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EFFLUX TRANSPORTERS: Newly Appreciated Roles in Protection against Pollutants:

Cellular "bouncers" help keep toxicants out of cells, but anthropogenic chemicals can circumvent or overwhelm this defense

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Although your high-school biology teacher might not have told you, cells are like nightclubs. They have to make sure that the right individuals come in and that the bad individuals—those who might disrupt the normal activity inside the club—stay out. To that end, both nightclubs and cells employ "bouncers" to prevent unwanted characters from entering. For nightclubs the undesirable characters could be drunks; for cells the undesirable characters are noxious chemicals. And, as in a nightclub, the job of the cellular bouncer is to prevent any bad players from entering and to throw them out if they should happen to get in.

The cellular bouncers are efflux transporter proteins belonging to the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, one of the largest families encoded in the human genome (1). They sit in the membrane of cells, possess a common ATP-binding region referred to as the ABC cassette (hence, ABC transporters), and are present in all organisms, from bacteria to humans (2 , 3). In many cases, these transporters act as a first line of defense, preventing toxic chemicals from entering the cell. However, if a compound is not recognized by these transporters and enters the cytoplasm, detoxifying enzymes in the cell may modify the chemical to a more hydrophilic form. In this case, related cellular bouncers can again come into play, effluxing the modified products out of the cell.

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Protecting cells against toxicants

This article aims to better acquaint environmental scientists with these transporters and their relevance in protecting cells against toxicants. The role of these transporters is well described in the pharmacology and cell biology literature. However, in environmental toxicology, the bulk of research is on xenobiotic transformation. In fact, the efflux transporters are the first defense against toxicants, keeping them out of cells in the first place, and they can be the last defense via their role in expelling the toxicant metabolites.

This article also highlights ways in which anthropogenic chemicals can circumvent or overwhelm this defense. Anthropogenic chemicals that possess novel molecular structures may not be recognized by the cell's efflux transporters and may freely enter the cytoplasm where they can exert toxic effects. If this unrecognized chemical also cannot be metabolized, it will remain in the cell as a legacy chemical—a marker of the cell's exposure history.

Mixtures of xenobiotics can compromise this defense because competitive binding of different chemicals to the transporters can sabotage the activity of transporter binding sites or saturate them. In addition, some of the chemicals in the mixture might be direct inhibitors of the transporter activity. The resultant competition or inhibition, termed chemosensitization, can decrease transporter activity such that toxic substances normally excluded from the cell can now enter (4, 5). We will discuss how these weak links can have subtle but detrimental consequences.

Our focus is on the three ABC subfamilies that have toxicologically relevant efflux activity. These include members of the P-glycoprotein (P-gp) or ABCB family, the multidrug resistance protein (MRP) or ABCC family, and the breast cancer resistance protein (BCRP) transporter of the ABCG family. These proteins are commonly referred to as multidrug or efflux transporters. They provide important protection in all organisms. In humans, for example, they are active in the blood–brain barrier, intestines, liver, kidney, and placenta. Similar efflux transporters enable aquatic organisms to thrive and reproduce in challenging contaminated environments.

We first provide background on these transporters and their properties. We then describe their roles in determining availability and hence toxicity of pollutants. Next, we consider the functional limits of this defense mechanism, specifically its disruption by certain chemicals. We close with a consideration of the potential role of these transporters in the phenomenon of global contamination, as in the cases of DDT, PCBs, and perfluorochemicals.

Overview of transporters

The P-gp (ABCB) family

The majority of research on efflux transporters is on the drug resistance that sometimes follows cancer chemotherapy. Juliano and Ling (6) found that this resistance resulted from the cells' low accumulation of anticancer drugs and related it to elevated levels of a membrane glycoprotein, which they termed the permeability glycoprotein (P-glycoprotein, P-gp). By preventing sufficient anticancer drug accumulation, P-gp apparently conferred the phenotype of multidrug resistance (MDR).

Further evidence that P-gp caused drug resistance included transfecting cells with the P-gp gene; such cells exhibited decreased drug accumulation (7). Similarly, gene knockouts resulted in increased drug accumulation and sensitivity as compared with the wild-type organism (8). Subsequent studies have provided extensive information on the structure of the protein, its genes, and the regulation of gene activity (reviewed in Refs. 4 and 9).

P-gp is remarkably nonspecific with respect to its substrates—hence, the MDR descriptor. This lack of specificity is adaptive; it provides predators with a mechanism to keep pace with the evolution of new natural-defense molecules of their prey and fortunately provides protection against many novel anthropogenic products.

MDR provided by P-gp is a consequence of its remarkable nonspecificity with respect to its substrates. Much research has focused on understanding the seeming promiscuity of these transporters, indicating common characteristics among substrates, such as moderate hydrophobicity, small size, and positively charged domains (4, 10, 11). A downside to this multispecificity, which P-gp shares with some of the other efflux transporter families, is that the proper function of the system is vulnerable to the presence of multiple substrates that compete for substrate binding sites. Going back to the bouncer concept, if one distracts the bouncer (as with a competitive substrate), normally excluded and undesirable characters can now gain entry into the cell.

P-gp-mediated efflux does not involve the metabolism of the xenobiotic. Rather, the chemical is the substrate, indicating that cytoplasmic transformation is not a prerequisite for efflux. Indeed, localization of P-gp to the membrane suggests its action on the substrate before the xenobiotic can even enter the cytoplasm.

This idea can be tested by using calcein acetoxymethyl (calcein-AM) ester, a nonfluorescent P-gp substrate (Figure 1). If this ester gets into the cytoplasm, generalized esterases will hydrolyze the calcein-AM ester bond, producing highly fluorescent calcein. Because calcein is not a good substrate of the transporter, it becomes trapped in the cell. When cells overexpressing P-gp are exposed to calcein-AM, little fluorescent calcein is produced, indicating that little calcein-AM has entered the cytoplasm. However, in the presence of P-gp inhibitors, a large fluorescence signal is observed, indicating that calcein-AM has entered the cytoplasm where esterase activity produces fluorescent calcein (12).

This simple experiment shows that efflux activity is, in fact, a first line of defense against toxicants. Indeed, this activity can possibly determine the effective pharmacological dose of drugs or toxicants; the effective dose has to exceed the capacity of the transporter for that chemical before therapeutic (for drugs) or toxic (for toxicants) effects are seen.

This protection contrasts with the enzymatic transformation or detoxification of xenobiotics after they enter the cell (e.g., when the concentration exceeds the transporter capacity or if the efflux transporters do not recognize the substrate). Detoxification mechanisms typically include oxidation of xenobiotics by a P450 system (phase I; 13), followed by the conjugation of the now-modified xenobiotic to small polar moieties such as glucose, sulfate, or glutathione (phase II reactions). Then, the now-modified toxicant can be eliminated in phase III by another ABCC family of efflux transporters, which recognize the conjugated toxicant and then pump it out of the cell (see Figure 2 and the next section).

The MRP (ABCC) family

Some members of the MRP (ABCC) family also contribute to multidrug efflux by acting like P-gp to efflux unmodified xenobiotics. However, members of this family also act on endogenous substrates that are normal products of metabolism, or—and this is an important difference—they work on toxicants that have entered the cell and are modified as a part of the abovementioned phase I and phase II detoxification processes (14, 15). The resulting conjugated, negatively charged molecules are recognized by various ABCC transporters and then exported from the cell (15).

The MXR (ABCG) family

ABCG2, a member of the ABCG family commonly referred to as BCRP, also causes significant resistance to a limited group of chemotherapeutic treatments (substrate specificity is less broad than for ABCB and ABCC; 10) as well as effluxing dietary toxicants (16). A difference from the aforementioned transporter types, which are so-called full transporters (the gene product constitutes one structural unit), is that ABCG2 is a half transporter, in which two protein molecules are assembled to form a structural unit active as a homodimer.

ABCG2 is also highly expressed in the mammary gland during pregnancy and lactation where it may play a role in the translocation of essential compounds, such as riboflavin, into breast milk (17). This activity may be a double-edged sword: although transport of vitamins into milk is highly favorable, ABCG2 might also increase concentrations of toxicants in milk (18).

Environmental relevance

The above overview emphasizes the medical aspects that have been the major focus of efflux transporter research. The broader goal of this work is to increase drug access to cells; this can be achieved by, for example, inhibiting transporter activity so that otherwise excluded drugs can now accumulate in cells at therapeutically effective levels (4, 5, 11, 19, 20).

In contrast to the medical goals, the environmental focus is to understand how these transporters keep toxicants out of cells and to ensure that the transporters operate optimally to protect cells from environmental contaminants. This environmental work focuses on properties of the efflux transporters and the identification of any chemicals that might elude or inhibit them, such that the transporters cannot carry out their protective function.

The environmental relevance of these transporters was first recognized by Kurelec and collaborators, who showed that P-gp (ABCB) transporters protected aquatic organisms from several pollutants. They named this protection multixenobiotic resistance (MXR) to highlight its ability to protect cells from or provide resistance to foreign chemicals (21).

Evidence for environmental protection

Efflux activity in living cells is typically measured by quantifying substrate uptake into cells in the absence and presence of specific efflux transporter inhibitors. If activity is present, little uptake of the substrate will occur, but if an inhibitor of the transporter is added, the uptake increases (Figure 1). Thus, the ratio of substrate accumulation in a cell in the absence and presence of inhibitors provides a measure of transport activity (12).

Test substrates could be radioactive, such as ^{14}C vinblastine, or fluorescent, such as rhodamine or calcein-AM. Cyclosporin, its derivative PSC833, and verapamil are often used as relatively specific inhibitors of the P-gp family, and MK571 is considered the most effective inhibitor of the MRP family. However, all of these chemicals can act on other types of ABC transporters, making their nonspecificity a key issue in interpreting experimental data. Clearly, more specific reagents would be of great use.

Numerous studies on mammalian cells demonstrate increased substrate accumulation when the efflux transporters are inhibited (4). Similarly, sea urchin embryos take up little calcein-AM. However, in the presence of PSC833 (P-gp inhibitor), the accumulation is increased 20-fold, and in the presence of MK571 (MRP inhibitor), the accumulation is increased 80-

fold (22). In mussel gills, accumulation of the pesticide dimethyl tetrachloroterephthalate (Dacthal) is increased 2.5-fold by verapamil (inhibitor of both P-gp and MRP; 23).

One can assess the protective role of the transporter by comparing the effects of a toxicant on a cellular process in the presence and absence of transporter inhibitors. For example, the effect of the microtubule-destabilizing drug vinblastine on cell division in sea urchin embryos is enhanced 13-fold when the MRP activity is inhibited. The effective concentration for 50% of the population is 3.3 μM in the control situation but drops to 0.25 μM when the transporter is inhibited with MK571 (22).

Response of the transporter system to cellular stress

An important adaptation to environmental stress is increased transcription and translation of protective genes. This defense, termed the cellular stress response (24, 25), is initiated by stressors such as xenobiotics, thermal stress, inflammation, and hypoxia (26) and can directly mitigate the damage or prevent further injury if the stress remains. The response includes up-regulation of a suite of protective and detoxifying genes, including some ABC efflux transporters, P450 detoxification proteins, and associated conjugating enzymes (9).

Interestingly, the up-regulation of P-gp activity in response to xenobiotics is not large (1–2-fold [27] as compared with 10–100-fold for some of the cytochrome P450s [28]) and indicates that increased P-gp levels are not an important protective response, that the level of transporter activity is already set to the expected historical load of xenobiotics, or that a small increase is adequate to protect the organism. Alternatively, it might indicate that an increase in activity has negative consequences, such as the export of essential cell constituents.

Sabotage of the transporter defense

We have emphasized that P-gp (ABCB) and some MRP (ABCC) efflux transporters act as first lines of defense against xenobiotics. However, toxicants *do* enter cells, indicating that the transporter defense is fallible. Several factors allow this defense to be breached.

Environmental concentrations and substrate specificity

The two major factors limiting the efficacy of efflux transporters are toxicant concentration and molecular structure of the substrate. With regard to concentration and using the bouncer analogy, if too many undesirable characters (i.e., high concentration of xenobiotics) try to enter at the same time, some may slip past the bouncer. Molecular structure is also critical. In the sea urchin embryo, for example, the transporter keeps calcein-AM out of the cell at levels between 10^{-9} and 10^{-5} M, a 10^4 range of concentration. With vinblastine, however, the transporter protects against 0.5×10^{-6} M vinblas-tine but is overwhelmed at 5×10^{-6} M (22).

Perhaps to compensate for these limitations, every organism has several transporters. The sea urchin, for example, has 35 different ABCC transporters in its genome (25), nearly twice the number in any other deuterostome genome examined.

What is the significance of this diversification of protective proteins? Are different types expressed in different tissues? Are the proteins aimed at different types of substrates? Do they have different affinities for the same substrates? Further research should reveal the meaning of variation in transporter sequences and their role in the organism (29). Surprisingly, humans have only one P-gp transporter involved in toxicant efflux. Is this because of a limited diet or limited environmental exposure? Does this mean that humans are more vulnerable to novel chemicals? Or are these transporters particularly effective?

Chemosensitization and sabotage of the efflux transporters

As previously noted, many chemicals can inhibit the activity of the transporters, a phenomenon referred to as chemosensitization (the chemicals that cause this effect are called chemosensitizers; 30). One consequence of chemosensitization is enhancement of toxicity (19); the inhibition of transporter activity decreases efficacy such that previously excluded toxicants can now enter the cell.

This could explain the seeming paradox in toxicology whereby toxic effects are unexpected or unexplainable because the levels of known toxic substances are less than the established toxic thresholds. Often the enhanced effect is assumed to result from bioaccumulation, which brings concentrations sufficiently high to be toxic, or from synergistic interactions of the potpourri of chemicals that exists in the real world (31). Chemosensitization from interaction of multiple chemicals could be a part of this puzzle, with increased deleterious effects resulting from sensitization by chemicals that by themselves are not even toxic.

Several studies with aquatic organisms demonstrate the likelihood of this scenario. In vivo experiments with clams, mussels, sponges, marine worms, sea urchin embryos, and so on demonstrate that exposure of cells to mixtures of model substrates and P-gp inhibitors, or to model substrates plus polluted water or extracts from polluted waters, leads to enhanced accumulation of the model substrates (30, 32).

One example of a chemosensitizer is synthetic musk fragrances, which accumulate in humans from direct dermal contact and in aquatic organisms from exposure to effluents from sewage treatment plants. These chemicals by themselves are not considered directly toxic (33), but they inhibit the major efflux transporters of marine mussels. Interestingly, the inhibition is long-lived and persists for at least 24 hours after removal of the musks (34).

A second group of chemosensitizers are the long-chain perfluoroalkyl acids (PFAs) used in such products as stain repellents, nonstick cookware, and firefighting foams (35). The carbon–fluorine bond makes them largely resistant to photo- and biodegradation, which ensures their persistence. Although not very fat soluble, they accumulate in humans and wildlife, including albatross and even polar bears (36). Such global pollution by a water-soluble chemical was unexpected, raising many questions about mechanisms of dispersal and accumulation.

Using gill tissue of marine mussels, Stevenson et al. (37) found that PFAs are taken up in the mussels' gills, with a preference for molecules with C7–C9 chain lengths. Verapamil, which inhibits the known transporters in the gill, does not enhance the accumulation of PFAs, suggesting that PFAs are not substrates of the verapamil-sensitive transporters. The observed uptake may reflect a propensity to bind to proteins or to dissolve in the lipids of the cell (PFAs have detergent-like properties).

Interestingly, these long-chain congeners inhibit the P-gp transporter activity. Because they are not substrates of the transporters (e.g., the aforementioned lack of effect on accumulation by the transport inhibitor verapamil), they might be inhibiting activity by some indirect effect on the transport mechanism, most likely via an effect on the plasma membrane; other effects on membranes have been reported.

The inhibition of transporter activity raises the question of whether other anthropogenic chemicals might have similar effects. An unknown factor is how extensive chemosensitization will be when the concentrations of candidate compounds are very low. Will there be subtle effects with small but cumulative consequences? Or is chemosensitization only a problem when chemosensitizer levels are high?

A final consideration is whether the accumulation of persistent chemicals such as DDT, PCBs, and PFAs ensues because the transporters do not recognize these chemicals when they initially enter the cell. If not recognized, these pollutants will not be removed from the cells. An analogy would be that the transporters are garbage collectors of the cell; if they can't recognize something as garbage, it will remain there.

Conclusions and prognosis

The efflux transporters, well-studied for their medical relevance, are also critical players in environmental toxicology. Some provide a first line of defense against toxicants, and others export the toxicants after they have entered the cell and have been modified by detoxification enzymes. This defense is effective at concentrations from the nano- to micro-molar range. The transporters' efficacies vary with different substrates and concentrations, most likely reflecting adaptation to past history of the species and in anticipation of chemicals they will commonly encounter (38). The defense, however, can be sabotaged by natural and anthropogenic chemicals that can reduce transporter activity, inhibit the protein(s), or affect the membrane structure.

Two aspects demand prompt attention. The first is chemosensitization: seemingly benign environmental chemicals can inhibit efflux activity, thereby exposing the cells to toxicants that would normally be excluded. This is a masked or hidden aspect of toxicology. Such impairment of transport activity could be manifested ultimately as a negative effect on wildlife population structure or as an unexplained health or epidemiological effect in humans. Confirming or denying the reality of this phenomenon should be given a high research priority.

The second aspect warranting further attention is the possibility that the limited action of these transporters could account for an unappreciated aspect of global contamination. Clearly, the primary factors leading to accumulation of chemicals such as DDT, PCBs, and PFAs are persistence and permeability. However, if no mechanism for the removal of these chemicals exists (i.e., the bouncers of the cells do not recognize these chemicals), they will remain there as legacies of exposure. If this conclusion is correct, assessing recognition of high-volume chemicals by efflux transporters would provide an additional criterion for the identification of potential global pollutants and thus prevent global contamination.

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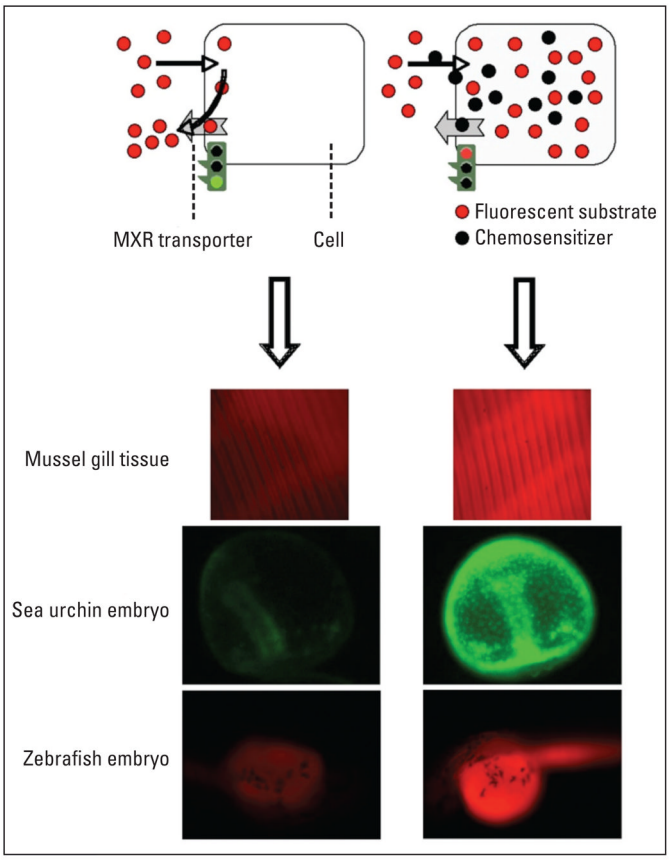

FIGURE 1. Inhibiting transport activity

Efflux transporters prevent fluorescent dyes from entering the cytoplasm and provide a simple means of assaying activity. The sequence on the left shows effective exclusion of either rhodamine B (mussel and zebrafish) or calcein-AM (sea urchin) from cells and tissues of three different aquatic organisms. The sequence on the right depicts the consequences of inhibiting transport activity in the same material. As shown, only a small amount of the dye enters unless the transporter is inhibited.

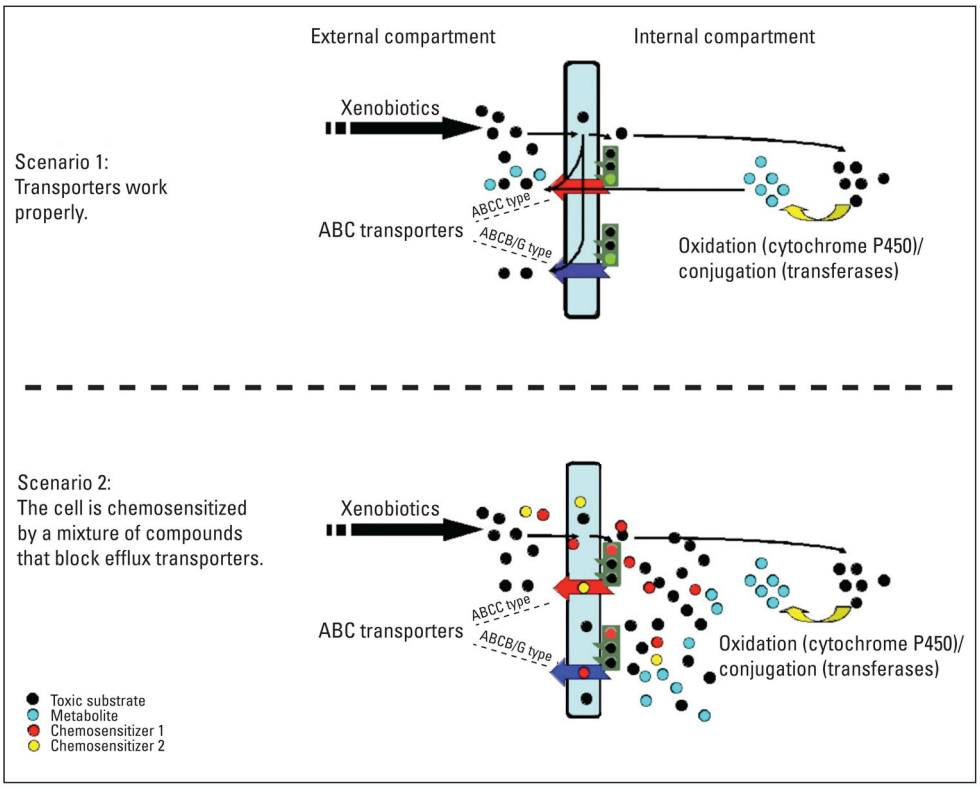

FIGURE 2. Scenarios of transporter function in a cell

Scenario 1. All transporters work properly. Few toxicants enter the cell, and those that do are modified by oxidation and conjugation (phases I and II) and then extruded. Scenario 2. The cell is exposed to a mixture of chemicals, including some that act as chemosensitizers. Their inhibition of the transporter activity allows previously excluded chemicals to now enter the cytoplasm.