REVIEWS

Multiple molecular mechanisms for multidrug resistance transporters

Christopher F. Higgins[1]†

The acquisition of multidrug resistance is a serious impediment to improved healthcare. Multidrug resistance is most frequently due to active transporters that pump a broad spectrum of chemically distinct, cytotoxic molecules out of cells, including antibiotics, antimalarials, herbicides and cancer chemotherapeutics in humans. The paradigm multidrug transporter, mammalian P-glycoprotein, was identified 30 years ago. Nonetheless, success in overcoming or circumventing multidrug resistance in a clinical setting has been modest. Recent structural and biochemical data for several multidrug transporters now provide mechanistic insights into how they work. Organisms have evolved several elegant solutions to ridding the cell of such cytotoxic compounds. Answers are emerging to questions such as how multispecificity for different drugs is achieved, why multidrug resistance arises so readily, and what chance there is of devising a clinical solution.

Since the discovery of effective antibiotics in the 1940s, a false sense of security has pervaded the public consciousness: the assumption that new drugs will increasingly conquer disease. This has proved over-optimistic, nowhere more so than the re-emergence of antibiotic-resistant infections such as tuberculosis. The problem is not restricted to antimicrobials—around 40% of human tumours develop resistance to chemotherapeutic drugs. With hindsight, it is not surprising that drug resistance is selected and spreads rapidly through cell populations. What is, perhaps, surprising is the phenomenon of multidrug resistance—the simultaneous acquisition of resistance to many chemically unrelated compounds to which the cell has never been exposed. Multidrug resistance is, in large part, the story of membrane transporters.

Cellular resistance to a single class of cytotoxic drugs can arise in many ways, including alteration of the target protein, decreased membrane permeability and drug metabolism. In contrast, the principal mechanism of multidrug resistance is the active transport of drugs out of the cell. Typically, each active transport protein is highly specific for its substrate, be it an amino acid, sugar or polypeptide. Unusually, however, multidrug transporters have broad specificity for a wide range of chemically unrelated molecules. Multidrug transporters, whether from *Escherichia coli* or an elephant, have similar (but not identical) multispecificity for many relatively lipophilic, planar molecules of molecular weight less than around 800 Da that are often, but not exclusively, weakly cationic (Fig. 1). These characteristics mirror those of many biologically active drugs and it is, therefore, not surprising that multidrug transporters in humans influence drug delivery and pharmacokinetics[1].

Active membrane transporters, whatever their substrate, fall into a relatively small number of protein superfamilies. Transporters within each superfamily are related with regards to amino acid sequence, structure and evolutionary origin. Intriguingly, multidrug transporters occur within several of these superfamilies[2] and must, therefore, have evolved several times, independently, in the context of very different protein backbones. These transporters present many intellectual and experimental challenges. How do they pump lipophilic drugs vectorially across lipid membranes? How can the very different architectures of different families of transporter each be adapted to multidrug transport? How is multispecificity achieved? What is the normal physiological function of these transporters?

Structures have now been obtained for multidrug transporters from four distinct transporter superfamilies (Fig. 2): (1) the ABC family (ATP-binding cassette: Sav1866 from *Staphylococcus* and mammalian P-glycoprotein); (2) the MFS family (major facilitator superfamily: EmrD from *E. coli*); (3) the RND family (resistance-nodulation-division: AcrB from *E. coli*); (4) the SMR family (small multidrug resistance: EmrE from *E. coli*). Together with structures of soluble, drug-binding transcription factors these data now provide satisfying, if incomplete, insights into the mechanisms and biology of multidrug resistance.

ABC transporters: Sav1866 and P-glycoprotein

ATP-binding cassette (ABC) transporters are present in all cells of all organisms and use the energy of ATP binding/hydrolysis to transport

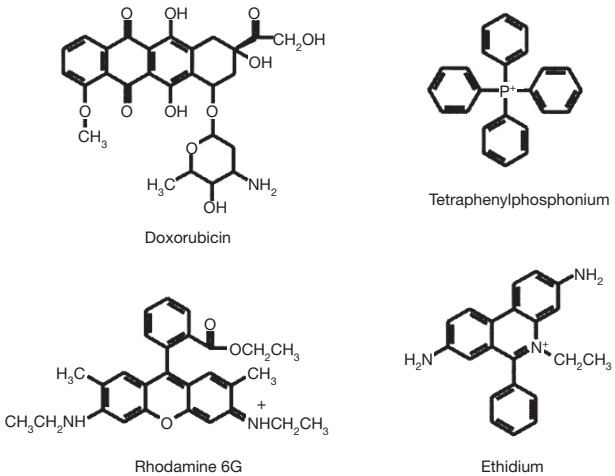

Figure 1 | Substrates of multidrug transporters. Multidrug transporters have subtly different multispecificities. However, their substrates share a number of features in common: planar, heterocyclic, lipophilic compounds of molecular mass less than 800 Da and, often, weakly cationic.

[1]MRC Clinical Sciences Centre, Imperial College, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, UK.
†Present Address: Vice-Chancellor's Office, Durham University, The University Offices, Old Elvet, Durham DH1 3HP, UK.

substrates across cell membranes[3]. Typically, they are specific for a given ligand that can be an inorganic ion, amino acid, sugar, polypeptide, or any one of a number of other classes of molecule. However, a few ABC transporters have evolved a broad specificity for hydrophobic molecules. Mammalian P-glycoprotein (ABCB1) is, arguably, the best characterized of all ABC transporters and, when overexpressed, confers resistance of cancer cells to a variety of chemotherapeutic drugs (for example, doxorubicin, Taxol, etoposide)[4,5]. Multidrug ABC transporters have also been implicated in antibiotic resistance, drug resistance in fungi and parasitic protozoa, and herbicide resistance in plants[6]. Intriguingly, the bacterial transporter LmrA, when expressed in mammalian cells, confers multidrug resistance indistinguishable from that of mammalian P-glycoprotein[7]. The minimal functional unit of all ABC transporters consists of four domains[8] (Fig. 2). Two cytoplasmic, nucleotide-binding domains (NBDs) bind and hydrolyse ATP and share a common protein fold distinct from that of other ATP-binding proteins. Two transmembrane domains (TMDs) each consist of multiple (generally six) membrane-spanning α-helices and form the pathway through which substrates cross the membrane. These four domains can be fused into multidomain polypeptides in a variety of ways. Bacterial multidrug transporters (for example, Sav1866) are most commonly homodimers of molecules comprising one NBD and one TMD, whereas mammalian P-glycoprotein has all four domains fused into a single polypeptide.

P-glycoprotein was the first multidrug transporter for which structural data were obtained, albeit at low-to-medium resolution[9–12]. These remain the only structural data for any mammalian multidrug transporter. Recently, a high-resolution structure of a homologous bacterial multidrug ABC transporter, Sav1866 from *Staphylococcus aureus*, was determined[13] (Fig. 3). The Sav1866 structure is consistent with the lower resolution structures[9–12] and biochemical cross-linking data[14,15] for P-glycoprotein. The two TMDs form a chamber in the membrane which, at least in the equivalent to the ATP-bound state (see below), is open extracellularly. This chamber is lined by hydrophobic and aromatic amino acids contributed by several transmembrane α-helices. The two NBDs form a head-to-tail 'sandwich' dimer in the intact protein, aligned such that each NBD contacts both TMDs. Two ATP-binding pockets are formed at the NBD dimer interface with amino acids from each monomer contributing to each ATP-binding pocket[16].

Structures for two substrate-specific ABC transporters from bacteria have also been determined: the vitamin B_{12} transporter BtuCD[17] and a metal-chelate transporter, HI1470-1 (ref. 18). Three putative structures for MsbA, a lipid A transporter, have been retracted[19]. As expected for homologous proteins, the structures of BtuCD and HI1470-1 are closely related to each other, and each transporter has a total of 20 transmembrane α-helices. However, although their overall architecture is similar to that of Sav1866/P-glycoprotein, there are significant differences in detail. The NBD dimer is similar in the BtuCD/HI1470-1 and Sav1866/P-glycoprotein pairs but the TMDs have unrelated folds. Furthermore, in BtuCD/HI1470-1 each NBD contacts only one of the two TMDs, whereas in Sav1866/P-glycoprotein each NBD contacts both TMDs. Biochemical cross-linking for P-glycoprotein confirms that each NBD interacts with two TMDs in this sub-group of proteins[20]. Thus, it seems that the two families of ABC transporters—the ABCB sub-family (Sav1866 and P-glycoprotein) and the BtuCD/HI1470-1 sub-family—have similar NBD dimers coupled to structurally and evolutionarily distinct pairs of TMDs. Consistent with this, ABC dimers are also known to couple ATP binding/hydrolysis to other very different classes of protein including DNA repair enzymes[21].

These structural data, together with extensive biochemical and genetic characterization, have led to the ATP-switch model for transport[21]. The driving force for drug transport is a switch between two principal conformations of the NBD dimer: ATP binding induces rigid body rotation of domains within each NBD with respect to each other and formation of a closed dimer with two molecules of ATP sandwiched at the dimer interface. ATP hydrolysis and inorganic phosphate (P_i)/ADP release return the dimer to its open configuration. The close proximity of the two NBDs in structures of intact ABC transporters suggests that the structural differences between the open and closed dimers are probably subtle rather than complete dimer dissociation. The kinetics of the switch can differ between transporters depending on the extent of cooperativity between the two nucleotide-binding pockets and signals from the transmembrane domains. ATP-binding by the NBDs and formation of the closed dimer induce substantial conformational changes in the TMDs[10,22] that mediate substrate translocation—a reduction in the drug-binding affinity[23–26] and reorientation of the binding site so that it is exposed to the extracellular face of the membrane[27] and drug can be released. ATP hydrolysis and P_i/ADP release restore the open dimer and the transporter to its starting configuration. The functional role of ATP binding and closed dimer formation is illustrated by studies of another ABC protein, CFTR, where, instead of mediating transport the ATP switch opens a chloride channel[28]. The nature of the conformational changes in the TMDs of ABC transporters is unknown. However, biochemical data, comparison of the ATP-bound and ATP-free forms of P-glycoprotein, and comparison of the BtuCD/HI1470-1 pair—which are thought to be in the ATP-bound (closed dimer) and ATP-free (open dimer) conformations, respectively—imply relatively small-scale tilting and rotation of several individual transmembrane α-helices with respect to each other to expose alternately the central chamber and drug-binding site(s) to the extracellular and cytoplasmic faces of the membrane[21].

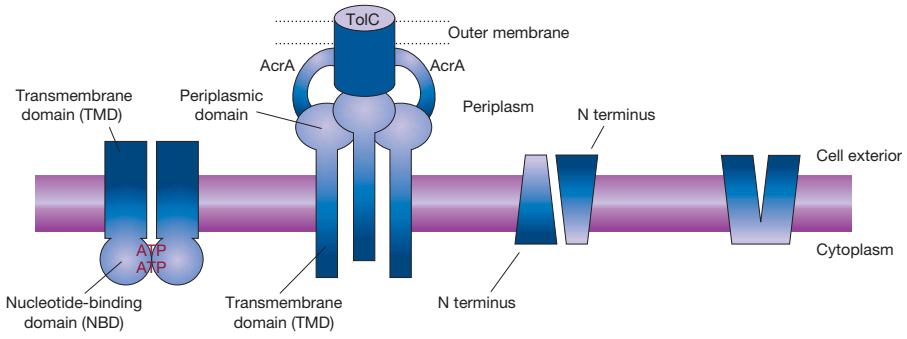

Figure 2 | Schematic diagram of domain organization of multidrug transporters. Examples of each of the four major families of transporters that include multidrug transporters and for which structural data are available.

As no structures of ABC transporters with bound substrate have been obtained, the nature of the substrate-binding site(s) can only be inferred. Drugs bind to a high-affinity site(s) on the protein from the inner leaflet of the lipid bilayer[29,30]. Mutations that alter drug-binding specificity of P-glycoprotein implicate several α-helices that line the central chamber[21,31]. It is reasonable, therefore, to suppose that the drug-binding site(s) is located in this chamber. Competitive and non-competitive drug-binding interactions[23,32], the observation that one drug can stimulate transport of another[33,34], and the demonstration that two drug molecules can bind per protein molecule[27,35], implicate multiple drug-binding sites. However, these data are also compatible with a single, large, flexible pocket that can bind more than one drug molecule simultaneously. On the basis of data for other multidrug-binding proteins this now seems to be the most plausible hypothesis (see below).

RND transporters: AcrB

Resistance-nodulation-division (RND) proteins are found in both prokaryotic and eukaryotic cells and have diverse substrate specificities and physiological roles. However, there are relatively few RND transporters and they are secondary transporters, energized not by ATP binding/hydrolysis but by proton movement down the transmembrane electrochemical gradient. Few RND proteins have been well characterized. The family includes NPC-1, which is defective in Niemann–Pick disease and modulates subcellular lipid/cholesterol distribution[36], and Dispatched in *Drosophila* and mammals which is required for the export of the cholesterol-modified signalling peptide Hedgehog[37]. Neither NPC-1 nor Dispatched, however, have formally been demonstrated to transport their putative substrates. By far the best characterized RND protein is AcrB from *E. coli* that can increase resistance to a variety of antibiotics by several orders of magnitude[38]. Recent structures of AcrB provide insights into the mechanism by which it, and presumably other RND transporters, works[39–42].

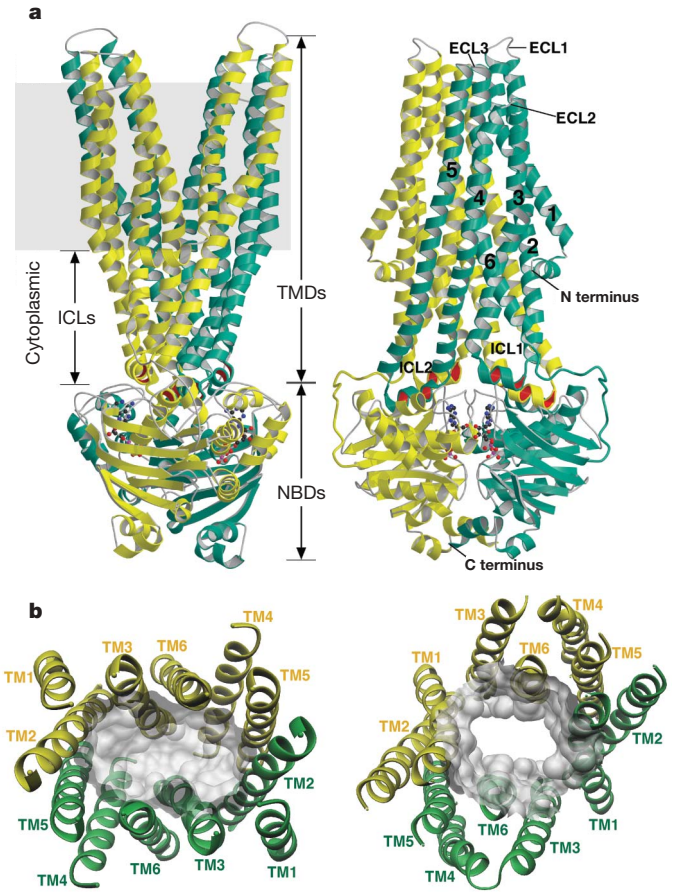

Figure 3 | Structure of ABC multidrug transporters. The backbone structure of Sav1866 is shown in ribbon representation. Sav1833 is a homodimer and the two monomers are coloured yellow and turquoise. **a**, View perpendicular to the cell membrane, in two orientations at right angles to each other. The TMDs span the lipid bilayer and consist of a total of 12 membrane-spanning α-helices. The NBDs are exposed at the cytoplasmic face of the membrane, linked to the TMDs by intracellular loops (ICLs). The six transmembrane α-helices of one subunit are numbered. The grey box indicates the probable position of the lipid membrane bilayer. **b**, View in the plane of the membrane showing the substrate translocation pathway, from the intracellular (left panel) and extracellular (right panel) faces of the membrane. The transmembrane (TM) helices are numbered and the cavity is shown as grey shading. Figure adapted from ref. 13.

Gram-negative bacteria including *E. coli* have two cell membranes—a cytoplasmic membrane and an outer membrane—separated by the periplasmic space. Many antibiotic targets are located in the periplasmic space (for example, the cell wall components targeted by β-lactam antibiotics). Thus, to confer resistance against a broad spectrum of antibiotics, active transporters must not only pump them out of the cytoplasm but also across the outer membrane. As the outer membrane is unable to maintain an electrochemical gradient or access ATP, energy input requires proteins located in the cytoplasmic membrane. AcrB is one such transporter (Fig. 2). AcrB consists of a transmembrane domain with 12 membrane-spanning α-helices and a large periplasmic domain. The functional transporter is a trimer with a total of 36 membrane-spanning α-helices. Two 'helper' proteins, AcrA and TolC, are required for AcrB to pump antibiotics out of the cell. AcrA is thought to have a role in membrane fusion but also has a more active, but poorly understood, role in the transport event itself[38,43]. TolC is a pore-like molecule comprising a 100 Å α-helical pore that spans the periplasm and a 40 Å β-barrel that spans the outer membrane[44]. AcrB translocates drugs into the TolC pore through which they cross both the periplasm and outer membrane. As TolC can couple to many different transporters, besides AcrB, it serves a generic role and has little or no function in determining the specificity or directionality of transport.

The structure of the AcrB trimer is shown in Fig. 4. The periplasmic domain adopts a subtly different conformation in each subunit of the trimer[40,42]. Each contains a potential substrate-recognition site and structures with bound drugs have been obtained[40]. However, only one site (the binding site; Fig. 4) is occupied by substrate at any given time. A second site (the extrusion site) is closed to the periplasm but open to the TolC docking domain, suggesting that it has just released substrate into the TolC pathway to exit the cell. In this site the drug pocket is smaller and the phenylalanine side chains are realigned so drug cannot enter or form stacking interactions—exactly as predicted for a low-affinity 'release' site. The third site (the access site) is closed to the TolC pathway but open to the periplasm, apparently ready to accept substrate. It does not take much imagination to envisage an 'alternating sites' model in which each of the three periplasmic domains adopts each of the three conformations in turn, passing substrate through the periplasmic domains to the TolC pathway and out of the cell. The movement of substrate through each periplasmic domain has been described as peristaltic[42]. Although analogous to the F_1F_0 ATPase, AcrB has no rotating subunit and the conformational changes must be induced directly by protons passing across the membrane down their electrochemical gradient. Three charged residues (Asp 407, Asp 408 and Lys 940) in the transmembrane domains that are conserved among all RND proteins and essential for function[39] probably mediate proton movement. Thus, like ABC transporters, energy transduction mediated by one domain (the transmembrane domain) is transduced by way of a conformational change to a second domain (the periplasmic domain) that mediates vectorial drug transport through changes in the affinity and orientation of a substrate-binding site. Without further data the molecular basis of energy transduction remains obscure.

As for other multidrug transporters, AcrB transports a plethora of hydrophobic compounds out of the cell. AcrB has been crystallized, separately, with two different bound substrates: minocycline and doxorubicin[40]. Despite their chemical differences both drugs bind in the same cavity in the periplasmic domain. This cavity is lined by hydrophobic and aromatic amino acids but also includes two polar residues, Gln 176 and Asn 274, that help to neutralize the charge of cationic drugs. The two drugs bind in different although overlapping places within this cavity and interact with different amino acid side chains. The finding that the substrate-binding site is in the periplasmic domain implies that AcrB 'picks up' drug from the outer (periplasmic) leaflet of the membrane to transport it out of the cell. This raises an apparent paradox: does AcrB also transport drugs from the cytoplasm? It is possible that another transporter facilitates drug 'flopping' from the inner to the outer leaflet from which AcrB pumps drug out of the cell. However, the three transmembrane domains of AcrB form a large (30 Å) hydrophobic cavity that appears to span much of the lipid bilayer and was originally suspected to form the drug translocation pathway. Indeed, an early structure showed three ligand molecules bound in this pathway[41]. Although subsequent data are inconsistent with these being sites from which transport occurs, it seems likely that AcrB also transports drugs directly from the cytoplasm, because a homologous protein, AcrD, has been shown to transport aminoglycosides from the cytoplasm[44].

SMR transporters: EmrE

Small multidrug resistance (SMR) proteins are a relatively small family of transporters, restricted to prokaryotic cells. They are also the smallest multidrug transporters, with only four transmembrane α-helices and no significant extramembrane domain, although as they function as dimers the minimal functional unit is a bundle of eight α-helices (Fig. 2). The paradigm SMR transporter, EmrE, is an electrogenic antiporter from *E. coli* that can confer resistance to a wide variety of hydrophobic cationic molecules, including antibiotics[45].

A structure obtained by cryo-electron microscopy to 7 Å resolution[46], together with genetic and biochemical data[47], shows that EmrE is a homodimer (Fig. 5). Two putative X-ray structures that were inconsistent with these data have recently been retracted[19]. Unusually, the two identical subunits appear to be oriented oppositely (antiparallel) in the membrane, although the folds of each monomer are subtly different. Although an antiparallel arrangement has been challenged by protein cross-linking studies[48], antiparallel homodimers and heterodimers are increasingly being recognized among membrane proteins and evolutionary and topological mapping now support an antiparallel arrangement for the subunits of EmrE[49].

EmrE is, in essence, a simple bundle of eight transmembrane α-helices that forms a pathway across the membrane. This pathway is lined primarily by hydrophobic and aromatic amino acids from several of the α-helices and is potentially accessible from both sides of the membrane (Fig. 5). The structure with bound TPP^+ (tetraphenylphosphonium) shows a relatively large, open pocket within the core of the hydrophobic transmembrane pathway and close to the middle of the membrane bilayer[46]. The involvement of multiple α-helices in forming the binding site/translocation pathway is consistent with extensive mutagenesis and other studies[47]. Glu 14, known to be essential for binding cationic drugs, is appropriately located. This residue has also been implicated in proton movement and, although the mechanism of proton coupling remains unknown, the involvement of Glu 14 in both substrate- and proton-binding suggests that the mechanisms of energy coupling in this family may differ from RND and MFS transporters[50]. The precise nature of the drug-binding site and the proton-induced conformational changes that presumably expose the site alternately to opposite faces of the membrane to achieve transport, remain unknown.

MFS transporters: EmrD

Major facilitator superfamily (MFS) transporters and ABC transporters comprise the two largest and most functionally diverse of the transporter superfamilies. However, MFS transporters are distinct from ABC transporters in both their primary sequence and structure (Fig. 2) and in the mechanism of energy coupling. As secondary transporters they are, like RND and SMR transporters, energized by the electrochemical proton gradient. Only in 2003 were the first X-ray structures for MFS transporters determined, for LacY[51] and GlpT[52] from *E. coli*. Subsequently, the structure of a single multidrug transporter from this superfamily, EmrD from *E. coli*, was determined to 3.5 Å resolution[53]. EmrD extrudes a range of cytotoxic molecules from the cell, although it is otherwise not well characterized. Fortunately, however, EmrD is homologous to two other MFS multidrug transporters that have been characterized biochemically in some detail: LmrP from *Lactococcus lactis* and MdfA from *E. coli*.

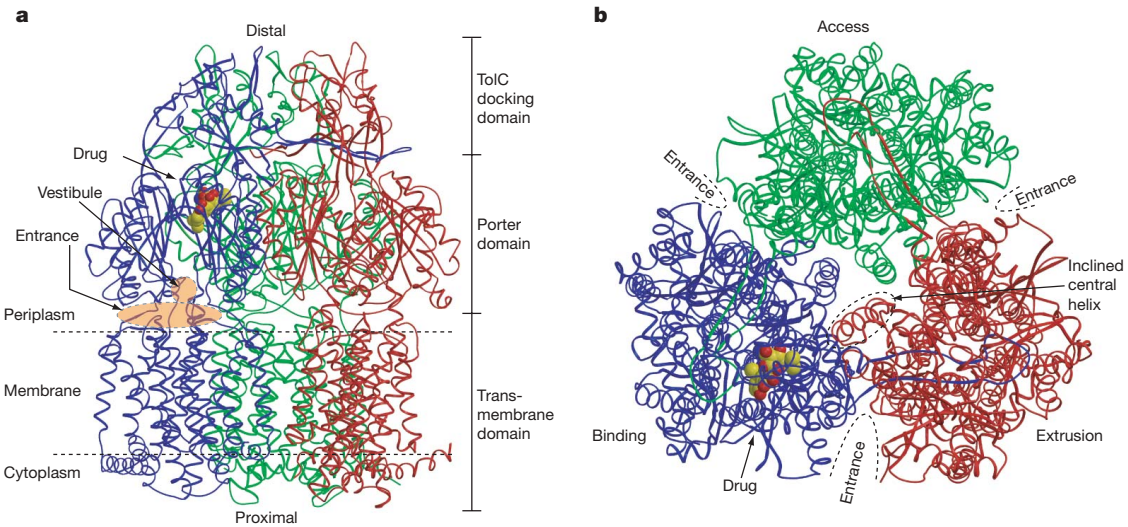

Figure 4 | Structure of RND multidrug transporters. The structure of AcrB in complex with minocycline is shown. AcrB is a trimer and the three monomers are coloured blue, red and green. The bound substrate minocycline is shown in the periplasmic domain as coloured balls and is present in one of the monomers only. **a**, View perpendicular to the membrane plane. The approximate position of the membrane bilayer is indicated. **b**, View parallel to the membrane plane, from the periplasmic side. The three periplasmic domains differ subtly in structure and the 'binding', 'access' and 'extrusion' subunits are indicated. Figure adapted from ref. 40.

EmrD is a compact protein with twelve membrane-spanning α-helices organized as two bundles of six that form a hydrophobic cavity within the plane of the bilayer (Fig. 5). The external α-helices (helices 3, 6, 9, 12) adopt a similar configuration to their equivalents in LacY and GlpT. The more internal α-helices deviate in their arrangement and form a larger internal cavity, presumably because of the different substrate specificities—LacY and GlpT are very substrate-specific whereas EmrD has broad multispecificity. This suggests that this internal cavity forms part of the drug transport pathway. No structure with bound drug has yet been obtained and there is no direct evidence that drugs bind in, or are transported through, this cavity. Nevertheless, mutational data for MdfA[54] and LmrP[55] imply that residues lining this central cavity are indeed involved in substrate recognition and translocation. These are, primarily, hydrophobic and aromatic amino acids but also include some polar residues. Notably, mutagenesis data suggest that many residues contribute to substrate binding, with different residues being more or less significant for different substrates. A good example is E26, which is important for the transport of cationic drugs but much less so for neutral ones[54]. Different drugs show complex competitive and non-competitive interactions with LmrP, leading to the suggestion that there may be multiple binding sites[56]. Indeed, MdfA appears to be able to bind chloramphenicol and TPP^+ simultaneously[57]. However, as for P-glycoprotein (see above), these data are also compatible with the simpler interpretation that there is a single large and flexible drug-binding site (see below).

Apart from its structure and the known requirement for the electrochemical gradient, little is known about the mechanisms of energy coupling or drug transport by EmrD. However, the related LacY protein is, arguably, the most intensively studied of all transporters[58] and a clear kinetic model has been established. For each molecule of lactose transported, a proton is transferred across the membrane via conserved and essential acidic residues. The proton and substrate (lactose) pathways seem to be distinct. Proton movement induces a conformational change that exposes the lactose-binding site to the external face of the membrane, reducing the affinity for lactose binding and facilitating its consequent release. The proton is then released and the transporter returns to its basal state with a high-affinity lactose-binding site exposed to the cytoplasm. It is likely that the principles established for LacY apply to the less-extensively studied drug transporters. However, there may be some adaptations to enable drugs of different charge to be accommodated. For example, in LmrP the two acidic residues (D142 and E327) involved in proton translocation are not essential but are individually replaceable, influencing the proton/substrate stoichiometry[55]. The relative importance of $\Delta\psi$ and ΔpH also seems to depend on the charge of the drug. As no structures of an MFS transporter at different stages of the transport cycle have yet been determined, the conformational changes that occur during transport can only be speculative.

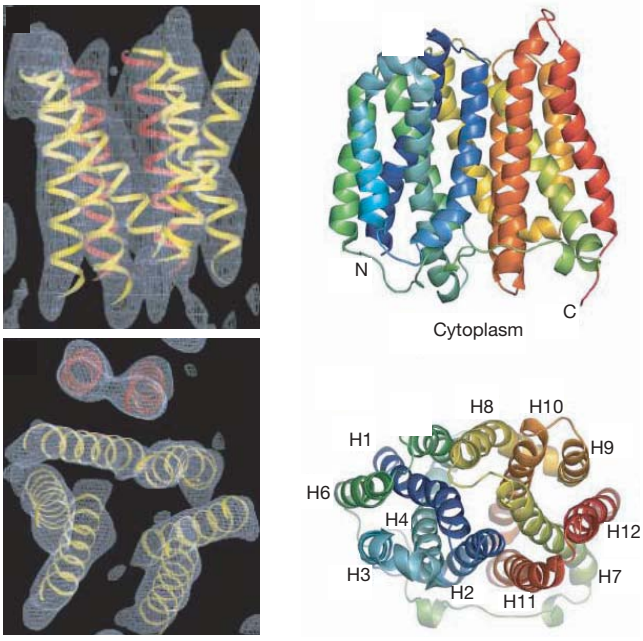

Figure 5 | Structures of SMR and MFS multidrug transporters. Left panels: structure of EmrE from *E. coli* determined by cryo-electron microscopy, viewed perpendicular to the membrane plane (top) and parallel to the membrane plane (bottom). The two antiparallel subunits each have four membrane-spanning α-helices. Taken, with permission, from ref. 46. Right panels: structure of EmrD from *E. coli*, viewed perpendicular (top) and parallel (bottom) to the plane of the membrane from the cytoplasmic side. The 12 membrane-spanning α-helices are numbered. Taken, with permission, from ref. 53.

Drug-binding transcription factors: QacR and BmrR

Structures of multidrug transporters with bound drug are limited. Nevertheless, important insights into the nature of multispecific drug-binding sites have been gained from studies of multidrug-binding transcription factors. In bacteria, expression of several multidrug transporter genes is induced by their cognate drug substrates. Soluble transcription factors bind drug and mediate this response, and the multispecificity of drug binding by these transcription factors is, unsurprisingly, similar to that of their partner transporters. These transcription factors have been much more amenable to structural study than the membrane-bound transporters. Elegant studies of two of them have been particularly informative: BmrR from *Bacillus subtilis*[59,60] and QacR from *Staphylococcus aureus*[61,62]. Structures with and without bound drug show that, although the folds of their multidrug-binding domains differ, the two proteins bind drugs in a similar manner.

BmrR and QacR each have a relatively large drug-binding pocket that can accommodate the entire spectrum of drug ligands—there is no need for multiple pockets to explain multispecificity[63]. The architecture of the drug-binding pockets allows different ligands to adopt different orientations within the pocket and interact with different sets of amino acids. For example, in QacR two chemically diverse ligands (rhodamine and ethidium) occupy distinct, almost non-overlapping sites within the binding pocket and interact with different amino acid side chains. A structure has also been obtained in which two different drugs (ethidium and proflavin) are bound simultaneously[62] (Fig. 6). Regions of the pocket not occupied by ligand are occupied by water molecules, as initially described for the polyspecific oligopeptide-binding protein of *Salmonella typhimurium*[64]. The pocket wall is flexible and can change conformation upon ligand binding, increasing promiscuity. Nevertheless, flexibility is limited, not unexpectedly for a folded protein, explaining why addition of a specific side chain to some drugs can reduce binding affinity.

The drug-binding pockets shield bound drug from the aqueous phase and drug binding is stabilized by van der Waals interactions with the surrounding hydrophobic and aromatic amino acid side chains. Sequestration of drug in a hydrophobic pocket provides sufficient energy to stabilize binding, as it negates the disruption of hydrogen bonds between water molecules otherwise caused by drug molecules in aqueous solution. Binding affinity for cationic drugs is augmented by electrostatic attraction between the positively charged ligand and negative charges on the protein. The hydrophobic environment of the drug-binding pocket makes electrostatic attraction an especially powerful stabilizing factor as water dipoles are avoided. Notably, this electrostatic interaction does not require perfect alignment of the positive and negative charges. For example, for BmrR the closer the positive charge of the bound drug to the single glutamate (Glu 134) in the binding pocket the higher the drug-binding affinity[59]. In QacR, the four glutamates exposed in the drug-binding pocket stabilize the binding of different drugs, depending on how the specific drug is aligned in the pocket[61]. In the unliganded proteins

the charged residues are shielded from the aqueous phase, for example in BmrR by a flexible α-helical arm that is displaced when drug binds[59].

Although not linked to transport, the structures of three other proteins that bind multiple hydrophobic ligands illustrate a similar mechanism of multispecific binding[63]. The drug-metabolizing enzyme P450 not only has a binding site with similar characteristics to those of BmrR and QacR, but a structure has been obtained with two identical drug molecules in the same pocket, explaining homotopic cooperativity[65]. The PXR transcriptional regulator[66] has a similar binding site, as does the mammalian odorant-binding protein, which, intriguingly, is constructed from β-sheets rather than α-helices[67].

Insights from comparison of multidrug transporters

It has proved unusually difficult to obtain structures of drug transporters, in part owing to the general problems of purifying and crystallizing membrane proteins. Multispecific transporters may be particularly problematical as they are required to be flexible in order to translocate relatively large molecules across the bilayer—flexibility can lead to anomalous crystal contacts when the protein is removed from the lipid environment. This is illustrated by two of the first multidrug transporter X-ray structures obtained (MsbA[68] and EmrE[69] from *E. coli*), which, even disregarding software difficulties[19], appear to be anomalously folded proteins. Thus, once a structure has been determined it should not necessarily be assumed to reflect the physiological fold. Structure determination in the absence of demonstrable biochemical activity should be strenuously avoided. More importantly, it is critical that multiple structures are obtained, compared and tested against independent biochemical and genetic data to give a level of reassurance about biological relevance before more detailed mechanistic interpretation.

As we have seen, multidrug transporters have evolved several times, apparently independently, on very different protein backbones. These transporters perform similar functions yet achieve this by very different means. Nevertheless, several common principles emerge.

First, multidrug transporters are conventional enzymes that can be exemplified by simple kinetic schemes, as first proposed by Mitchell nearly 50 years ago[70]. Although this now seems self-evident, the unusual properties of multidrug transporters have led to alternative speculations as to how drugs cross the membrane, including formation of discontinuities in the lipid bilayer and 'slippery' protein–lipid interfaces. We do not yet have a complete set of structures for any transporter at different stages in the transport cycle, but the current body of structural and biochemical data, *in toto*, shows that substrates are bound by a defined high-affinity site exposed to one face of the membrane. Conformational changes induced by ATP binding/hydrolysis or proton movement down the electrochemical gradient convert this site to a low-affinity 'release' site exposed to the alternative face of the membrane. These conformational changes can be within a protein domain (as for MFS and SMR transporters) or transmitted between domains (as for ABC and RND transporters).

Second, substrates cross the bilayer through a pathway formed within the core of the transporter, largely shielded from the surrounding lipid phase. For some multidrug resistance transporters the evidence comes directly from structures with bound drug, although for others it is based on indirect mutagenesis studies. This finding is, perhaps, not surprising, as most multidrug resistance transporters are closely related to transporters that transport hydrophilic substrates that must clearly be shielded from the lipid phase. The pathway is not strictly a channel, as it is not open to both faces of the membrane simultaneously but instead alternately during the transport cycle. In all transporters the pathway is constructed from

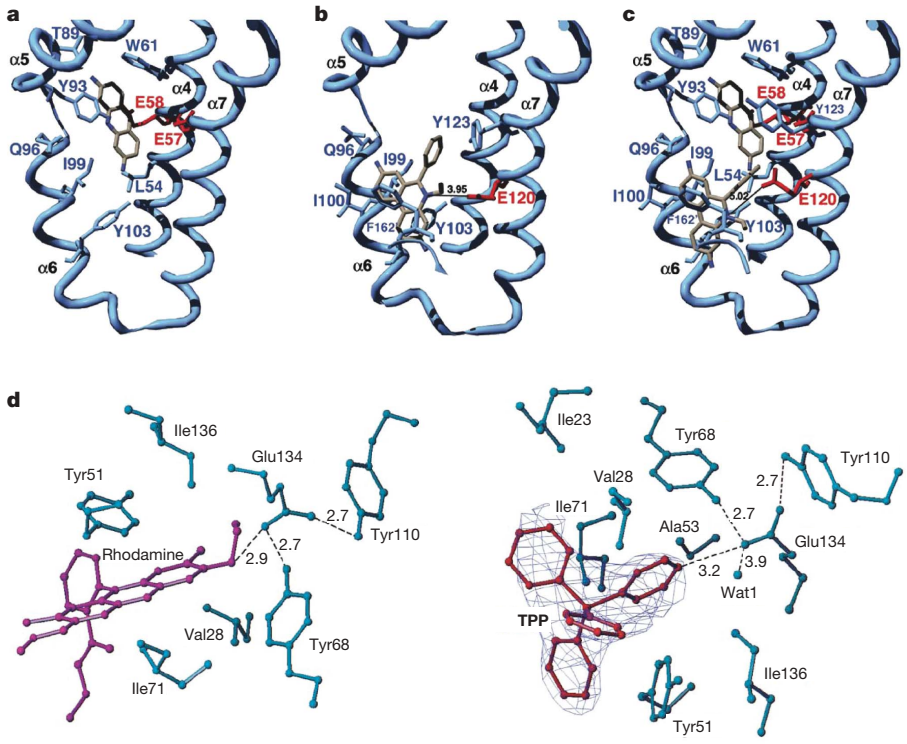

Figure 6 | Drug binding by soluble bacterial transcription factors. **a**–**c**, Binding of three different drugs by QacR. **a**, QacR with bound proflavin; **b**, QacR with bound ethidium; **c**, QacR with both proflavin and ethidium bound simultaneously in a tertiary complex. Only the key drug-binding residues are shown. Acidic residues involved in neutralization of cationic drugs are shown in red. Taken, with permission, from ref. 62. **d**, Drug binding by BmrR. The key residues involved in drug binding are shown in ball-and-stick configuration. Left: structure-based model with bound rhodamine (purple balls and sticks). The key neutralizing acidic residue, Glu 134, is shown. Right: structure with bound TPP^+ (in red) together with a bound water molecule (Wat 1). Values shown are in angstrom. Dashed lines indicate H bonds. Taken, with permission, from ref. 59.

multiple α-helices. However, because the TolC pore through which drugs cross the bacterial outer membrane is formed by β-sheets—as is the drug-binding site of the olfactory receptor—the involvement of α-helices may say more about the constraints of building a membrane transporter than anything profound about the specific requirements for a drug transport pathway per se.

Third, multidrug transporters extract their substrates from the inner leaflet of the bilayer, analogous to phospholipid flippases/floppases, which generate lipid asymmetry in the membrane[29]. AcrB is the exception that proves this rule, accessing drugs from the outer leaflet of the bilayer in order to remove them from the periplasm. Given this, it is perhaps not unexpected that multidrug transporters are related to lipid flippases/floppases. For example, the most closely related transporter to P-glycoprotein is the phosphatidylcholine floppase ABCB4 (ref. 71). This does not mean that multidrug transporters are themselves lipid flippases/floppases—just that they are mechanistically similar. The acquisition of substrate from a specific leaflet of the membrane has a number of potential advantages. (1) Hydrophobic substrates partition into the bilayer and so are at increased concentration compared with the aqueous phase; (2) drugs in the membrane diffuse in two- rather than three-dimensions, facilitating interactions with the transporter; (3) extraction of substrate from the membrane ensures that broad-specificity transporters do not expel normal cellular constituents that remain in the cytoplasm; and (4) capturing drugs from the inner leaflet, before they enter the cytoplasm, is the most effective means of ensuring that they do not interact with their cytoplasmic target[72], although for drugs for which flip-flopping between leaflets is rate-limiting, extraction from the periplasmic leaflet is most efficient.

The fourth common principle is that structures of soluble drug-binding proteins demonstrate that multispecificity is achieved by a single, large, flexible hydrophobic pocket in which drug is essentially shielded from both lipid and aqueous phase. These pockets can, in some cases, accommodate two identical or different drug molecules at once. The pockets are lined primarily by hydrophobic/aromatic amino acids that bind drugs via van der Waals and stacking interactions. Polar residues can negate nearby positive charges of weakly cationic drugs. Critically, these multispecific drug-binding sites are very different from the usual 'lock-and-key' mechanisms of enzymes with hydrophilic substrates that rely on a perfect spatial alignment between ligand and side chains in the binding site to overcome the energetic disadvantage of disrupting hydrogen bonds between ligand and water. In contrast, simply the removal of hydrophobic drugs from the aqueous environment is energetically favourable and the multiple, weak interactions between a hydrophobic ligand and hydrophobic amino acids are sufficient to generate high affinity.

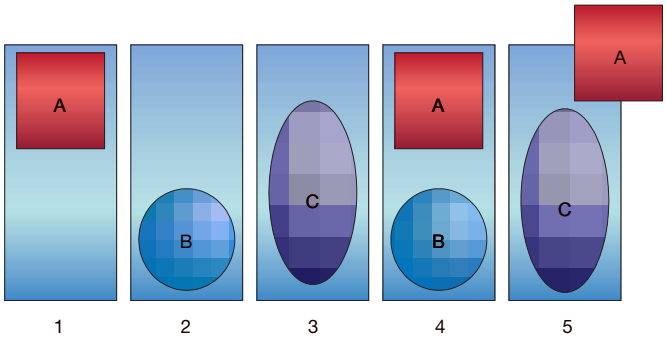

Figure 7 | One or multiple drug-binding sites? A single, large, drug-binding site can accommodate drug A (panel 1), drug B (panel 2) or drug C (panel 3). It can also bind drugs A and B simultaneously (panel 4), equivalent to two pharmacologically distinct sites, but is unable to bind drugs A and C simultaneously (panel 5), equivalent to a pharmacologically single site. MRP1 can bind drug and glutathione separately or as a conjugate[76,77], showing that the question of whether there are one or two sites is, to some extent, semantic.

The limited available structures of multidrug transporters with bound substrate (AcrB and EmrE) reveal drug-binding pockets that conform to this model. All other multidrug transporters have a similar hydrophobic pocket that appears to be the drug-binding site based on mutagenesis data. Although pharmacological and kinetic data showing both competitive and non-competitive drug interactions are consistent with multiple, interacting drug-binding sites, a single, large, flexible pocket can equally explain these data and now seems the most plausible model for all multidrug transporters (Fig. 7).

The biology of multidrug transporters

The ability of a cell to protect itself against environmental toxins is an essential biological function. Many organisms produce toxins to repel ecological competitors, and plants, which cannot run away from predators, rely on toxic secondary metabolites to make themselves unappetizing. Many antibiotics are derivatives of natural bacterial or fungal products, and many anticancer dugs (for example, Taxol, vinca alkaloids) are natural plant products. These molecules intercalate into lipid bilayers and are often deleterious to membrane function, altering fluidity, curvature or the activity of membrane proteins. To survive these natural chemical onslaughts, most organisms have evolved multidrug transporters to prevent cytotoxic molecules entering cells and to clear membranes of unwanted agents. This is self-evidently essential for *Streptomyces* strains that must protect antibiotic-producing cells from the very molecules they themselves synthesize. In mammals, the normal cellular function of P-glycoprotein is also protective, illustrated by the *mdr* (also known as *Abcb1b*) (P-glycoprotein) knockout mouse, which has no overt phenotype except altered drug pharmacokinetics and sensitivity to neurotoxins that are otherwise prevented from crossing the blood–brain barrier[2].

On the other hand, a plethora of proteins have been designated as multidrug transporters on the basis of sequence homologies, often with little or no biological evidence. In many cases this is likely to be a misnomer. Indeed, several proteins that transport and/or confer resistance to drugs when overexpressed have rather specific physiological roles when expressed at physiological levels. For example, MRP2 (ABCC2) can confer drug resistance when overexpressed yet is actually a leukotriene C_4 transporter[73]. The Blt 'multidrug transporter' of *Bacillus subtilis* confers drug resistance when overexpressed, yet its expression is normally co-induced with enzymes involved in spermidine/spermine metabolism and it is actually a spermidine transporter[74]. Overexpression of MdfA confers resistance of *E. coli* to many antibiotics, yet deletion of the *mdfA* gene barely alters cellular resistance. Instead, the real physiological role of MdfA turns out to be as a $Na^+(K^+)/H^+$ antiporter, which enables cells to maintain a constant intracellular pH under alkaline conditions[75].

It is now clear why drug resistance in nature most frequently involves multispecific membrane transporters. Resistance to a single drug can be achieved by mutation of its target. This is difficult to achieve without adversely altering the function of the target itself, and could not confer resistance to drugs with different targets. Similarly, enzymes designed to degrade a specific drug cannot metabolize unrelated molecules—57 cytochrome P450 genes are necessary for mammals to metabolize multiple cytotoxic drugs. Given the multiplicity of natural cytotoxic entities with different targets, the simplest means to achieve resistance is to take advantage of a common property of these molecules: the need to cross the cell membrane. Most natural toxins, like synthetic drugs, are small, planar, lipophilic molecules precisely because they have to cross the lipid bilayer to exert their toxic effects (with the rare exception of those that gain entry to the cell by 'piggy-backing' on a specific transporter or have an extracellular target). It turns out, as we have seen, that the evolution of a broad-specificity transporter for chemical entities with the characteristics required to cross the cell membrane is rather straightforward. This is the strategy adopted across the natural kingdom, and it is these natural resistance mechanisms that are frequently brought

into play when we try to interfere with nature by developing and using cytotoxic drugs.

Clinical implications

Over the past 20 years two general approaches have been adopted, relatively unsuccessfully, to overcoming drug resistance in the clinic. As discussed above, it is no coincidence that most clinically useful drugs are substrates for multidrug transporters. Given what we now know, what are the prospects of circumventing multidrug resistance in the clinic? Depending on perspective, views may either be 'glass-half-full' or 'glass-half-empty'.

First, there have been considerable efforts to modify drugs and antibiotics chemically so that they are no longer substrates for multidrug transporters. In the absence of structural data on the nature of multidrug-binding sites this has been undertaken 'blind' and has proved unsatisfying. Unlike conventional enzyme-substrate-binding sites, small changes in a drug rarely result in a substantial reduction in its affinity for transport. We now understand that this is because drug-binding sites are large and flexible and because the precise alignment of the ligand with respect to amino acids on the binding pocket is not required for high-affinity binding. Any modification to a drug that substantially reduces its affinity for a transporter also tends to reduce its 'druggability'—its ability to cross the cell membrane and bind to its target. It has, perhaps naively, been assumed that understanding the structure of multidrug-binding sites on transporters would enable drugs to be modified, rationally, to circumvent resistance. Instead, the current picture suggests that the very nature of drug-binding sites on transporters makes this a difficult proposition—certainly far more difficult than modifying hydrophilic ligands of cytosolic enzymes.

The second approach to overcoming multidrug resistance—the development of specific inhibitors of transporters—has also proved unsatisfactory[5]. There is frequently more than one multidrug transporter that can confer resistance to a specific drug or antibiotic, and so more than one inhibitor may be required. By trial and error, rather than rational design, several high-affinity and relatively specific inhibitors of human P-glycoprotein have been developed. These work well in the laboratory setting. However, they have proved difficult to assess in the clinic because of side-effects caused by inhibiting P-glycoprotein's normal physiological function in healthy tissues, altering the pharmacokinetics of the co-administered cytotoxic drug and enabling it to cross the blood–brain barrier.

Perhaps most worrying is the relative ease with which multidrug transporters can arise to confound our efforts at therapy. Most bacterial and mammalian cells have multidrug transporters to protect themselves against natural cytotoxic and membrane-disruptive compounds. It seems that selection for overexpression, or heterologous expression in different cell or tissue types, is relatively straightforward in response to a therapeutic or antimicrobial drug. Because of the nature of drug-binding sites, new multidrug transporters can evolve relatively easily from substrate-specific transporters by mutation. Indeed, some substrate-specific transporters can handle a variety of drugs without mutation, conferring multidrug resistance simply upon overexpression. Finally, cytotoxic drugs themselves upregulate multidrug transporters both in bacteria and in mammalian cells, often as part of a more general stress response.

Concluding remarks

Recent rapid advances in our understanding of multidrug transporters have not yet provided solutions to pressing clinical problems. These advances have, however, shown us why apparently straightforward approaches to overcoming multidrug resistance have been less effective than might otherwise have been expected. Increased understanding will inevitably enhance the chances of clever and effective solutions. In the meantime, profligate use of antibiotics has, unquestionably, led to the spread of antibiotic resistance. Similarly, chemotherapeutic drugs induce expression of drug resistance pathways. Should we perhaps stop assuming we can beat multidrug resistance and instead implement strategies to avoid it?

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Acknowledgements This review is dedicated to Alex Neyfakh (1959–2006) who contributed much to our understanding of the multispecificity of drug binding.

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