTs, the methodological considerations for their evaluation, and the future directions for TIC discovery.

Keywords: ABC transporter; allosteric; chemosensitization; endogenous substrate competition; environmental; mixtures; signaling interference; SLC transporter; small molecule transporter; transporter-interfering chemicals

Introduction–Evolution and function of the small molecule transporter system

Selective transport of diverse small molecules across the plasma membrane is central to intercellular communication and the interaction of organisms with their environment. These molecules include toxic xenobiotics in the environment, such as the byproducts of microbial metabolism, like secondary bile acids and short chain fatty acids, and/or endobiotics such as the diverse signal molecules including uric acid, prostaglandins, and cyclic nucleotides, necessary for coordinating cell behavior [1–8]. The major transporters responsible for these molecular movements are members of the ATP-binding cassette (ABC) and solute carrier (SLC) families (Fig. 1). These small molecule transporters (SMTs) are expressed at environmental barriers such as the epithelial cells lining the gut, where they can export toxic compounds for excretion [9,10]. They are also highly expressed in stem cells and embryos [11–17].

In humans, there are more than 800 transporters, including 393 SLC and 81 ABC-type transporter proteins [18]. Seven, comprised of two ABC-type (ABCB1 and ABCG2) and five SLC (OAT1, OAT3, OCT2,

Abbreviations
ABC, ATP-binding cassette; DDI, drug–drug interaction; IMV, inverted membrane vesicle; MDR, multidrug resistance; SLC, solute carrier; SMT, small molecule transporters; TIC, transporter-interfering chemicals.

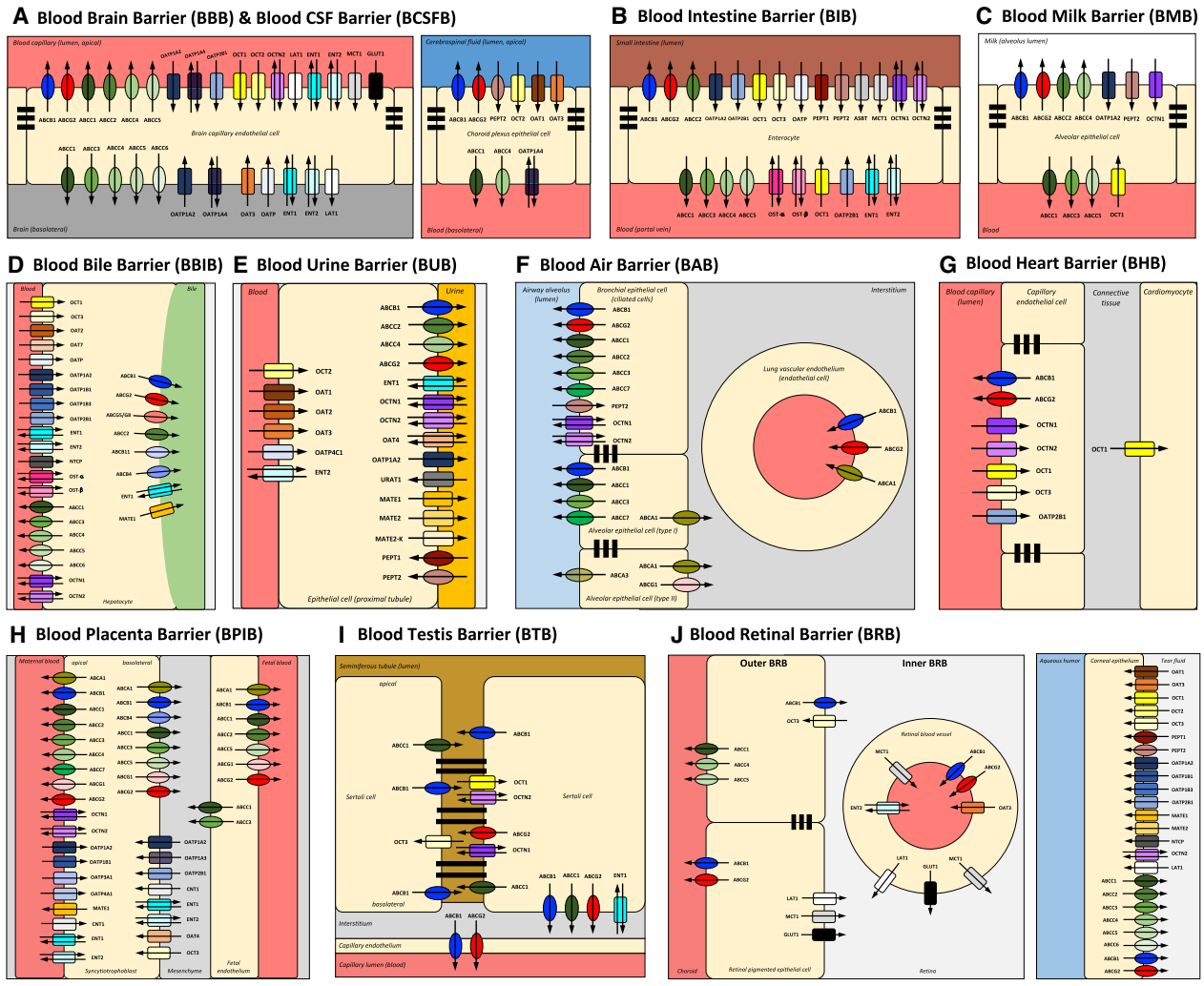


Fig. 1. Subcellular localizations of ABC- and SLC-type SMTs in 10 different biological barriers. Apical and basolateral membrane localization of ABC and SLC transporters in the indicated cell type. The anticipated direction of substrate and co-substrate flow are marked with arrows. Tight junctions are displayed as a group of three black bars in each cell type. (A) Blood–brain barrier and blood–cerebrospinal fluid barrier [20,201,217–225]. (B) Blood–intestine barrier [10,20,201,225–228]. (C) Blood–milk barrier in mammary glands [229–231]. (D) Blood–bile barrier in the liver [20,201,225,232–236]. (E) Blood–urine barrier in the kidney [20,201,237–240]. (F) Blood–air barrier in lung epithelial and endothelial cells [241–244]. (G) Blood–heart barrier [245–248]. (H) Blood–placenta barrier [73,225,249–254]. (I) Blood–testis barrier [255–260]. (J) Blood–retinal barrier in the eye [225,261–264]. Note that the common names for SLC-type transporters are used and the HUGO nomenclature for ABC-type transporters (https://www.genenames.org).

OATP1B1, and OATP1B3) transporters, are already known to be of major importance in clinical drug interactions and relevance to toxicity [9]. The list has been growing to include additional transporters of emerging importance, including the multidrug and toxin extrusion transporters (MATEs), multidrug resistance-associated proteins (MRPs), and the bile salt export pump BSEP (ABCB11) [19,20].

Among the key features of many of these proteins is a broad substrate specificity—sometimes termed 'polyspecificity'—that enables the interaction of a single transporter with numerous substrates [21–24]. As a result of this substrate promiscuity, SMTs also interact with the panoply of anthropogenic small molecule pollutants to which humans and other organisms are exposed. Indeed, the idea that environmental chemicals interact with transporters was demonstrated almost 30 years ago [25,26] and later elaborated on in numerous studies using purified proteins, model organisms, and cell lines [14,27–35]. The results pointed to a diverse range of ligands including pesticides, flame retardants, oil hydrocarbons, stain repellents, personal

care products (PCPs), and numerous other ubiquitous environmental chemicals. Importantly, as these chemicals are regularly detected in the environment, humans and wildlife are continuously exposed.

These chemicals pose a possible threat to health through their potential to interfere with the functioning of the SMT system. While there is a large and growing literature on the interaction of transporters with environmental chemicals, the mechanisms of interaction and the implications considered remain limited.

In analogy to treatment of multidrug resistant cancers with drug transporter inhibitors, most of the previous studies examined the role of environmental chemicals as competitive inhibitors that limit detoxification capabilities of organisms, thereby acting as 'chemosensitizers' [26,36,37]. However, as we will elaborate upon in this review, emerging structural and functional studies of transporters reveal that the interactions of chemicals with drug transporters can be complex, due to the existence of multiple ligand binding sites in these proteins and additional allosteric interactions [38,39]. Indeed, modern drug discovery and development efforts already seek to evaluate and validate transporter substrates, inhibitors, or noninteracting compounds in the context of various confounding factors, including the type of assay system, physicochemical properties of the test compound, and mixture effects on the overall transport kinetics [40–46].

Here, we posit that the interactions of 'drug' transporters with environmental chemicals are likely to be more intricate than simply dose-dependent inhibition of transporter function. A number of additional effects including stimulation, partial inhibition, and/or interference with transporter-mediated signaling could lead to a range of adverse effects including unanticipated drug interactions and developmental defects through physiological disruptions. We discuss the potentially unanticipated mechanisms and implications of transporter-interfering chemicals (TICs).

Identity of transporter-interfering chemicals

TICs—more than just inhibitors

Considering the promiscuity of SMTs for their ligands, it is not surprising that there are a diverse range of natural and anthropogenic chemicals that interact with these transporters (Table 1). Several terms have been used to describe TICs in the prior literature, perhaps most frequently with authors referring to them as transporter 'inhibitors' or 'chemosensitizers'. However, as will be elaborated upon in this review, this partially reflects the fact that most assays to study these environmental chemicals are best suited to revealing inhibitory interactions. As has been shown in numerous structural studies [21,47–52], many of the key SMTs, such as ABCB1 have large binding pockets capable of binding the same ligand in different locations or even multiple different ligands simultaneously, leading to nonmonotonic dose–response relationships of transporters with their ligands [53–56].

Transporter inhibition and ATPase stimulation can be properties of the same compound. For example, the potent ABCB1 transporter stimulators, verapamil and nicardipine, can also act as inhibitors to uncouple the ATP-dependent translocation mechanism at high concentrations [47]. Other compounds have been shown to be both substrate and inhibitor for drug transporters, including zearalenone and tariquidar for ABCG2 [48,49]. Similarly, the pesticide methoxychlor has been shown to both stimulate and inhibit P-glycoprotein activity [30,32]. Likewise, progesterone and verapamil can bind to high affinity sites in P-glycoprotein to stimulate ATPase activity at low concentrations and inhibit at higher concentrations by binding to a low affinity site [38]. Interestingly, this nonmonotonic concentration dependence of effect may be analogous to what is seen in several endocrine disrupting compounds [50,51], and would suggest that TICs may have different effects on organisms depending on the concentration encountered.

In addition, since real-world exposures typically involve multiple ligands, TICs can interact with multiple independent binding sites that can be simultaneously occupied by inhibitors and substrates [52]. Depending on substrate and inhibitor affinities for each of those sites, transport of a substrate could be only partially inhibited when the inhibitor binds to the primary sites, while the secondary sites could still transport the substrate. As such, chemicals can interact with SMTs as single compounds or in concert to alter transport function. Understanding the molecular mechanisms and effects of drug mixtures on transport has long been a goal in clinical pharmacology, yet methods to clearly discriminate effects of more than two compounds remain challenging [57–59].

Transporter-interfering chemicals and their conserved modes of interaction

Given what is known about these diverse modes of ligand interaction with transporters, a broader definition of the TICs is proposed here. Known TICs include a wide range of persistent legacy and emerging compounds and as such are ubiquitous in the

Table 1. List of drugs and environmental chemicals and their known modes of interactions with selected SMT transporters. The table summarizes literature data on 40 compounds and their interactions with 10 different transporters. Physicochemical (MW, Log K_{ow}) and kinetic (IC_{50}, K_m) parameters are provided. Interactions according to [28,29,31,104,189,265–285]. —, not available; IND, induction (no expression value determined); INH, inhibitor (no IC50 determined); SI, synergistic inhibition; SUB, substrate (no K_m or EC50 value determined); WI, weak interaction.

						ABCB1		*ABCG2*		*ABCC1*		*ABCC4*				*URAT 1*				
	Chemical	Class	Pubchem CID	MW	Log K_{ow}	IC_{50} (μM)	K_m (μM)	IC_{50} (μM)	K_m (μM)	IC_{50} (μM)	K_m (μM)	IC_{50} (μM)	K_m (μM)	*OATP1A1* $K_{i,app}$ (μM)	*OATP1A2* IC_{50} (μM)	IC_{50} (μM)	*MATE1* EC_{50} (μM)	*OCT2* EC_{50} (μM)	*OAT4* IC_{50} (μM)	References
Inhibitors	Endosulfan[f]	Insecticide	3224	406.9	3.62(β), 3.83(α)	2.8[g], 11.4, 33.6	17	6.9	—	—	—	—	—	—	—	—	—	—	—	Smital *et al.* [28], Pivcevic and Zaja [31], Bain and LeBlanc [81], Buss *et al.* [82], Sreeramulu *et al.* [83], Bircsak *et al.* [84]
	Endrin	Pesticide	12358480	380.9	5.34	1.1	—	——	—	—	—	—	—	—	—	—	—	—	—	Nicklisch *et al.* [30]
	Heptachlor	Insecticide	3589	373.3	6.1	INH	—	—	—	—	—	—	—	—	—	—	—	—	—	Bain and LeBlanc [81]
	Malathion	Insecticide	4004	330.4	2.36	INH	—	—	—	—	—	—	—	—	—	—	—	—	—	Smital *et al.* [28]
	Musk ketone (MK)	Synthetic musk	6669	294.3	4.3	0.74[h]	—	—	—	—	—	—	—	—	—	—	—	—	—	Luckenbach and Epel [29]
	Musk xylene (MX)	Synthetic musk	62329	297.3	4.9	0.97[h]	—	—	—	—	—	—	—	—	—	—	—	—	—	Luckenbach and Epel [29]
	p,p′-DDE	Insecticide	3035	318.03	6.51	31.3	—	4	—	—	—	—	—	—	—	—	—	—	—	Bircsak *et al.* [84], Nicklisch *et al.* [30]
	p,p′-DDT	Insecticide	3036	354.49	6.36, 6.91	3.8–25.6	—	5	—	—	—	—	—	—	—	—	—	—	—	Bircsak *et al.* [84], Nicklisch *et al.* [30]
	PBDE-100	Flame Retardant	154083	564.69	7.24	23.2	—	—	—	—	—	—	—	—	—	—	—	—	—	Nicklisch *et al.* [30]
	Perfluorodecanoic acid (PFDA)	Perfluorochemicals	9555	514.08	6.3[a]	7.1[h]	—	—	—	—	—	—	—	26.8	INH	INH	—	—	INH	Stevenson *et al.* [189], Yang *et al.* [265], Yang *et al.* [266]
	Perfluorononanoic acid (PFNA)	Perfluorochemicals	67821	464.08	5.48	4.8[h]	—	—	—	—	—	—	—	44.6	INH	INH	—	—	INH	Stevenson *et al.* [189], Yang *et al.* [265], Yang *et al.* [266]
	Permethrin	Insecticide	40326	391.3	6.5	INH	—	7	—	—	—	—	—	—	—	—	—	—	—	Bircsak *et al.* [84], Bain and LeBlanc [81]
	Tetrabromobisphenol A (TBBPA)	Flame Retardant	6618	543.9	4.75[b]	22.9	—	INH	—	INH	—	24	—	—	—	—	—	—	—	Dankers *et al.* [267]
Substrates	Cyperquat (MPP^+)	Herbicide	39484	170.23	2.7[a]	—	SUB	—	—	—	—	—	—	—	—	—	—	—	—	Lacher *et al.* [268]
	Diazinon[e]	Insecticide	3017	304.35	3.81	—	9.7	—	—	—	—	—	—	—	—	—	—	—	—	Lacher *et al.* [268]
	Indinavir	Antiviral	5362440	613.8	2.9, 3.49	—	SUB	—	—	—	SUB	—	—	—	—	—	—	—	—	Van der Sandt etal.[269]
	Ivermectin[e]	Antiparasitic	6321424	875.1	3.2[c]	0.1	9[g]	—	—	—	—	—	—	—	—	—	—	—	—	Buss *et al.* [82], Schinkel *et al.* [85], Eneroth *et al.* [86], Griffin *et al.* [87]
	Methoxychlor	Insecticide	4115	345.6		—	—	—	—	—	SUB	—	—	—	—	—	—	—	—	Tribull *et al.* [271]

Table 1. (Continued).

	Chemical	Class	Pubchem CID	MW	Log K_{ow}	*ABCB1*		*ABCG2*		*ABCC1*		*ABCC4*		*OATP1A1*	*OATP1A2*	*URAT 1*	*MATE1*	*OCT2*	*OAT4*	References
						IC_{50} (μM)	K_m (μM)	IC_{50} (μM)	K_m (μM)	IC_{50} (μM)	K_m (μM)	IC_{50} (μM)	K_m (μM)	$K_{i,app}$ (μM)	IC_{50} (μM)	IC_{50} (μM)	EC_{50} (μM)	EC_{50} (μM)	IC_{50} (μM)	
					4.68, 5.08															
	Paraquat	Herbicide	15939	186.25	−4.22, −4.5[d]	—	SUB	—	—	—	—	—	—	—	—	—	SUB	SUB	—	Chen *et al.* [271], Wen *et al.* [272]
	Parathion methyl[f]	Insecticide	4130	263.21	2.86	3.4	7.3	—	—	—	—	—	SUB[i]	—	—	—	—	—	—	Sreeramulu *et al.* [83], Nornberg *et al.* [273], Aurade *et al.* [274]
	Ritonavir[e]	Antiviral	392622	720.9	3.9	—	SUB	—	—	—	SUB	—	—	—	—	—	—	—	—	Van der Sandt *et al.* [269], Alsenz *et al.* [275], Srinivas *et al.* [276]
Inducers (expression)	Diazinon[e]	Insecticide	3017	304.35	3.81	—	IND	—	—	—	—	—	—	—	—	—	—	—	—	Lecoeur *et al.* [277]
	p,p′-DDE	Insecticide	3035	318.03	6.51	—	IND	—	—	—	—	—	—	—	—	—	—	—	—	Shabbir *et al.* [278]
	p,p′-DDT	Insecticide	3036	354.49	6.36, 6.91	—	IND	—	—	—	—	—	—	—	—	—	—	—	—	Shabbir *et al.* [278]
	Prochloraz	Fungicide	73665	376.7	4.1	—	—	—	IND	—	—	—	—	—	—	—	—	—	—	Halwachs *et al.* [279]
	Rifampicin	Antibiotic	135398735	822.9	2.7	—	IND	—	—	—	—	—	—	—	—	—	—	—	—	Westphal *et al.* [280]
	Ritonavir	Antiviral	392622	720.9	3.9	—	IND	—	—	—	IND	—	—	—	—	—	—	—	—	Perloff *et al.* [281]
	St. John's Wort	Supplement	46507869	NA	NA	—	IND	—	—	—	—	—	—	—	—	—	—	—	—	Hennessy *et al.* [282]
	TCDD	Dioxin	15625	322	6.8	—	—	—	IND	—	—	—	—	—	—	—	—	—	—	Halwachs *et al.* [327]
Weak interactors	Aminocarb	Insecticide	16247	208.26	1.73, 1.9	WI	—	WI	—	WI	—	—	—	—	—	—	—	—	—	Bain and LeBlanc [81], Gueniche *et al.* [283]
	BPA (Bisphenol A)	Plastic monomer	6623	228.29	3.32	WI	—	INH	—	WI	—	WI	—	—	—	—	—	—	—	Dankers *et al.* [267], Nicklisch *et al.* [30]
	DEHP (Bis(2-ethylhexyl) phthalate)	Plasticizer	8343	390.6	5.03, 7.6	WI	—	WI	—	WI	—	WI	—	—	—	—	—	—	—	Dankers *et al.* [267], Nicklisch *et al.* [30]
	MEHP	DEHP metabolite	20393	278.34	4[a]	WI	—	WI	—	WI	—	WI	—	—	—	—	—	—	—	Dankers *et al.* [267]
	Warfarin	Anticoagulant	54678486	308.3	2.7	WI	—	—	—	—	—	—	—	—	—	—	—	—	—	Palmeira *et al.* [284], Gschwind *et al.* [285]
Synergistic inhibitors	Diazinon[e]	Insecticide	3017	304.35	3.81	SI	—	—	—	—	—	—	—	—	—	—	—	—	—	Pivcevic and Zaja [31]
	Endosulfan	Insecticide	3224	406.9	3.62(β), 3.83(α)	SI	—	—	—	—	—	—	—	—	—	—	—	—	—	Pivcevic and Zaja [31]
	Fenamiphos	Insecticide	31070	303.36	3.23	—	—	—	—	—	—	—	—	—	—	—	—	SI	—	Chedik *et al.* [79]
	Phosalone	Insecticide	4793	367.8	4.38	SI	—	—	—	—	—	—	—	—	—	—	—	—	—	Pivcevic and Zaja [31]
	Phosmet	Insecticide	12901	317.3	2.78	—	—	—	—	—	—	—	—	—	—	—	—	SI	—	Chedik *et al.* [79]
	Propiconazole	Fungicide	43234	342.2	3.72	SI	—	—	—	—	—	—	—	—	—	—	—	—	—	Pivcevic and Zaja [31]

[a]Estimated (Pubchem).; [b]Ionizable compound.; [c]Lumaret *et al.* 326.; [d]Polar compound.; [e]Also inhibits transporter efflux.; [f]Also stimulates transporter ATPase.; [g]EC50.; [h]Muscle gill transporters (predominantly ABCB1).; [i]Zebrafish ABCC4.

environment, meaning that virtually all humans and wildlife are exposed. Exposure to TICs is unintentional, and environmental or dietary preferences can have a large impact on the overall chemical intake [60–72]. Effects of TICs will be dependent on both the dynamic regulation of the SMT system during development [13,14,73–76], and the modulated transporter activities due to polymorphism in specific ethnic populations [77,78].

Many environmental chemicals have known molecular interactions with the drug transporters (Table 1). We defined as inhibitors, compounds that competitively or noncompetitively inhibits ATPase activity or the direct transport of a probe substrate across a membrane. Substrates are defined as compounds that have been directly transported across a membrane in an assay system. Inducers are compounds that induce the expression of a transporter. And weak interactors are compounds that have been shown to be either weak inhibitors or substrates of transporters in a given assay. A more detailed definition of TIC modes of interaction with transporter can be found in the Glossary (Box 1).

To date, only a few studies exist that test multi-compound mixture interactions on drug transporters [30,31,79,80]. Super-additive (synergistic) effects of binary combinations of pesticides have been shown for inhibiting ABCB1 [31] and an SLC drug transporter [79]. For instance, a mixture of the two pesticides fenamiphos and phosmet showed synergistic and additive effects on OCT2 transporter inhibition over a wide range of concentrations (0.38–26.85 μM). Similarly, the binary combination of the pesticide diazinon together with either the drug verapamil or the pesticides phosalone, endosulfan, and propiconazole always showed synergistic inhibition of P-glycoprotein-mediated calcein-AM transport. Both additive and synergistic effects of TICs effectively reduce the concentration needed of a single compound to interfere with transport function.

Interestingly, while those listed transporter interactions have been evaluated using different assay systems and drug transporters from different organisms, some interactions are conserved across assays. For instance, the insecticide endosulfan has been shown to inhibit human, hamster and mussel ABCB1 when tested for inhibition of transport or ATPase activity in gills, stably transfected cell lines and as purified membrane fraction [28,31,81–84]. Similarly, the antiparasitic compound ivermectin has been shown to be transported by human, canine, and mouse ABCB1 using knockout mouse models and whole cell monolayer assays [82,85–87].

Environmental levels of TICs

A major route of human exposure to TICs is through consumption of contaminated food. Large-scale assessments of food contaminants have continuously detected elevated levels of several persistent TICs such as polybrominated flame retardants (PBDEs) and polychlorinated biphenyls (PCBs) were detected in dairy, meat, and fish [62,67,69,70]. Lipid normalized levels of a single flame retardant and TIC, BDE-47, were 58.9 nanomolar in sardines [88] and as high as 175 nanomolar in tuna [62]. Importantly, the cumulative lipid-based concentrations of the 10 most potent TICs were as high as 3.3 μM, while all pollutants were 12.7 μM, respectively [62]. Similarly, the same persistent pollutants can be detected at high concentrations in human blood and urine [89,90] and breast milk [91–93]. For instance, the flame retardants BDE-47 and BDE-99 had lipid-based concentrations in US mothers' milk up to 559 nanomolar and 197 nanomolar [92]. For the organochlorine pesticide and TIC, p,p′-DDE, concentrations of up to 314 μM have been reported in breast milk fat from South African women [93].

Another possibility is that TICs could act indirectly on upstream regulators like the nuclear receptors AhR, PXR, or CAR to reduce transporter expression and further facilitate the retention of TICs and other persistent compounds [94–99]. However, recent studies have shown that while the transporters may not be able to eliminate these chemicals, TICs are nonetheless able to bind and interfere with transporter function. Notably, the brominated flame-retardant and TIC, BDE-100, was shown to tightly bind to the ligand binding sites in ABCB1 and to inhibit the function in mice and humans [30]. The binding occurs at evolutionarily conserved residues, indicating the potential for effects in a wide range of organisms (Fig. 2).

As we will discuss further below, TICs could also act in concert with other drugs and food ingredients, and both a continuous assessment of levels of environmental chemicals in food and a detailed analysis of their additive and uper-additive effects is necessary to provide appropriate dietary and food safety guidelines [100].

Mechanisms and approaches for evaluating TIC interactions

The categorization of environmental chemicals as TICs requires careful consideration. Unlike analytical chemistry with its 'gold standard' approved chromatography and mass spectrometry methods, there is no single assay for each TIC effect. Importantly, the choice of assay will influence the investigators' ability to discern TIC

Box 1. Glossary—Modes and effects of environmental chemical interactions with SMTs

A Types of environmental chemical interactions

Inducers. Compounds that upregulate SMT function at the level of expression.
Inhibitors. Compounds that bind to SMTs and inhibit transporter activity and function.
Modulators. Compounds that bind to orthosteric or allosteric sites in SMTs without being transported and alter the specificity toward inhibitors or substrates.
Stimulators. Compounds that bind and activate SMTs but do not necessarily get transported. ABC transporter activation in absence of transport can be determined using ATPase assays.
Substrates. Compounds that bind to SMTs and get transported.
Weak interactors. Compounds that are not recognized or weakly interact with SMTs and do not alter transporter activity or function.

B Molecular mechanisms and interactions

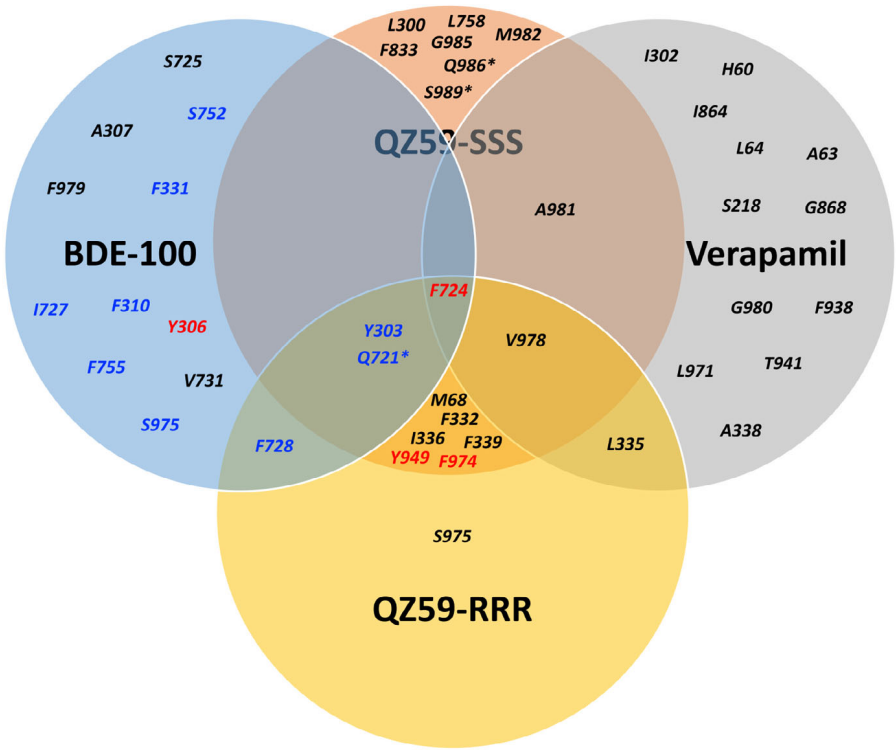
Additive interactions. Concerted binding of two or more compounds to SMTs modulates transporter function equal to the sum of the compounds' separate effects.
Allosteric interaction. Compounds that bind to SMTs at sites distinct from the ligand binding site(s) and modulate transporter function.
Antagonistic interaction. Concerted binding of two or more compounds to SMTs negates or modulates transporter function to a lesser degree than the sum of each individual effect.
Cooperative interaction. Binding of compound(s) to one site in SMTs influences the interaction of the same or different compound(s) at another functional site.
Orthosteric interaction. Compounds that bind to functional site(s) in SMTs and modulate function by competitive interactions with other ligands.
Synergistic interaction. Concerted binding of two or more compounds to SMTs modulates function to a higher degree than the sum of each individual effect.

C Cellular and organismal effects

Chemical defense priming. Continuous exposure to xenobiotics alters SMT function by inducing transient or permanent, compensatory upregulation at the physiological, transcriptional or epigenetic level.
Chemosensitization. Interaction of compound(s) with SMTs increases sensitivity of a cell or organism toward a (toxic) substrate.
Endogenous substrate competition. Interaction of compound(s) with SMTs that interferes with physiological substrate transport and cellular homeostasis.
Energy depletion. Increase in cellular energy (e.g., ATP) consumption due to constant exposure to compounds that activate SMTs.
Futile cycling. Ineffective transport of (high permeability) compounds that immediately re-enter membranes/cells for another transport cycle.
Signaling interference. Interactions of compounds with SMTs disrupt cell signaling and/or signal transduction.

effects. To date, multiple *in vitro* and *in vivo* assays have been used to determine the nature of drug TICs [30,101,102]. However, many of the assays used in the field of TICs are best suited for discovery of inhibitors.

For SLC-type transporters, the majority of assays is determining the (competitive) inhibition of intracellular accumulation of a reference substrate by a test compound [79,103]. For ABC-type transporters, TIC interactions are often determined indirectly by inhibition of the prestimulated ATPase activity [84,104]. Alternatively, competitive uptake inhibition of reference compounds into membrane vesicles or competitive efflux inhibition and transport across cell monolayers have been employed.



Human ABCB1	LLIYASYALAFWYGTTLVLSGEYSIGQVLTVFFSVLIGAFSVGQASPSIEAFANARGAAYEIFKIIDNKPSIDS	377
Mouse ABCB1a	LLIYASYALAFWYGTSLVISKEYSIGQVLTVFFSVLIGAFSVGQASPNIEAFANARGAAYEVFKIIDNKPSIDS	373
Chicken ABCB1	LLIYASYALAFWYGTTLILANEYSIGNVLTVFFSVLIGAFSIGQTAPSIEAFANARGAAYAIFNIIDNEPEIDS	385
Clawed frog ABCB1	LMIYAAYSLAFWYGTTLIIDGGYTIGSVLTVFFAVIIGAFAVGQTSPNIEAFANARGAAYTIFNIIDNQPKIDS	387
Zebrafish ABCB4	FMIYMSYALAFWYGSTLILGGEYTIGMLLTIFFAVLIGAFGLGQTSPNIQTFSSARGAAHKVFQIIDHEPKINS	382
Human ABCB1	PVSFWRIMKLNLTEWPYFVVGVFCAIINGGLQPAFAIIFSKIIGVFTRIDDPETKRQNSNLFSLLFLALGIISF	767
Mouse ABCB1a	PASFWRILKLNSTEWPYFVVGIFCAIINGGLQPAFSVIFSKVVGVFTNGGPPETQRQNSNLFSLLFLILGIISF	763
Chicken ABCB1	PVSFLKLMKLNKNEWPYFVAGTFCAIVNGALQPAFSVIFSEIIGIFSETDQ-KVLREKSNLYSLLFLALGIISF	775
Clawed frog ABCB1	PVSFFKVMKLNKPEWPYFVVGVICAMINGATQPAFAIIFSRIIGVFA--GPVSQMRSESSMYSLLFLALGGVSF	775
Zebrafish ABCB4	NVSFLTVLKLNYPEWPYMVVGILCATINGGMQPAFAVIFSKIIAVFAEPDQ-NLVRQRCDLYSLLFAGIGVLSF	765
Human ABCB1	KAHIFGITFSFTQAMMYFSYAGCFR-FGAYLVAHKLMSFEDVLLVFSAVVFGAMAVGQVSSFAPDYAKAKISAA	1006
Mouse ABCB1a	KAHVFGITFSFTQAMMYFSYAACFR-FGAYLVTQQLMTFENVLLVFSAIVFGAMAVGQVSSFAPDYAKATVSAS	1002
Chicken ABCB1	KAHIFGFCFSLSQAMMFFTYAGCFR-FGAYLVVNGHIEYKTVFLVFSAVVFGAMALGQTSSFAPDYAKAKISAA	1014
Clawed frog ABCB1	KAHLHGLTYGLSQAHHVLCLCWVFSVLGAYLVVEGLMKLDEVFLVSSAIVLGAMALGQTSSFAPDYTKAMISAA	1015
Zebrafish ABCB4	KAHVFGLTFSFSQAMIYFAYAGCFK-FGSWLIEQKLMTFEGVFLVISAVVYGAMAVGEANSFTPNYAKAKMSAS	1004

Fig. 2. Similar residues in vertebrate ABCB1 interact with pharmaceutical inhibitors and the TIC and flame-retardant BDE-100. The Venn diagram displays all residues in mouse ABCB1a that interact with flame-retardant BDE-100 and known inhibitors verapamil, QZ59-SSS and QZ59-RRR according to [104] and [21]. Residues marked with an asterisk represent the 'lower' binding site of QZ59-SSS. Residues marked in red are assumed to be involved in inhibition of ATP hydrolysis and transport function according to [200]. The amino acid alignment shows that 11 (marked in blue and red) of the 15 residues interacting with BDE-100 are conserved across model vertebrate species.

Strengths and limitations of current methods and assays to evaluate TICs

Interactions of small molecule drugs with SMTs have been a major focus of pharmaceutical and toxicological sciences over the past four decades. A wide array of *in vitro* and *in vivo* assays to evaluate those interactions have been developed since then to quantify ATPase activity in a variety of assay systems [37,105–109] or to determine (competitive) transport inhibition [93–98], bidirectional transport across cell monolayers [40,110,111] or the binding affinities (Table 2) of different drugs and small molecules to the transporters [112–115]. Some of the most prominent assays have sparked commercial interest and are readily available as purified protein kits, membrane fractions or drug transporter-expressing cell lines.

In these assays, ABC transporter inhibition is often measured indirectly by competitively inhibiting the

efflux or uptake of a fluorescent or radiolabeled substrate or directly by ATPase stimulation or inhibition with a model drug compound. Arguably, the three most common assays to measure and quantify TICs with drug transporters are ATPase, unidirectional vesicular transport and bidirectional cell monolayer assays (i.e., transwell assays).

ATPase assays

ATPase assays were first developed for small soluble ATPases [116] and later applied to determine the activity of ABC drug transporters [117]. These assays offered the advantage of using the liberated orthophosphate from ATP hydrolysis as an indirect reporter for ATPase stimulation and hence activation of the transporters. Initial drug transporter purification attempts focused on plasma membrane preparations of drug-resistant cancer cell lines [118] and large-scale protein production has been traditionally performed heterologously in bacterial and yeast systems [119,120]. Sophisticated methods have been developed over the years, determining the ATPase activity in drug transporter expressing cell lines, lipid vesicles, membrane patches, artificial membranes, and with purified and detergent-solubilized protein [37,106,108,121,122]. It is commonly accepted that substrate translocation requires both ATP binding and hydrolysis [122–124], enabling the development of fluorescent and colorimetric assays to stoichiometrically relate Pi liberation to transporter activation. ATPase assays can be conducted in activation mode by measuring phosphate liberation with the drug alone or in inhibition mode by prestimulating the ATPase activity with a model stimulator and following the 'knock-off' kinetics by inhibitory test compounds. Interactions of drugs with ABCB1 in an ATPase assay have been characterized with solubilized protein, reconstituted protein, and heterologous and homologous expressed protein (Table 2). In those cases, ATPase activity was measured prestimulated with different ratios and types of stimulator or nonstimulated (basal activity).

A wide range of factors can introduce variation into the results of ATPase assays. In some cases, it is not known if the basal activity has been properly subtracted from the final values due to lack of experimental details. Another confounding factor is the type of protein concentration assay used to calculate the specific ATPase activity of each protein. Another major factor is the use of different detergents and lipids to purify and reconstitute P-gp and other drug transporters. Some authors had to 'activate' P-gp with a lipid/deterrent mixture to become fully amenable for drug interaction assays [125]. Furthermore, the amount of ATP and reducing agent (DTT, BME) can vary dramatically. Buffer type, ions, and capacity have also not been standardized and assay pH can range from pH 7.0–8.0 [104,126–128]. Depending on the pKa of the TIC, the assay pH can influence both overall charge and membrane permeability of a given compound tested. Finally, the assay temperature and time course will affect overall kinetics and parameters. A decrease in assay temperature from 37 to 25 °C has been previously shown to decrease ATPase activity [129], while an increase in the assay temperature above 37 °C could inactivate the transporter [130].

It is important to note that these ATPase assays have some common limitations. First, they favor discovery of inhibitory effects [30,31,81,83,84,131], thereby skewing our potential understanding of TIC effects. Second, in a solubilized protein ATPase assay, the protein conformation does not resemble the native conformation in a membrane environment, and instead allows access to ATPase and other protein domains typically embedded in the membrane. Such non-native conformational changes could influence transporter kinetics [132]. Finally, low permeability compounds that typically cannot cross the membrane in an *in vivo* system, can interact with the ligand binding sites in a solubilized SMT and be falsely identified as TICs.

By measuring ATPase activity of SMTs in a membrane environment, the active conformation can be preserved and nonspecific binding to protein domains otherwise embedded in the membrane can be prevented. In this case, clear knowledge of apical or basolateral localization of the transporter under study and the tissue geometry are necessary to decide whether the compound would be able to interact with the transporter under physiological conditions.

Vesicular transport assays

Vesicular transport assays can be divided into two main systems: artificial (proteo-) liposomes and inverted membrane vesicles (IMVs) made from living cells. One of the first preparations of inverted membranes was done with human red blood cells [133]. The unique feature of these vesicles for the analysis of drug efflux transporters is the fact that most of the cell membranes overexpressing the transporter of interest will get inverted during the preparation. This inside-out orientation of the ABC drug transporters allows access to the ATPase domains and ligand binding sites to study uptake of substrates into the enclosed vesicles. The uptake of fluorescently or radiolabeled control

Table 2. List of common in vitro and in vivo assays to determine interactions of small molecules with transporters. The table summarizes the current arsenal of biochemical, biophysical and cell-based assays that have been developed to interrogate drug and environmental chemical affinity and potency toward SMTs. Assays according to [13,21,54,101,104,117,122,146,157,164,165,176,279,287–325]. 6-AIPP-forskolin, 6-O-[[2-[3-(4-azido-3-[125I]iodophenyl)propionamido]ethyl]carba-myl]forskolin; COL, colchicine; DOX, Doxorubicin; E217βG, 17β-Estradiol 17β-D-glucuronide; IAAP, Iodoarylazidoprazosin; MIANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; PheoA, Pheophorbide A; QUI, Quinidine; sNBDL-CSA, [N-ε-(4-nitrobenzofurazan-7-yl)-D-Lys8] cyclosporin A; SR101, Sulforhodamine 101; TPP+, Tetraphenylphosphonium; VBL, vinblastine; VCR, vincristine; VER, Verapamil.

Assay type	Assay system	Species: transporter(s)	Reporter molecule	Original references
ATPase Assays	IMVs in KB-V1 and KB-3-1	Human: ABCB1	VBL	Horio *et al.* [122]
	Proteoliposomes (protein from CH^RC5)	Hamster: ABCB1	COL	Sharom *et al.* [286]
	Membrane Nanodiscs	Human: ABCB1	Nicardipine	Ritchie *et al.* [287]
	Styrene-maleic acid lipid particles (SMALPs)	Human: ABCB1, ABCC1, ABCC4, ABCG2; Mouse: ABCC7 (CFTR)	MIANS, Estrone sulfate, PheoA	Gulati *et al.* [288]
	Isolated membranes (from Sf9 cells)	Human: ABCB1	VER, VBL, 5-Fluorouracil, Trifluoperazine	Sarkadi *et al.* [117]
	Amphipols	Mouse: ABCB1a (MDR3); Human: ABCB1	N/A	Lee *et al.* [289], Alam *et al.* [325]
	Purified protein (protein from CH^RC5)	Hamster: ABCB1	VER, VBL, COL, Nifedipine, Daunomycin	Doige *et al.* [290]
Competitive dye transport assays (unidirectional accumulation)	Invertebrate embryos (*Strongylocentrotus purpuratus*)	Sea urchin: ABCB1	Rhodamine	Toomey and Epel [291]
	BEWO (choriocarcinoma)	Human: ABCB1, ABCC1	CAM, VBL, Fluorescein	Utoguchi *et al.* [292]
	Huh-7 (human hepatocellular carcinoma)	Human: ABCB1, ABCC1, ABCG2	Rho123, Hoechst 33342	Jouan *et al.* [293]
	HeLa cells (Henrietta Lacks cervical cancer)	Human: ABCB1	Rho123, CAM	Sauna et al [294]
	K562 cells (human bone marrow chronic myelogenous leukemia)	Human: ABCB1, ABCG2	Hoechst 33342, DyeCycle Violet	Nerada *et al.* [168]
	A431 cells (human skin epidermoid carcinoma)	Human: ABCB1, ABCG2	Hoechst 33342, DyeCycle Violet	Nerada *et al.* [168]
	CHO K1 (Chinese Hamster Ovary)	Hamster: ABCB1, ABCC1, ABCG2	CAM, eFluxx-ID, CMFDA, PheoA	Lebedeva *et al.* [295]
	A549 (human lung carcinoma)	Human: ABCB1, ABCC1, ABCG2	eFluxx-ID, CMFDA, PheoA, DiOC2(3)	Lebedeva *et al.* [295]
	HL-60/MX1 (human acute promyelocytic leukemia)	Human: ABCB1, ABCC1, ABCG2	CAM, eFluxx-ID, PheoA	Lebedeva *et al.* [295]
	HCT-8 and HCT-15 (human ileocecal colorectal carcinoma)	Human: ABCB1, ABCC1	CAM, eFluxx-ID, CMFDA, DiOC2(3)	Lebedeva *et al.* [295]
	HepG2 cells (human liver hepatocellular carcinoma)	Human: ABCB1	Rho123	Shabbir *et al.* [278]
	NIH/3T3 murine fibroblasts	Human: ABCB1	CAM	Homolya *et al.* [165], Hollo *et al.* [164]
	PLHC-1/dox cell lines (Poeciliopsis Lucida hepatocellular carcinoma)	Clearfin Livebearer: ABCB1	CAM, Rho123	Zaja *et al.* [101], Caminada *et al.* [296]
	MDCK-II (Madin-Darby canine kidney strain II cells)	Human: ABCB1	CAM	Gannon *et al.* [297]
	NCI-H441 (human lung adenocarcinoma)	Human: ABCB1	Rho123	Salomon *et al.* [299]
	KB-V1 and KB-3-1 cells (Cervix carcinoma - HeLa derivative)	Human: ABCB1	CAM	Ansbro *et al.* [299]
	Renal proximal tubules	Killifish: ABCB1	NBDL-CSA	Schramm *et al.* [300]

Table 2. (Continued).

Assay type	Assay system	Species: transporter(s)	Reporter molecule	Original references
	Brain capillaries	Rat: ABCB1a/b	NBDL-CSA, BODIPY-Prazosin, SR101	Hartz *et al.* [301]
Monolayer assay (bidirectional transport)	Caco-2 (Caucasian Colon Carcinoma)	ABCB1, ABCG2, MRP2	VBL	Hunter *et al.* [302]
	LLC-PK1 (Epithelial-like pig kidney cell line)	Human: ABCB1	DOX, Rho123, QUI, VER	Van Der Sandt *et al.* [157], Riede *et al.* [159]
	MDCK-II (Madin-Darby canine kidney strain II)	Human: ABCB1	Rho123	Haemmerle *et al.* [303]
	IPEC-J2 (Pig Ileum Epithelial cells)	Human: ABCB1	Digoxin, VER, Citalopram, VBL, VCR	Saaby *et al.* [304]
	Calu-3 (Human lung adenocarcinoma)	Human: ABCB1	CAM, Rho123	Hamilton *et al.* [305]
	hCMEC/D3 (brain microvascular epithelial cell line)	Human: ABCB1	eFLUXX-ID Gold	Noack *et al.* [306]
Cytotoxicity assays	CR1R12 (CHO subline)	Hamster: ABCB1	COL	Aller et al [21]
	2-cell embryos (*S. purpuratus*)	Sea urchin: MRP-like	VBL	Hamdoun *et al.* [13]
	Functional complementation (*Saccharomyces cerevisiae*)	Yeast: Human ABCB1	Valinomycin	Kuchler and Thorner [307]
	Competitive growth inhibition (*Saccharomyces cerevisiae*)	Yeast: Mouse ABCB1a (MDR3)	DOX	Nicklisch *et al.* [30], Jeong *et al.* [308]
Fluorescence anisotropy/ polarization	Proteoliposomes	*Mycobacterium tuberculosis*: TBsmr	Ethidium bromide, TPP+	Basting *et al.* [309]
	Purified protein	*Escherichia coli*: EmrE	Ethidium bromide, TPP+	Chen *et al.* [310]
	Purified protein	*Lactobacillus lactis*: LmrP	Propidium and ethidium dyes	Schaedler and Veen [311]
	Purified protein	*Escherichia coli*: AcrB	Rho6G, Ethidium, Proflavine, Ciprofloxacin	Su *et al.* [312]
	Purified protein	*Staphylococcus aureus*: MepA	Acriflavine, Rho6G, Ethidium	Banchs *et al.* [313]
Drug binding affinity	Surface plasmon resonance (SPR)	Human: ABCB1	MRK16, UIC2 mAB	Ritchie *et al.* [314], Chen *et al.* [315]
	FRET analysis in Hek293T	Human: ABCC1	E217βG, ATP, Vanadate	Osa-Andrews et al [316], Iram et al [317], Swartz et al [318]
	Intrinsic Trp fluorescence quenching in CH^RB30 (CHO derivative)	Hamster: ABCB1	Tryptophan	Liu et al [319]
	Site-directed fluorescence labeling & quenching in CH^RB30	Hamster: ABCB1	MIANS label	Liu and Sharom [320]
Biochemical assays (binding sites)	Photo-affinity labeling in KB-3-1 (HeLa derivative)	Human: ABCB1	Azidopine, IAAP, 6-AIPP-forskolin	Bruggemann *et al.* [321], Greenberger [322]
	Cys & thiol reactive labeling in HEK293 cells	Human: ABCB1	Dibromobimane, MTS-VER	Loo and Clarke [323], Loo and Clarke [324]
	Nucleotide trapping assays in CR1R12 (CHO derivative)	Hamster: ABCB1	Vanadate	Urbatsch and Senior *et al.* [125]
	Radioligand binding in CH^RB30 (CHO derivative)	Hamster: ABCB1	VBL, XR9576	Martin *et al.* [54]

substrates and test compounds can then be determined using LC/GC mass spectrometry or by fluorescent microscopy, flow cytometry, or spectrophotometry.

Proteoliposomes are a type of artificial lipid vesicle, where the protein of interest gets reconstituted into preformed liposomes, often made from total membrane extracts of *Escherichia coli* or yeast, chicken eggs, or pig total brain. One of the first membrane protein reconstitutions was carried out with bacteriorhodopsin in chicken egg phospholipids [134,135]. A key advantage of proteoliposomal systems are the almost unlimited types of natural or synthetic lipids available that can be combined to form unilamellar and multilamellar vesicles of any size [136–138]. Functional reconstitution and correct orientation of membrane proteins in liposomes depends on numerous factors, including protein stability, lipid quality, and detergent suitability, and usually requires rigorous optimization [139–141]. Using proteoliposomes, the effects of lipid type, charge and size, buffer conditions and protein composition on the interactions of TICs with SMTs can be conveniently evaluated and compared.

To preserve proper mammalian protein folding and posttranslational modification for structural and kinetic analysis, drug transporters are often expressed in insect cells [142–145] or human cell lines [146–149] to form inside-out vesicles. Such vesicles provide a native membrane environment in the absence of cytoplasmic proteins and enzymes that could interfere with the assay. Using these IMVs, the effects of TICs on SMTs embedded in a natural cell membrane can be evaluated on two dimensions in the same system: the stimulation or inhibition of ATPase activity and the actual transport of TIC substrates into the vesicle lumen [150–153].

A potential drawback is that vesicle-based assays do not perform well with highly permeable chemicals since they likely cross the membrane by simple diffusion. This in turn would overestimate the actual uptake of compounds into the vesicles and possibly promote a futile cycle when highly hydrophobic compounds rapidly re-enter the lipid environment for another transport cycle [154–156]. In this case, the use of control membrane vesicles that lack the transporter under study should be used to estimate and subtract false positive transport values.

Transwell monolayer assays

Transwell assays measure transcellular transport across polarized epithelial or endothelial cell monolayer expressing the transporter of interest. The transwell assay is considered the gold standard for assessing drug transport and drug permeability [9]. The bidirectional transport of a substrate across a polarized cell layer can be measured by adding the test substrate to the apical (upper) or basolateral (lower) chamber and quantifying the compounds in the opposite chambers using GC or LC mass spectrometry. The derived drug permeability coefficient (Papp) and the efflux ratios ($Papp_{B\text{-}to\text{-}A}$, $Papp_{A\text{-}to\text{-}B}$) can provide a wealth of information, including directionality of drug transport, the involved drug transporters, the specificity of substrates, inhibitors and modulators, and the prediction of drug permeability. The three most commonly used cell monolayers are formed from human Caco-2 cells, dog MDCK-II cells, and pig LLC-PK1 cells, the latter two cell lines expressing nonhuman endogenous transporters and often used to express human isoforms of transporters [40,157]. The same type of cell lines is also used in unidirectional fluorescent substrate transport assays with stably transfected transporters [158,159].

Both of these assays have important limitations to consider. For instance, when transfecting the common cell lines LLC and MDCK-II with the studied drug transporter genes, these cells show markedly lower expression of the endogenous transporters versus wild-type cells, leading to underestimation of substrate transport in transfected cells [160]. The differences in background transporter expression levels in these cells has also been suggested to be responsible for the high variability of IC_{50} values for ABCB1 inhibitors [161,162]. Furthermore, in order to measure efflux by an apically localized transporter, the compound needs to first cross the basolateral membrane (either by another transporter, or by passive diffusion). Since low permeability compounds cannot cross the basolateral membrane in a polarized cell system in the absence of a suitable uptake transporter, the compound cannot interact with the efflux transporter.

Limitations of fluorescent dye assays include the availability, specificity, and dynamic range of substrates to measure (competitive) inhibition of drug transporters in cells. While numerous fluorescent small molecules are transported by ABC and SLC transporters [163], very few have proven as robust as calcein-AM pioneered by Homolya *et al.* in the early 1990s [164,165]. Reasons for this are many and include the high basal permeability of some substrates, low quantum yield of the fluorophores, intracellular compartmentation, and fluorescence quenching [158]. Another challenge for dye uptake assay is the fact that numerous cell level studies have shown that there is considerable overlap in fluorescent substrates among transporters [158,163,166] and it may depend on the

cellular background whether a given fluorescent compounds can be a specific reporter for a monitored transporter activity. However, a handful of fluorescent substrates, along with specific inhibitors, have been useful for understanding the three key drug transporters ABCB1, ABCC1, and ABCG2, over the past 20 years [158,167,168].

Evaluating molecular interactions of TICs

While the detailed molecular mechanism underlying TIC bioaccumulation is still unknown, the interactions of those chemicals with small molecule uptake and efflux transporters at epithelial barriers have been suggested to be a key step in entering the body via systemic circulation. Hydrophobic TICs could either inhibit ABC-type drug efflux systems to promote their passive transport into cells or—by mimicking beneficial nutrient structures—bind with higher affinity to a SLC-type nutrient and metabolite uptake system, or both.

To begin to understand how TICs can interact with SMTs and how to best evaluate those interactions, multiple molecular interactions and binding sites within the transporter and its immediate membrane environment have to be considered. Similar to drug interactions with receptors and transporters, such interactions can be broadly divided into inhibitory or stimulatory effects. Inhibitory effects can be further discriminated based on the binding location. For instance, orthosteric compounds bind in the ligand binding site of a transporter and can competitively inhibit its function. The inhibition of verapamil-stimulated ATPase activity by cyclosporine A is a well-known example of competitive ABCB1 inhibition [38,52,169,170]. However, hydrophobic TICs could also bind specifically or nonspecifically within the hydrophobic parts of the membrane spanning domains to cause transporter inhibition. Such noncompetitive inhibition can occur either at a defined allosteric site or a nonspecific site within the SMT. For instance, the drugs daunorubicin, colchicine and vinblastine are known to allosterically inhibit verapamil-stimulated ABCB1 ATPase activity [38]. Allosteric noncompetitive inhibition has also been shown for the ABCB1-mediated multidrug resistance (MDR) reversal agent XR9576 (tariquidar) [47,171]. Tariquidar and the acridone carboxamide derivative GF120918 (elacridar) are also a competitive inhibitor for drug efflux transporter ABCG2 but do not inhibit ABCC1 [49,172,173].

Noncompetitive inhibition of ABC-type transporters can also occur by interfering with ATP binding [174]. TICs could act directly at the two ATPase domains (i.e., NBDs) of these ABC transporters, inhibiting both ATPase activity and drug binding capacity [175–177]. A less explored option for TICs to disrupt MRP-type transporters would be noncompetitive inhibition of the GSH binding site in these transporters [178–182]. Several drugs, including vincristine and daunorubicin, critically depend on GSH binding and/or co-transport to be effectively eliminated [183,184].

A different kind of allosteric effect on the SMT activity regulation is the influence by its local membrane environment and in particular cholesterol interactions [132,185,186]. Early experiments with ABCB1 showed that the ATPase activity was not stimulated by the canonical drug substrates vinblastine, colchicine, or daunomycin when reconstituted in *E. coli* lipids versus sheep brain or bovine liver extracts [118,125]. Similarly, the photo-affinity labeling of ABCB1 using the substrate [^{3}H]azidopine was increased when increasing amounts of cholesterol were incorporated into liposomes [187]. In contrast, in cell lines expressing human ABCB1, the addition of cholesterol inhibited the efflux of daunorubicin [188].

This has several implications of TICs. First in an analogous way, long-chain, lipid-like TICs could change the immediate membrane environment of drug transporters, thereby changing transporter activity. Such noncompetitive inhibition of ABCB1-mediated Rhodamine B efflux from mussel gills has been shown for synthetic perfluorochemicals that have high structural resemblance to fatty acids [189]. Second, the native lipid environment could affect assay results [132].

Finally, stimulatory effects of compounds can be exerted on the SMTs when binding to a modulatory site. A special case of these stimulatory effects are positively cooperative interactions between two or more compounds that either bind at overlapping or different modulatory sites within the SMT [58]. Such cooperative stimulation is a versatile and noninvasive mechanism to transiently modulate transporter activity and current clinical efforts focus on the discovery and development of modulating small molecules [174,190,191]. For instance, prolonged ABC transporter stimulation could be costly in terms of dramatically increasing the ATP usage of a (cancerous) cell and ultimately trigger apoptosis [154,192,193].

A more standardized set of assays is needed to probe for transporter- and possibly organism-specific evaluation of their modes of interaction due to known variations in drug transporter substrate recognition and differences in protein stability across species [194–197]. Since interaction of TICs with SMTs can occur at different ligand binding sites, the use of multiple reference probes with different binding sites could help to capture unknown TIC interactions. Finally, since real-

world exposures to environmental chemicals typically involve multiple compounds, the standardized assay criteria have to be expanded to probe for additive, synergistic, and antagonistic effects of chemical mixtures.

Insights from structural biology

To fully elucidate the intricate network of intramolecular interactions of environmental chemicals with SMT proteins, a detailed knowledge of protein structure and dynamics is essential. Until recently, the exact mode of SMT transport inhibition by environmental chemicals was unknown. The co-crystal structure of mouse ABCB1 in complex with the flame-retardant BDE-100 revealed for the first time, that TICs can specifically bind within the ligand binding site of the transporter and inhibit its function [30]. In general, to be able to successfully resolve a transporter-ligand co-crystal structure, a transporter has to bind its ligand with high affinity and specificity (i.e., high level of occupancy) and in a stable conformation for crystal packing [198,199]. Thus, the co-crystal reveals that binding of the flame retardant to specific sites in the ligand binding pocket of ABCB1 could be responsible for competitive inhibition observed in the corresponding ATPase and yeast growth inhibiting assays.

When comparing the residues in mouse ABCB1 that have been shown to interact with BDE-100 and other known ABCB1 inhibitors, the flame-retardant shares the critical aromatic residue phenylalanine 724 (F728 in human ABCB1) with all three other inhibitors (Fig. 2). It has been shown recently for the human ABCB1 transporter that the aromatic residue pairs F728-Y310 and F978-Y953 can form important hydrogen bonds with the third-generation inhibitors zosuquidar, elacridar, and tariquidar, which in turn mediates the inhibition of ATP hydrolysis and transport function [200]. One of those corresponding residue pairs in mouse ABCB1 is F724-Y306, which has been shown to interact with BDE-100 in the crystal structure (Fig. 2). Hence, inhibition of ATP hydrolysis could be the major mode of action for TICs to interfere with ABCB1 function and possibly other ABC transporter. Interestingly, nine additional residues interacting with BDE-100 in mouse ABCB1 are conserved in five model vertebrates, indicating a structural basis for predicting TIC interactions across species.

Conclusions and future directions

Nearly 45 years ago, the first multidrug transporter, P-glycoprotein (ABCB1), was identified and shown to increase drug resistance in cancer cells. Since then, a race for the detailed elucidation of its structure, function and molecular mechanism of ligand interactions has started and fueled academic, governmental, and industrial efforts to identify the common pharmacophore to develop transporter inhibitors or therapeutic drugs that are not recognized by these types of MDR efflux pumps. Pharmacological studies on ABCB1 and other drug transporters have done pioneering work for a basic understanding of its drug recognition and interactions. Multiple 'generations' of synthetic and natural inhibitors and substrates have been synthesized or identified, but a clear understanding of how small molecules are recognized and interact with these types of transporters is still mysterious.

Given the scale of the environmental chemical problem, high-throughput assays to determine interactions of the multitude of emerging environmental chemicals with SMTs are urgently needed. More importantly, SMT interactions with chemical mixtures, representing real-world combinations of drugs, food ingredients, and chemicals, have to be tested to predict individual and combined chemical uptake and disposition in humans. Existing TIC data have been collected through a wide variety of *in vitro* assays and approaches, and there is urgent need to standardize the conditions for establishing environmental chemicals as TICs. Some of the key criteria for the establishment of such standardized methods would include assay accuracy, specificity, and reproducibility, both between measurements and analysts in the same laboratory and when performed in different laboratories. The International Transporter Consortium (ITC) has been pioneering such *in vitro* assay standardization with clinically important transporters for identifying drug–drug interactions (DDIs) that may inform clinical studies in drug development [9,20,201]. A similar approach could be applied to TICs so as to identify and predict possible adverse drug-TIC and TIC-TIC interactions with SMTs. The results of these approaches could also serve as guidelines for the design of environmental chemicals that do not interfere with the SMT system and are better eliminated from the body [202,203].

An alternative and emerging approach to narrow down drug and chemical candidates to test for transporter interactions in the wet laboratory is the combination of *in vitro* or *in vivo* assays and *in silico* analysis. Such data-driven, predictive approaches that combine computational methods with pharmacokinetic and exposome data sets are essential for developing a holistic understanding of transporter interactions with drugs and xenobiotics. The main advantages of these *in silico* tools are the ability to rapidly analyze large data sets, to prioritize chemicals, to develop predictive models, and to

guide the selection for pharmacokinetic and toxicokinetic laboratory analysis [51,204,205]. Together with recent advances in the application of machine learning algorithms combined with network analysis tools in biological science [206–211], *in silico* tools could prove valuable for predicting and deciphering novel DDIs, drug–food interactions, and drug–environmental chemical interactions with SMTs. The ultimate goal would be to use *in silico* analysis as a high throughput, noninvasive SMT : chemical interaction tool to identify SMT interactions with small molecules and to predict chemical accumulation potential and chemical toxicities in humans and other organisms.

Finally, to better understand and validate the organismal effects of TIC:SMT interactions, including cell signaling disruption and chemosensitization, the development of animal knock-out models is necessary. Emerging model systems should include food organisms across multiple trophic levels to investigate the role of SMT disruption in environmental chemical bioaccumulation, trophic transfer and ultimately (dietary) exposures to humans [62,212–216]. Collectively, these advances in TIC research are likely to help us better predict how environmental chemicals bioaccumulate and how they cause harm to humans and wildlife.

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Author contributions

SCTN and AH conceived and wrote the manuscript.

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