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

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Abstract

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

Abbreviations: TIC, transporter interfering chemicals; SMT, small molecule transporters; SLC, solute carrier; ABC, ATP-binding cassette; MDR, multidrug resistance; IMV, inverted membrane vesicle; DDI, drug-drug interaction; DFI, drug-food interaction; DECI, drug-environmental chemical interaction; BBB, blood brain barrier; BCSFB, blood cerebrospinal fluid barrier; BBIB, blood bile barrier; BUB, blood urine barrier; BIB, blood intestine barrier; BMB, blood milk barrier; BAB, blood air barrier; BPB, blood placenta barrier; BTB, blood testis barrier; BRB, blood retinal barrier; BHB, blood heart barrier.

Introduction – Evolution and function of the Small Molecule Transporter (SMT) 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 (Figure 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, 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 non-interacting 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)

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 non-monotonic 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 [32,57]. 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 non-monotonic 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 [58–60].

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 Transporter-Interfering Chemicals is proposed here. Known TICs include a wide

range of persistent legacy and emerging compounds and as such are ubiquitous in the 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 [61–73]. Effects of TICs will be dependent on both the dynamic regulation of the SMT system during development [13,14,74–77], and the modulated transporter activities due to polymorphism in specific ethnic populations [78,79].

Many environmental chemicals have known molecular interactions with the drug transporters (Table 1). We defined as inhibitors, compounds that competitively or non-competitively 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.

To date only a few studies exist that test multi-compound mixture interactions on drug transporters [31,57,80,81]. Super-additive (synergistic) effects of binary combinations of pesticides have been shown for inhibiting ABCB1 [31] and an SLC drug transporter [80]. 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 Transporter-Interfering Chemicals 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 [28,31,82–85]. Similarly, the antiparasitic

compound ivermectin has been shown to be transported by human, canine and mouse ABCB1 using knock-out mouse models and whole cell monolayer assays [83,86–88].

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 [63,68,70,71]. Lipid normalized levels of a single flame retardant and TIC, BDE-47, were 58.9 nanomolar in sardines [89] and as high as 175 nanomolar in tuna [63]. Importantly, the cumulative lipid-based concentrations of the ten most potent TICs were as high as 3.3 μM, while all pollutants were 12.7 μM, respectively [63]. Similarly, the same persistent pollutants can be detected at high concentrations in human blood and urine [90,91] and breast milk [92–94]. 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 [93]. For the organochlorine pesticide and TIC, p,p'-DDE, concentrations of up to 314 μM have been reported in breastmilk fat from South African women [94].

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 [95–100]. 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 [57]. The binding occurs at evolutionarily conserved residues, indicating the potential for effects in a wide range of organisms (Figure 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 super additive effects is necessary to provide appropriate dietary and food safety guidelines [101].

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 effects. To date multiple *in vitro* and *in vivo* assays have been used to determine the nature of drug Transporter-Interfering Chemicals [57,102,103]. 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 [80,104]. For ABC-type transporters, TIC interactions are often determined indirectly by inhibition of the pre-stimulated ATPase activity [85,105]. Alternatively, competitive uptake inhibition of reference compounds into membrane vesicles or competitive efflux inhibition and transport across cell monolayers have been employed.

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 [37,106–110] or to determine (competitive) transport inhibition [94–99], bidirectional transport across cell monolayers [40,111,112] or the binding affinities (Table 2) of different drugs and small molecules to the transporters [113–116]. 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 [117] and later applied to determine the activity of ABC drug transporters [118]. 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 [119] and large-scale protein production has been traditionally performed heterologously in bacterial and yeast systems [120,121]. 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 [37,107,109,122,123]. It is commonly accepted that substrate translocation requires both ATP binding and hydrolysis [123–125], 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 pre-stimulating 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, heterologous and homologous expressed protein (Table 2). In those cases, ATPase activity was measured pre-stimulated with different ratios and types of stimulator or non-stimulated (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 [126]. 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 [105,127–129]. 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 37C to 25C has been previously shown to decrease ATPase activity [130], while an increase in the assay temperature above 37C could inactivate the transporter [131].

It is important to note that these ATPase assays have some common limitations. First they favor discovery of inhibitory effects [31,57,82,84,85,132], 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 [133]. 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 non-specific 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 if 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 [134]. 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 radio-labeled control 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 *E. 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 [135,136]. 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 [137–139]. 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 [140–142]. 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 [143–146] or human cell lines [147–150] 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 inverted membrane vesicles (IMV), the effects of TICs on small molecule transporters 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 [151–154].

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 [155–157]. 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 [158]. 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 non-human endogenous transporters and often used to express human isoforms of transporters [40,159]. The same type of cell lines are also used in unidirectional fluorescent substrate transport assays with stably transfected transporters [160,161].

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 wildtype cells, leading to underestimation of substrate transport in transfected cells [162]. 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 [163,164]. Furthermore, in order to measure efflux by an apically localized transporter, the compound need 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 [165], very few have proven as robust as calcein-AM pioneered by Homolya and colleagues in the early 1990s [166,167]. 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 [160]. 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 [160,168,169] 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 [170–172].

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 [38,52,173,174]. However, hydrophobic TICs could also bind specifically or non-specifically within the hydrophobic parts of the membrane spanning domains to cause transporter inhibition. Such non-competitive inhibition can occur either at a defined allosteric site or a non-specific site within the SMT. For instance, the drugs daunorubicin, colchicine and vinblastine are known to allosterically inhibit verapamil-stimulated ABCB1 ATPase activity [38]. Allosteric non-competitive inhibition has also been shown for the ABCB1-mediated MDR reversal agent XR9576 (tariquidar) [47,175]. Tariquidar and the acridone carboxamide derivative GF120918 (elacridar) are also a competitive inhibitor for drug efflux transporter ABCG2 but do not inhibit ABCC1 [49,176,177].

Non-competitive inhibition of ABC-type transporters can also occur by interfering with ATP-binding [178]. TICs could act directly at the two ATPase domains (i.e. NBDs) of these ABC transporters, inhibiting both ATPase activity and drug binding capacity [179–181]. A less explored option for TICs to disrupt MRP-type transporters would be non-competitive inhibition of the GSH binding site in these transporters [182–186]. Several drugs, including vincristine and daunorubicin, critically depend on GSH binding and/or co-transport to be effectively eliminated [187,188].

A different kind of allosteric effect on the SMT activity regulation is the influence by its local membrane environment and in particular cholesterol interactions [133,189,190]. 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 [119,126]. Similarly, the photoaffinity labeling of ABCB1 using the substrate [^{3}H]azidopine was increased when increasing amounts of cholesterol were incorporated into liposomes [191]. In contrast, in cell lines expressing human ABCB1, the addition of cholesterol inhibited the efflux of daunorubicin [192].

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 non-competitive inhibition of ABCB1-mediated Rhodamine B efflux from mussel gills has been shown for synthetic perfluorochemicals that have high structural resemblance to fatty acids [193]. Second, the native lipid environment could affect assay results [133].

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 [59]. Such cooperative stimulation is a versatile and non-invasive mechanism to transiently modulate transporter activity and current clinical efforts focus on the discovery and development of modulating small molecules [178,194,195]. 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 [155,196,197].

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 [198–201]. 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 [57]. 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 [202,203]. 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 (Figure 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 [204]. 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 (Figure 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 multidrug resistance (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 lab 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 that may inform clinical studies in drug development [9,20,205]. 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 [206,207].

An alternative and emerging approach to narrow down drug and chemical candidates to test for transporter interactions in the wet lab 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 lab analysis [51,208,209]. Together with recent advances in the application of machine learning (ML) algorithms combined with network analysis tools in biological science [210–215], *in silico* tools could prove valuable for predicting and deciphering novel drug-drug interactions (DDIs), drug-food interactions (DFIs) and drug-environmental chemical interactions (DECIs) with SMTs. The ultimate goal would be to use *in silico* analysis as a high throughput, non-invasive 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 [216–221]. 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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Conflict of interest

The authors declare no conflict of interest.

Author contributions

SCTN and AH conceived and wrote the manuscript.

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Figure legends

Figure 1: Subcellular localizations of ABC- and SLC-type small molecule transporters in ten 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 (BBB) and blood-cerebrospinal fluid barrier (BCSFB) [20,205,222–230]. (B) Blood-intestine barrier (BIB) [10,20,230–234]. (C) Blood-milk barrier (BMB) in mammary glands [235–237]. (D) Blood-bile barrier (BBIB) in the liver [20,230,231,238–242]. (E) Blood-urine barrier (BUB) in the kidney [20,231,243–246]. (F) Blood-air barrier (BAB) in lung epithelial and endothelial cells [247–250]. (G) Blood-heart barrier (BHB) [251–254]. (H) Blood-placenta barrier (BPB) [74,230,255–260]. (I) Blood-testis barrier (BTB) [261–266]. (J) Blood-retinal barrier (BRB) in the eye [230,267–270]. Note that the common names for SLC-type transporters are used and the HUGO nomenclature for ABC-type transporters (https://www.genenames.org).

Figure 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 [105] and [271]. 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 [204]. 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.

Table Legends

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 ten different transporters. Physicochemical (MW, Log K_{ow}) and kinetic (IC_{50}, Km) parameters are provided. Interactions according to [28,29,31,80,82–88,105,272–293].

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 towards small molecule transporters. Assays according to [13,21,54,102,105,118,123,147,159,166,167,180,286,294–332].

Glossary – Modes and effects of environmental chemical interactions with small molecule transporters (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 towards 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 towards 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 reenter membranes/cells for another transport cycle.

Signaling interference. Interactions of compounds with SMTs disrupt cell signaling and/or signal transduction.

Assay type	Assay System	Species: Transporter(s)	Reporter Molecule	Original References
ATPase Assays	Inverted membrane vesicles (IMVs) in KB-V1 and KB-3-1	Human: ABCB1	VBL	Horio et al. 1988
	Proteoliposomes (protein from $CH^{R}C5$)	Hamster: ABCB1	COL	Sharom et al. 1993
	Membrane Nanodiscs	Human: ABCB1	Nicardipine	Ricthie et al. 2009
	Styrene-maleic acid lipid particles (SMALPs)	Human: ABCB1, ABCC1, ABCC4, ABCG2; Mouse: ABCC7 (CFTR)	MIANS, Estrone sulfate, PheoA	Gulati et al. 2014
	Isolated membranes (from Sf9 cells)	Human: ABCB1	VER, VBL, 5-Fluorouracil, Trifluoperazine	Sarkadi et al. 1992
	Amphipols	Mouse: ABCB1a (MDR3); Human: ABCB1	N/A	Lee et al. 2002 , Alam et al. 2017
	Purified protein (protein from $CH^{R}C5$)	Hamster: ABCB1	VER, VBL, COL, Nifedipine, Daunomycin	Doige et al. 1992
Competitive dye transport assays (unidirectional accumulation)	Invertebrate embryos (*S. purpuratus*)	Sea urchin: ABCB1	Rhodamine	Toomey and Epel 1993
	BEWO (choriocarcinoma)	Human: ABCB1, ABCC1	CAM, VBL, Fluorescein	Utoguchi et al. 1999
	Huh-7 (human hepatocellular carcinoma)	Human: ABCB1, ABCC1, ABCG2	Rho123, Hoechst 33342	Jouan et al. 2016
	HeLa cells (Henrietta Lacks cervical cancer)	Human: ABCB1	Rho123, CAM	Sauna et al 2002
	K562 cells (human bone marrow chronic myelogenous leukemia)	Human: ABCB1, ABCG2	Hoechst 33342, DyeCycle Violet	Nerada et al. 2016
	A431 cells (human skin epidermoid carcinoma)	Human: ABCB1, ABCG2	Hoechst 33342, DyeCycle Violet	Nerada et al. 2016
	CHO K1 (Chinese Hamster Ovary)	Hamster: ABCB1, ABCC1, ABCG2	CAM, eFluxx-ID, CMFDA, PheoA	Lebedeva et al. 2011
	A549 (human lung carcinoma)	Human: ABCB1, ABCC1, ABCG2	eFluxx-ID, CMFDA, PheoA, DiOC2(3)	Lebedeva et al. 2011
	HL-60/MX1 (human acute promyelocytic leukemia)	Human: ABCB1, ABCC1, ABCG2	CAM, eFluxx-ID, PheoA	Lebedeva et al. 2011
	HCT-8 and HCT-15 (human ileocecal colorectal carcinoma)	Human: ABCB1, ABCC1	CAM, eFluxx-ID, CMFDA, DiOC2(3)	Lebedeva et al. 2011
	HepG2 cells (human liver hepatocellular carcinoma)	Human: ABCB1	Rho123	Shabbir et a. 2005
	NIH/3T3 murine fibroblasts	Human: ABCB1	CAM	Homolya et al 1993, Hollo et al. 1994
	PLHC-1/dox cell lines (Poeciliopsis Lucida hepatocellular carcinoma)	Clearfin Livebearer: ABCB1	CAM, Rho123	Caminada et al 2008, Zaja et al. 2011
	MDCKII (Madin-Darby canine kidney strain II cells)	Human: ABCB1	CAM	Gannon et al. 2009
	NCI-H441 (human lung adenocarcinoma)	Human: ABCB1	Rho123	Salomon et al. 2014
	KB-V1 and KB-3-1 cells (Cervix carcinoma - HeLa derivative)	Human: ABCB1	CAM	Ansbro et al. 2013
	Renal proximal tubules	Killifish: ABCB1	NBDL-CSA	Schramm et al. 1995
	Brain capillaries	Rat: ABCB1a/b	NBDL-CSA, BODIPY-Prazosin, SR101	Hartz et al. 2004
Monolayer assay (bidirectional transport)	Caco-2 (Caucasian Colon Carcinoma)	ABCB1, ABCG2, MRP2	VBL	Hunter et al. 1993
	LLC-PK1 (Epithelial-like pig kidney cell line)	Human: ABCB1	DOX, Rho123, QUI, VER	Van Der Sandt et al. 2000, Riede et al. 2019
	MDCKII (Madin-Darby canine kidney strain II)	Human: ABCB1	Rho123	Haemmerle et al. 2000
	IPEC-J2 (Pig Illeum Epithelial cells)	Human: ABCB1	Digoxin, VER, Citalopram, VBL, VCR	Saaby et al. 2016
	Calu-3 (Human lung adenocarcinoma)	Human: ABCB1	CAM, Rho123	Hamilton et al. 2000
	hCMEC/D3 (brain microvascular epithelial cell line)	Human: ABCB1	eFLUXX-ID Gold	Noack et al. 2016
Cytotoxicity assays	CR1R12 (CHO subline)	Hamster: ABCB1	COL	Aller et al 2009
	2-cell embryos (*S. purpuratus*)	Sea urchin: MRP-like	VBL	Hamdoun et al. 2004
	Functional complementation (*S. cerevisiae*)	Yeast: Human ABCB1	Valinomycin	Kuchler and Thorner 1992
	Competitive growth inhibiton (*S. cerevisiae*)	Yeast: Mouse ABCB1a (MDR3)	DOX	Jeong et al. 2007, Nicklisch et al. 2016
Fluorescence anisotropy/polarization	Proteoliposomes	*M. tuberculosis* : TBsmr	Ethidium bromide, TPP+	Basting et al. 2008
	Purified protein	*E. coli* : EmrE	Ethidium bromide, TPP+	Chen et al. 2007
	Purified protein	*L. lactis* : LmrP	Propidium and ethidium dyes	Schaedler and Veen 2010
	Purified protein	*E. coli* : AcrB	Rho6G, Ethidium, Proflavin, Ciprofloxacin	Su et al. 2007
	Purified protein	*S. aureus* : MepA	Acriflavine, Rho6G, Ethidium	Banchs et al. 2014
Drug binding affinity	Surface plasmon resonance (SPR)	Human: ABCB1	MRK16, UIC2 mAB	Ritchie et al. 2011, Chen et al. 1986
	FRET analysis in Hek293T	Human: ABCC1	E217βG, ATP, Vanadate	Osa-Andrews et al 2018, Iram et al 2015, Swartz et al 2013
	Intrinsic Trp fluorescence quenching in $CH^{R}B30$ (CHO derivative)	Hamster: ABCB1	Tryptophan	Liu et al 2000
	Site-directed fluorescence labeling & quenching in $CH^{R}B30$	Hamster: ABCB1	MIANS label	Liu and Sharom 1996
Biochemical assays (binding sites)	Photo-affinity labeling in KB-3-1 (HeLa derivative)	Human: ABCB1	Azidopine, IAAP, 6-AIPP-forskolin	Bruggemann et al. 1989, Greenberger 1998
	Cys & thiol reactive labeling in HEK293 cells	Human: ABCB1	Dibromobimane, MTS-VER	Loo and Clarke 1997, Loo and Clarke 2001
	Nucleotide trapping assays in CR1R12 (CHO derivative)	Hamster: ABCB1	Vanadate	Urbatsch et al. 1995
	Radioligand binding in $CH^{R}B30$ (CHO derivative)	Hamster: ABCB1	VBL, XR9576	Martin et al. 2000

VBL = vinblastine
VCR = vincristine
COL = colchicine
VER = Verapamil
DOX = Doxorubicin
QUI = Quinidine
PheoA = Phephorbide A

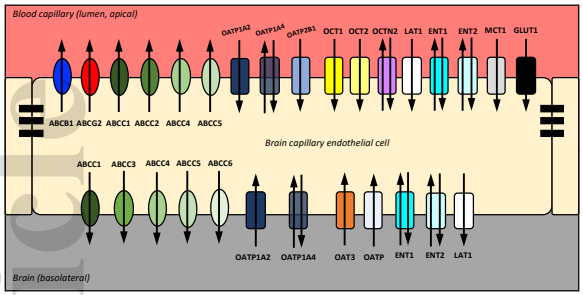
SR101 - Suforhodamine 101
MIANS = 2-(4'-maleimidylanilino)naphthalene-6- sulfonic acid
E217βG = 17β-Estradiol 17β-D-glucuronide
TPP+ = Tetraphenylphosphonium
NBDL-CSA = [N-ε-(4-nitrobenzofurazan-7-yl)-D-Lys8] cyclosporin A
IAAP = Iodoarylazidoprazosin
6-AIPP-forskolin = 6-O-[[2 -[3-(4-azido-3-[125I]iodophenyl)propionamido]ethyl]carba- myl]forskolin

	Chemical	Class	Pubchem CID	MW	Log K_{ow}	*ABCB1* IC_{50} (μM)	*ABCB1* K_m (μM)	*ABCG2* IC_{50} (μM)	*ABCG2* K_m (μM)	*ABCC1* IC_{50} (μM)	*ABCC1* K_m (μM)	*ABCC4* IC_{50} (μM)	*ABCC4* K_m (μM)	*OATP1A1* K_{cappi} (μM)	*OATP1A2* IC_{50} (μM)	*URAT 1* IC_{50} (μM)	*MATE1* EC_{50} (μM)	*OCT2* EC_{50} (μM)	*OAT4* IC_{50} (μM)	References
Inhibitors	Endosulfan**	Insecticide	3224	406.9	3.62(β), 3.83(α)	2.8 [1], 11.4, 33.6	17	6.9	---	---	---	---	---	---	---	---	---	---	---	Bircsak et al. 2013, Sreeramulu et al. 2007, Pivcevic and Zaja 2006, Bain and LeBlanc 1996, Buss et al. 2002, Smital et al. 2004
	Endrin	Pesticide	12358480	380.9	5.34	1.1	---	---	---	---	---	---	---	---	---	---	---	---	---	Nicklisch et al. 2016
	Heptachlor	Insecticide	3589	373.3	6.1	INH	---	---	---	---	---	---	---	---	---	---	---	---	---	Bain and LeBlanc 1996
	Malathion	Insecticide	4004	330.4	2.36	INH	---	---	---	---	---	---	---	---	---	---	---	---	---	Smital et al. 2004
	Musk ketone (MK)	Synthetic musk	6669	294.3	4.3	0.74 [2]	---	---	---	---	---	---	---	---	---	---	---	---	---	Luckenbach and Epel 2005
	Musk xylene (MK)	Synthetic musk	62329	297.3	4.9	0.97 [2]	---	---	---	---	---	---	---	---	---	---	---	---	---	Luckenbach and Epel 2005
	p,p'-DDE	Insecticide	3035	318.03	6.51	31.3	---	4	---	---	---	---	---	---	---	---	---	---	---	Bircsak et al. 2013, Nicklisch et al. 2016
	p,p'-DDT	Insecticide	3036	354.49	6.36, 6.91	3.8-25.6	---	5	---	---	---	---	---	---	---	---	---	---	---	Bircsak et al. 2013, Nicklisch et al. 2016
	PBDE-100	Flame Retardant	154083	564.69	7.24	23.2	---	---	---	---	---	---	---	---	---	---	---	---	---	Nicklisch et al. 2016
	Perfluorodecanoic acid (PFDA)	Perfluorochemicals	9555	514.08	6.3[d]	7.1 [2]	---	---	---	---	---	---	---	26.8	INH	INH	---	---	INH	Stevenson et al. 2006, Yang et al. 2010, Yang et al. 2009
	Perfluorononanoic acid (PFNA)	Perfluorochemicals	67821	464.08	5.48	4.8 [2]	---	---	---	---	---	---	---	44.6	INH	INH	---	---	INH	Stevenson et al. 2006, Yang et al. 2010, Yang et al. 2009
	Permethrin	Insecticide	40326	391.3	6.5	INH	---	7	---	---	---	---	---	---	---	---	---	---	---	Bircsak et al. 2013, Bain and LeBlanc 1996
	Tetrabromobisphenol A (TBBPA)	Flame Retardant	6618	543.9	4.75[b]	22.9	---	INH	---	INH	---	24	---	---	---	---	---	---	---	Dankers et al. 2013
Substrates	Cyperquat (MPP+)	Herbicide	39484	170.23	2.7[b]	---	SUB	---	---	---	---	---	---	---	---	---	---	---	---	Lacher et al. 2015
	Diazinon*	Insecticide	3017	304.35	3.81	---	9.7	---	---	---	---	---	---	---	---	---	---	---	---	Lacher et al. 2015
	Indinavir	Antiviral	5362440	613.8	2.9, 3.49	---	SUB	---	---	---	SUB	---	---	---	---	---	---	---	---	Van der Sandt 2001
	Ivermectin*	Antiparasitic	6321424	875.1	3.2[c]	0.1	9 [1]	---	---	---	---	---	---	---	---	---	---	---	---	Eneroth et al. 2001, Buss et al. 2002, Schinkel et al. 1994, Griffin et al. 2005
	Methoxychlor	Insecticide	4115	345.6	4.68, 5.08	---	---	---	---	---	SUB	---	---	---	---	---	---	---	---	Tribull et al. 2003
	Paraquat	Herbicide	15939	186.25	-4.22,-4.5[d]	---	SUB	---	---	---	---	---	---	---	---	---	---	SUB	SUB	Chen et al. 2007, Wen et al. 2014
	Parathion methyl **	Insecticide	4130	263.21	2.86	3.4	7.3	---	---	---	---	---	SUB [3]	---	---	---	---	---	---	Nomberg et al. 2015, Aurade et al. 2006, Sreeramulu et al. 2007
	Ritonavir*	Antiviral	392622	720.9	3.9	---	SUB	---	---	---	SUB	---	---	---	---	---	---	---	---	Van der Sandt 2001, Alsenz et al. 1998, Srinivas et al. 1998
Inducers (expression)	Diazinon*	Insecticide	3017	304.35	3.81	---	IND	---	---	---	---	---	---	---	---	---	---	---	---	Lecoeur et al. 2006
	p,p'-DDE	Insecticide	3035	318.03	6.51	---	IND	---	---	---	---	---	---	---	---	---	---	---	---	Shabbir et al. 2004
	p,p'-DDT	Insecticide	3036	354.49	6.36, 6.91	---	IND	---	---	---	---	---	---	---	---	---	---	---	---	Shabbir et al. 2004
	Prochloraz	Fungicide	73665	376.7	4.1	---	---	---	IND	---	---	---	---	---	---	---	---	---	---	Halwachs et al. 2012
	Rifampicin	Antibiotic	135398735	822.9	2.7	---	IND	---	---	---	---	---	---	---	---	---	---	---	---	Westphal et al. 2000
	Ritonavir	Antiviral	392622	720.9	3.9	---	IND	---	---	---	IND	---	---	---	---	---	---	---	---	Perloff et al. 2001
	St. John's Wort	Supplement	46507869	NA	NA	---	IND	---	---	---	---	---	---	---	---	---	---	---	---	Hennessy et al. 2001
	TCDD	Dioxin	15625	322	6.8	---	---	---	IND	---	---	---	---	---	---	---	---	---	---	Halwachs et al. 2012
weak interactors	Aminocarb	Insecticide	16247	208.26	1.73, 1.9	WI	---	WI	---	WI	---	---	---	---	---	---	---	---	---	Bain and LeBlanc 1996, Guenriche et al. 2020
	BPA (Bisphenol A)	Plastic monomer	6623	228.29	3.32	WI	---	INH	---	WI	---	WI	---	---	---	---	---	---	---	Dankers et al. 2013, Nicklisch et al. 2016
	DEHP (Bis(2-ethylhexyl) phthalate)	Plasticizer	8343	390.6	5.03, 7.6	WI	---	WI	---	WI	---	WI	---	---	---	---	---	---	---	Dankers et al. 2013, Nicklisch et al. 2016
	MEHP	DEHP metabolite	20393	278.34	4[b]	WI	---	WI	---	WI	---	WI	---	---	---	---	---	---	---	Dankers et al. 2013
	Warfarin	Anticoagulant	54678486	308.3	2.7	WI	---	---	---	---	---	---	---	---	---	---	---	---	---	Palmeira et al. 2011, Gschwind et al. 2013
synergist/inhibitor	Diazinon*	Insecticide	3017	304.35	3.81	SI	---	---	---	---	---	---	---	---	---	---	---	---	---	Pivcevic and Zaja 2006
	Endosulfan	Insecticide	3224	406.9	3.62(β), 3.83(α)	SI	---	---	---	---	---	---	---	---	---	---	---	---	---	Pivcevic and Zaja 2006
	Fenamiphos	Insecticide	31070	303.36	3.23	---	---	---	---	---	---	---	---	---	---	---	---	SI	---	Chedik et al. 2019
	Phosalone	Insecticide	4793	367.8	4.38	SI	---	---	---	---	---	---	---	---	---	---	---	---	---	Pivcevic and Zaja 2006
	Phosmet	Insecticide	12901	317.3	2.78	---	---	---	---	---	---	---	---	---	---	---	---	SI	---	Chedik et al. 2019
	Propiconazole	Fungicide	43234	342.2	3.72	SI	---	---	---	---	---	---	---	---	---	---	---	---	---	Pivcevic and Zaja 2006

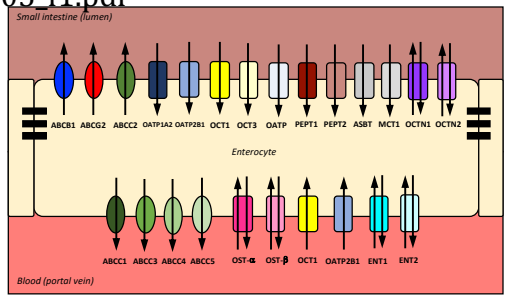
[a] estimated (Pubchem)
[b] ionizable compound
[c] Lumaret et al. 2012
[d] polar compound
* also inhibits transporter efflux
** also stimulates transporter ATPase

--- = not available
WI = weak interaction
INH = inhibitor (no IC50 determined)
SUB = substrate (no Km or EC50 value determined)
IND = induction (no expression value determined)
SI = synergistic inhibition
1 = EC50
2 = muscle gill transporters (predominantly ABCB1)
3 = Zebrafish ABCC4

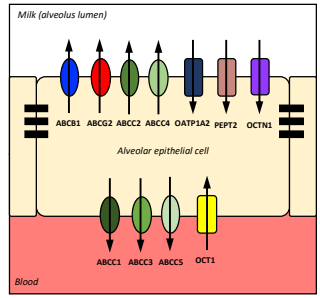
A. Blood Brain Barrier (BBB) & Blood CSF Barrier (BCSFB)



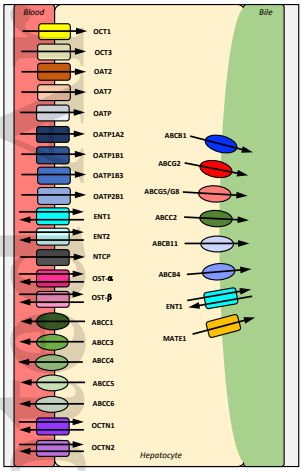
B. Blood Intestine Barrier (BIB)



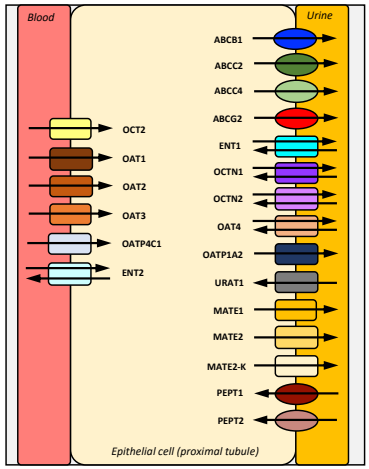
C. Blood Milk Barrier (BMB)



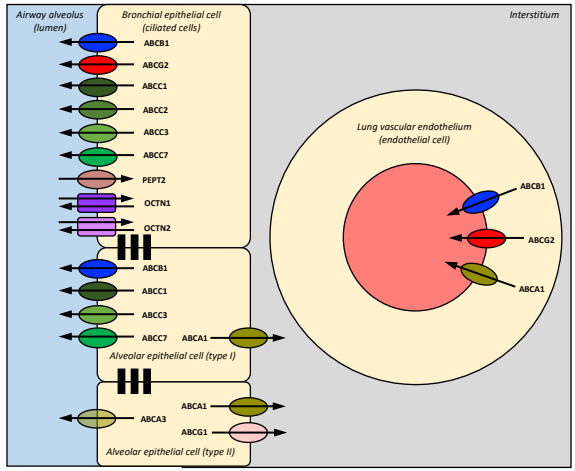
D. Blood Bile Barrier (BBIB)



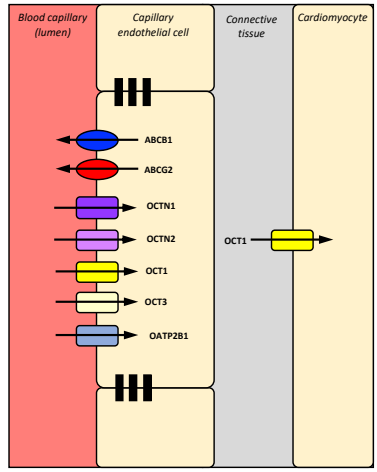
E. Blood Urine Barrier (BUB)



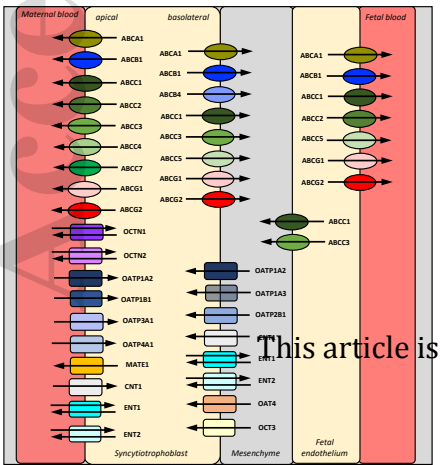
F. Blood Air Barrier (BAB)



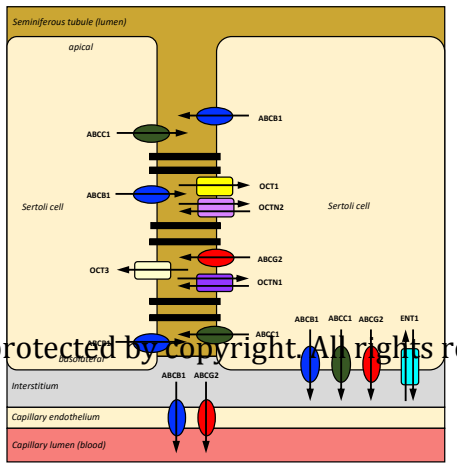
G. Blood Heart Barrier (BHB)



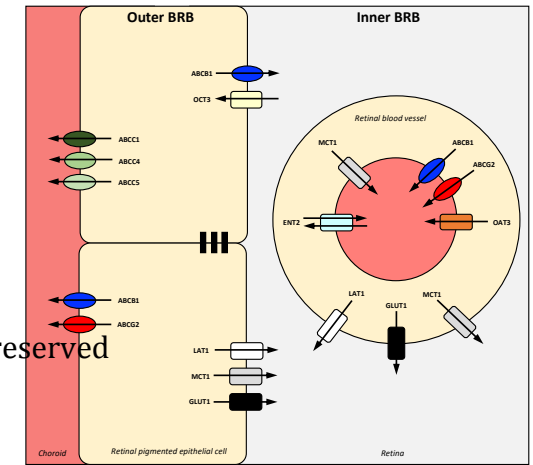
H. Blood Placenta Barrier (BPIB)

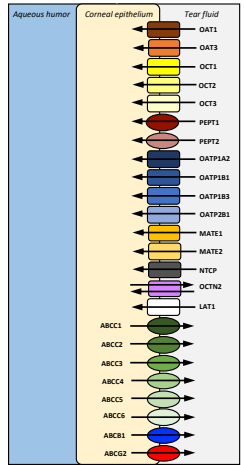


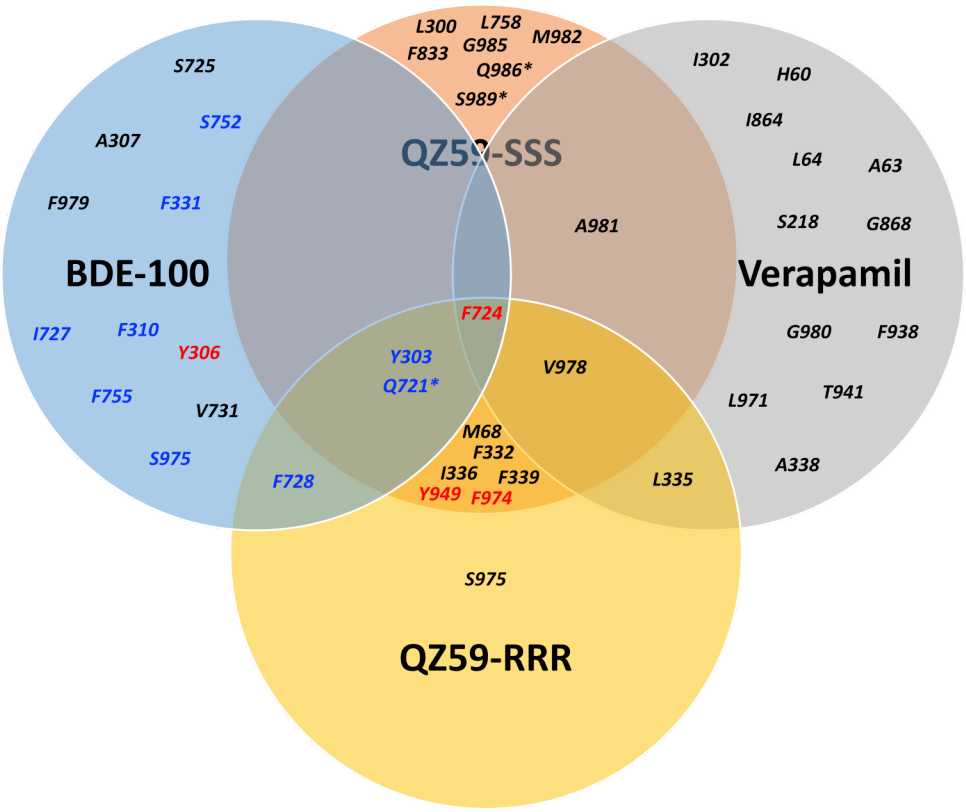
I. Blood Testis Barrier (BTB)



J. Blood Retinal Barrier (BRB)







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

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