REVIEW ARTICLE

Structure and function of efflux pumps that confer resistance to drugs

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Resistance to therapeutic drugs encompasses a diverse range of biological systems, which all have a human impact. From the relative simplicity of bacterial cells, fungi and protozoa to the complexity of human cancer cells, resistance has become problematic. Stated in its simplest terms, drug resistance decreases the chance of providing successful treatment against a plethora of diseases. Worryingly, it is a problem that is increasing, and consequently there is a pressing need to develop new and effective classes of drugs. This has provided a powerful stimulus in promoting research on drug resistance and, ultimately, it is hoped that this research will provide novel approaches that will allow the deliberate circumvention of well understood resistance mechanisms. A major mechanism of resistance in both microbes and cancer cells is the membrane protein-catalysed extrusion of drugs from the cell. Resistant cells exploit proton-driven antiporters and/or ATP-driven ABC (ATP-binding cassette) trans-

porters to extrude cytotoxic drugs that usually enter the cell by passive diffusion. Although some of these drug efflux pumps transport specific substrates, many are transporters of multiple substrates. These multidrug pumps can often transport a variety of structurally unrelated hydrophobic compounds, ranging from dyes to lipids. If we are to nullify the effects of efflux-mediated drug resistance, we must first of all understand how these efflux pumps can accommodate a diverse range of compounds and, secondly, how conformational changes in these proteins are coupled to substrate translocation. These are key questions that must be addressed. In this review we report on the advances that have been made in understanding the structure and function of drug efflux pumps.

Key words: antibiotics, ATP-binding cassette transporter (ABC transporter), cancer, drugs, efflux, resistance.

INTRODUCTION

One of the most frequently employed strategies in biological systems that demonstrate resistance to cytotoxic drugs is the extrusion or efflux of these compounds from the cell via membrane proteins. These membrane proteins act like bilge pumps, and decrease the intracellular concentration of the drug to subtoxic levels (Table 1) [1–4]. There is currently a great deal of research being undertaken on understanding the precise mechanisms that are involved in the efflux of drugs out of the cell. In time, and with a full understanding of these mechanisms, a situation may prevail in which we can rationally design efflux pump inhibitors. This would enable a renaissance of drugs that had previously become ineffective due to their efflux out of the cell. The key to understanding how these pumps operate involves the determination of the structures of representative pumps and the elucidation of the conformational changes that accompany drug translocation. Until recently, most of our knowledge of the structure of drug efflux pumps had come from secondary structure predictions based upon the primary structure of the proteins. These procedures usually involve scanning the primary structure for strings of 20 or more hydrophobic amino acids that could form an α -helix of sufficient length to span the membrane. The models produced are often tested by biophysical, biochemical and genetic analyses. Biophysical analyses typically

include CD (circular dichroism) and FTIR (Fourier-transform IR) spectroscopy: these techniques can be used to assess the α -helical content of the protein. A number of common biochemical techniques used for analysis include proteolytic cleavage to assess membrane topology, monoclonal antibodies to identify exposed polypeptide motifs or, similarly, cysteine scanning mutagenesis, which introduces thiol-containing cysteine residues that can be labelled and tested for their exposure. Genetic approaches, such as the construction of β -galactosidase (*lacZ* α) or alkaline phosphatase (*phoA*) fusions within the membrane protein, can be used to test the position of intracellular or extracellular loops connecting the membrane-spanning helices.

Unfortunately, even though these procedures provide valuable information, they only provide an indication of the membrane topology of the transporters, rather than their structure. Structural determination of these proteins to atomic resolution is, however, difficult, for a number of reasons. Membrane proteins are often difficult to overexpress in the quantities and purity required for 3D (three-dimensional) crystallization trials. Even when protein of a sufficient quantity and quality is available, producing crystals is not straightforward due to the amphiphilic nature of these proteins. In spite of these difficulties, several transporters have been overproduced and purified in sufficiently high yields to enable successful 3D crystallization trials [5–9]. Additionally, a range of novel techniques for crystallizing membrane proteins

Abbreviations used: ABC, ATP-binding cassette; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; CFTR, cystic fibrosis transmembrane conductance regulator; 2D, two-dimensional; 3D, three-dimensional; EC-MsbA, *Escherichia coli* MsbA; EtBr, ethidium bromide; hPXR, human pregnane xenobiotic receptor; ICD, intracellular domain; IMP, inner-membrane protein; MATE, multidrug and toxic compound extrusion; MDR, multidrug resistance; MF, major facilitator; MFP, membrane fusion protein; MIANS, 1-(4'-maleimidyl)anilino)naphthalene-6-sulphonic acid; MRP, multidrug resistance protein; NBD, nucleotide-binding domain; NBS, nucleotide-binding site; OMP, outer-membrane protein; P-gp, P-glycoprotein; R6G, rhodamine 6G; RND, resistance/nodulation/cell division; SMR, small multidrug resistance; TM1, transmembrane domain 1; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene iodide; TMD, transmembrane domain; TPP⁺, tetraphenylphosphonium; VC-MsbA, *Vibrio cholerae* MsbA.

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Table 1 Drug pumps: their origins and substrates

Substrate and ligand abbreviations: AC, acriflavin; AZ, azithromycin; CC, colchicine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CH, chlorhexidine; CHH, 2-chlorophenylhydrazine hydrochloride; Cip, ciprofloxacin; CL, clarithromycin; CP, chloramphenicol; CPC, cetylpyridinium chloride; CV, Crystal Violet; DAR, daunorubicin; DM, daunomycin; DOC, deoxycholate; DXR, doxorubicin; Ery, erythromycin; FQ, fluoroquinolones; Fus, fusidic acid; FZ, fluconazole; GM, gentamycin; H33342, Hoechst 33342; IPTG, isopropyl β -D-thiogalactosidase; IS, indolizine sulphones (e.g. SR33557); KM, kanamycin; KZ, ketoconazole; LTC₄, cysteinyl leukotriene; MMC, mitomycin; MV methyl viologen; MZ, miconazole; NA, nalidixic acid; NBD-PC, 7-nitrobenz-2-oxa-1,3-diazolophosphatidylcholine; NBD-PE, 1-myristoyl-2-[6-(NBD)aminocaproyl]phosphatidylethanolamine; Nor, norfloxacin; Nov, novobiocin; NQO, 4-nitroquinoline N-oxide; OL, oleandomycin; OM, oligomycin; PF, proflavine; PhMA, phenylmercuric acetate; PI, pentamidine isothionate; PM, puromycin; PY, pyronine Y; QA, quaternary amine compounds (including benzalkonium chloride and cetyltrimethylammonium bromide); R123G, rhodamine 123G; RB, rhodamine B, RP, rifampicin; SO, safranin O; SM, streptomycin; SP, spiramycin; SU, sulphonamides; TC, tetracycline; TL, thiolactomycin; TX100, Triton X-100; VB, vinblastin; VC, vincristine; VP, verapamil. Organism abbreviations: *B. subtilis*, *Bacillus subtilis*; *B. thetaiotaomicron*, *Bacillus thetaiotaomicron*; *B. fragilis*, *Bacteriodes fragilis*; *C. albicans*, *Candida albicans*; *E. coli*, *Escherichia coli*; *H. influenzae*, *Haemophilus influenzae*; *H. sapiens*, *Homo sapiens*; *L. lactis*, *Lactococcus lactis*; *L. monocytogenes*, *Listeria monocytogenes*; *M. fortuitum*, *Mycobacterium fortuitum*; *M. smegmatis*, *Mycobacterium smegmatis*; *M. tuberculosis*, *Mycobacterium tuberculosis*; *N. gonorrhoeae*, *Neisseria gonorrhoeae*; *P. falciparum*, *Plasmodium falciparum*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *P. putida*, *Pseudomonas putida*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *S. typhimurium*, *Salmonella typhimurium*; *S. aureus*, *Staphylococcus aureus*; *S. maltophilia*, *Stenotrophomonas maltophilia*; *S. peucetius*, *Streptomyces peucetius*; *S. rochei*, *Streptomyces rochei*; *V. cholerae*, *Vibrio cholerae*; *V. parahaemolyticus*, *Vibrio parahaemolyticus*.

Pump	Organism	Substrates/modulatory ligands	MFP/OMP	Ref.
SMR				
Smr/QacC	<i>S. aureus</i>	EtBr, CV, QA, MV, TPP ⁺		[26]
EmrE	<i>E. coli</i>	EtBr, AC, MV, TPP ⁺		[34]
EbrAB	<i>B. subtilis</i>	EtBr, AC, PY, SO, TPP ⁺		[48]
YkkCD	<i>B. subtilis</i>	EtBr, PF, CV, PY, MV, CPC, CP, SM, TC, PM, TPP ⁺		[47]
Tbsmr	<i>M. tuberculosis</i>	AC, EtBr, MV		[234]
Pasmr	<i>P. aeruginosa</i>	AC, EtBr, MV		[36]
QacE	Gram-negative organisms	EtBr, QA, SU		[235]
QacE Δ 1	Gram-negative and -positive organisms	Cip, EtBr, GM, QA, SU, TC		[235]
MF				
TetA	<i>E. coli</i>	TC		[26]
EmrB	<i>E. coli</i>	CCCP, NA, TL, CHH	EmrA/TolC	[137]
EmrD	<i>E. coli</i>	CCCP		[24]
MdIA/Cmr/CmlA	<i>E. coli</i>	EtBr, PM, TC, Ery, neomycin, Nor, CP, IPTG, TPP ⁺ , R6G, DAR, DXR, QA, RP, TC, PM		[52]
FarB	<i>N. gonorrhoeae</i>	Fatty acids	FarA/MtrE	[236]
NorA	<i>B. fragilis</i>	Ery, Nor, PM		[237]
VceB	<i>V. cholerae</i>	CCCP, NA, Cip, Ery, PhMA, DOC	VceA/TolC	[138]
TetK	<i>S. aureus</i>	TC		[238]
NorA	<i>S. aureus</i>	EtBr, AC, QA, FQ, R6G, TPP ⁺ , PM, CP		[219]
QacA	<i>S. aureus</i>	EtBr, QA, CH, PI		[166]
Bmr	<i>B. subtilis</i>	EtBr, AC, QA, FQ, R6G, TPP ⁺ , PM, CP		[239]
LmrP	<i>L. lactis</i>	EtBr, DM, H33342, TPP ⁺ /VP, nicardipin, quinine, TX100		[107]
MdrL	<i>L. monocytogenes</i>	EtBr, Ery, cefotaxime		[240]
Tap	<i>M. tuberculosis</i>	TC		[234]
Mmr	<i>M. tuberculosis</i>	EtBr, AC, Ery, TPP ⁺ , SO, PY		[234]
Tap	<i>M. fortuitum</i>	TC, gentamicin, 2-N'- and 2-N'-ethylnetilmicin, SM		[241]
LfrA	<i>M. smegmatis</i>	EtBr, AC, QA, FQ		[242]
CaMDR1	<i>C. albicans</i>	FZ, NQO, benomyl, methotrexate		[243]
RND				
AcrB	<i>E. coli</i>	EtBr, AC, CV, SDS, TX100, bile salts, β -lactams, Nov, Ery, Fus, TC, CP, MMC, FQ, NA, organic solvents, NBD-PC	AcrA/TolC	[5]
AcrB	<i>H. influenzae</i>	EtBr, CV, Ery, RP, Nov	AcrA/	[244]
MexB	<i>P. aeruginosa</i>	AZ, Cip, CV, RP, TX100	MexA/OprM	[36]
SmeE	<i>S. maltophilia</i>	EtBr, Ery, TC		[245]
AcrB	<i>S. typhimurium</i>	AC, DOC, Cip, Ery, Fus, NA		[246]
ArpB	<i>P. putida</i>	Nor, Nov, RP, SDS, TC		[247]
MtrD	<i>N. gonorrhoeae</i>	Cip, Ery, Nov, TC, Ery, TX100, β -lactams, azithromycin, vertebrate peptide antimicrobials, spermicides	MtrC/E	[248]
MATE				
BexA	<i>B. thetaiotaomicron</i>	AC, EtBr, MV		[249]
NorM	<i>V. parahaemolyticus</i>	CP, EtBr, Nor		[250]
YdhE	<i>E. coli</i>	AC, Cip, KM, Nor, SM, TPP ⁺		[250]
VcmA	<i>V. cholerae</i>	Nor, Cip, DAR, DXR, SM, KM, EtBr, H33342		[177]
YdhE	<i>B. fragilis</i>	Nor, Cip, EtBr		[251]
NorM	<i>N. gonorrhoeae</i>	AC, EtBr, berberine, Cip, Nor		[50]
NorM	<i>N. meningitidis</i>	AC, EtBr, berberine, Cip, Nor		[50]
ABC				
P-gp	<i>H. sapiens</i>	DAR, DXR, R6G, R123G, H33342, NBD-PE, NBD-PC, PM, VC, VB, CC, taxol, dexamethasone, steroids, VP, nicardipine, IS, antimalarials (e.g. quinine and quinidine), CA		[115]
MRP	<i>H. sapiens</i>	Similar specificity to P-gp + LTC ₄ and other glutathione S-conjugates		[59]

Table 1 (contd.)

Pump	Organism	Substrates/modulatory ligands	MFP/OMP	Ref.
ABC				
Pdr5	<i>S. cerevisiae</i>	R6G, R123G, RB, FZ, DAR, DXR, OM, cycloheximide, CP, tamoxifen, trifluoroperazine, steroids, NBD-PE/VP, VC, taxol, VP, ionophoric peptides, quinidine, CC	MacA/TolC	[252]
Cdr1	<i>C. albicans</i>	FZ, KZ, MZ, steroids		[243]
Pfmdr1	<i>P. falciparum</i>	Mefloquine, artemisinin, chloroquine		[253]
LmrA	<i>L. lactis</i>	EtBr, R6G, R123G, DAR, DXR, VB, VC, DM, CC, TPP ⁺ , H33342, NBD-PE/VP, nicardipine, quinine, quinidine, CA, IS		[109]
DrrAB	<i>S. peucetius</i>	DXR, DAR		[62]
MacB	<i>E. coli</i>	AZ, CL, Ery, OL		[63]
Msr	<i>S. rochei</i>	DXR, Ery, OL, SP, TC		[254]
YvcC	<i>B. subtilis</i>	? (P-gp homologue)		[183]
PGPA	<i>Leishmania</i> spp.	Pentostam, metal–thiol conjugates		[255]
MsbA	<i>E. coli</i>	Major transporter of lipids from cytoplasm to outer membrane		[8]
Pst	<i>M. smegmatis</i>	FQ		[256]

have recently emerged, which generally rely on masking the hydrophobic surfaces of the proteins using detergents [10], lipid/detergent micelles [11] or lipidic-cubic phases [12]. An alternative strategy to 3D crystallography is to use electron microscopy; this can determine the structure of membrane proteins incorporated, as well ordered 2D (two-dimensional) arrays, in a lipid bilayer [13]. Generally, these so called 2D crystals are produced by using dialysis to remove, or Bio-Beads to absorb, the detergent from a detergent/lipid/protein mixture. This allows the protein to be reconstituted into the vesicles that form spontaneously. A recent innovation of this technique involves immobilizing the detergent-solubilized protein on a detergent-resistant Ni²⁺–lipid monolayer, via the His tag used to purify the protein, and incorporating the protein into the bilayer that forms upon depletion of the detergent with Bio-Beads [14]. These advances are paving the way for structure determination of 3D and 2D crystals by X-ray diffraction and cryo-electron microscopy respectively.

PROTON-DRIVEN ANTIPORTERS

In prokaryotic organisms, the extrusion of drugs and other cytotoxic compounds is conferred largely by pumps in which the drug efflux process is coupled to the influx of a proton (H⁺). These pumps are often termed H⁺ antiporters, and can be categorized into a number of families: these include the MF (major facilitator) superfamily [15–17], the SMR (small multidrug resistance) family [18,19] and the RND (resistance/nodulation/cell division) family [20–22] (Figure 1). The DME (drug/metabolite efflux) family represent a recently described but as yet poorly defined family; these proteins have been shown to possess some similarity to the SMR family [23].

The predominance of these H⁺ antiporters in bacteria is shown clearly by *Escherichia coli*, which is predicted to have 19, seven and three drug transporters belonging to the MF, RND and SMR families respectively, but only seven belonging to the ABC (ATP-binding cassette) transporter family. All of these putative drug transporters have been expressed in a strain of *E. coli* that is hypersensitive to drugs, to test whether they could confer drug resistance. It was found that 21 of the H⁺ antiporters, including 11 MF, six RND and two SMR transporters, conferred drug resistance, but only one ABC transporter conferred drug resistance [24]. Of these drug pumps, some were shown to confer resistance to either a single drug [e.g. drug-specific transporters such as TetA(B)] or several unrelated drugs [e.g. multidrug transporters such as AcrB]. Indeed, the presence of a number of

resistance pumps with a variety of substrate(s) provides *E. coli* with the ability to pump out a wide range of substrates. This is a recurring theme found in many pathogenic bacteria (Table 1). The presence of multiple efflux pumps in pathogenic bacteria can only highlight the potential clinical significance that this mechanism of resistance has.

The MF family

The MF transporters form a superfamily that comprises a number of subfamilies; of these subfamilies, transporters of sugars and drugs are by far the most numerous. MF transporters are typically composed of approx. 400 amino acids that are putatively arranged into 12 membrane-spanning helices, with a large cytoplasmic loop between helices six and seven [15]. It is most likely that this structure has arisen by gene duplication, as the two halves of the transporter usually have related sequences. A smaller number of these transporters have a putative 14-helix topology; however, MF transporters of this type tend to have a much smaller cytoplasmic loop [15]. The MF drug transporters have been classified into two subfamilies – 12-helix and 14-helix transporters [e.g. TetA(B) and TetA(K), the class B and class K tetracycline transporters from *E. coli* and *Staphylococcus aureus* respectively].

TetA(B), a well characterized MF pump

The TetA(B) protein is one of the most extensively studied members of the MF family. All 400 residues of TetA(B) have been mutated to cysteines, allowing their exposure to be probed by labelling with *N*-ethylmaleimide [25]. These studies have provided a topological model of TetA(B) in which the amphiphilic helices 2, 5, 8 and 11 form the walls of a water-filled channel, the partially amphiphilic helices 1, 4, 7 and 10 are tilted away from the channel, with only their cytoplasmic or periplasmic halves facing it, and the hydrophobic helices 3, 6, 9 and 12 are located at the periphery of the helix bundle. The presence of a water-filled channel is not surprising when one considers that the transported substrate is cationic (e.g. [Mg-tetracycline]⁺). Although TetA(B) has been crystallized, with 2D crystals yielding a projection map to 17 Å, the resolution is insufficient to define the arrangement of the helices [26]. However, a projection map for a member of the MF superfamily, the oxalate-transporting OxlT transporter from *Oxalobacter formigenes*, has been determined to a higher resolution of 6 Å, revealing an oval monomer with overall dimensions of 48 Å × 32 Å (Figure 2) [27]. The projection map indicated the presence of 12 α-helices, with one set of six helices

Figure 1 Diagrammatic representation of the membrane topology of proton-driven drug pumps in Gram-negative bacteria

The helices of the pumps are depicted in green, spanning the lipid bilayer, and lipophilic antibiotics are depicted as red circles that freely permeate the lipid bilayer. The three classes of antiporters are shown: SMR, four-helix small multidrug transporters; RND, 12- and 14-helix RND transporters; MF, 12- and 14-helix MF transporters. These transporters are shown utilizing the protonmotive force generated by respiration to expel antibiotics and other drugs into the periplasmic space between the inner and outer membranes. MF and RND transporters can act in concert with a MFP and an OMP, such as TolC, to translocate drugs across both the inner and outer membranes, expelling them from the cell [134–136,141]. TolC acts as a protein channel that not only spans the outer membrane, as a β -barrel, but also possibly spans the periplasm, as an α -helical barrel [141]. The exact role of the MFP remains unknown, but it may act as a protein channel translocating drugs between the inner-membrane transporter and the helical barrel of TolC. An alternative hypothesis is that the MFP pulls the two membranes together, so that the inner-membrane transporter can pass drugs directly to TolC [150].

related to the other by an approximate internal two-fold axis; this encloses a central cavity, with those helices lying closest to the cavity tilted and those set further back perpendicular to the membrane. Perhaps tilting of the central helices around the central cavity is required to form the substrate-binding site that gates the movement of substrates through the transporter.

RND transporters

RND transporters are much larger than MF transporters, being composed typically of approx. 1000 amino acid residues [20]. Even with this obvious disparity in size, they are predicted to adopt a similar 12-helical structure (Figure 1). However, unlike MF transporters, they possess large periplasmic or extracytoplasmic domains between helices 1 and 2 and between helices 7 and 8 (Figure 1) [5,28–31]. In a similar fashion to the MF transporters, the sequence of the first half of RND transporters usually resembles that of the second half, again suggesting that these transporters have arisen by gene duplication [20].

Determination of the structure of AcrB

Of all the advances made recently on the understanding of drug pumps, the determination of the structure of the *E. coli* RND pump AcrB to 3.5 Å probably represents the most significant [5]. AcrB

is the inner-membrane component of a tripartite multidrug pump that consists of the outer-membrane protein (OMP) TolC and the periplasmic membrane fusion protein (MFP) AcrA (these proteins and how they interact with the inner-membrane component of drug pumps are discussed in detail later). The overall AcrB transporter structure was shown to be an AcrB trimer that has a superficial 'jellyfish'-like appearance. The 'jellyfish' comprises a periplasmic headpiece of dimensions $50 \text{ Å} \times > 100 \text{ Å}$, and a TMD (transmembrane domain) of dimensions $70 \text{ Å} \times > 80 \text{ Å}$. The headpiece is divided into two stacked parts, with the upper and lower parts being 30 Å and 40 Å thick respectively. A side-on view of the structure reveals an upper part that has a trapezoidal appearance, 70 Å wide at the bottom and 40 Å wide at the top. The view from above shows an upper part that has a funnel-like opening, with an internal diameter of 30 Å . The funnel is connected by a pore, between the headpieces of the three protomers, to a large central cavity at the interface of the headpiece and TMDs of the protomers. Within the membrane, the protomers are arranged into a ring, with a 30 Å hole between them. Consistent with previous predictions, the periplasmic headpiece is formed by protrusions between helices 1 and 2 and 7 and 8. The lower headpiece is arranged into four domains, each comprising a β -strand- α -helix- β -strand motif. The domains termed PN1 and PN2 correspond to the protrusion between helices 1 and 2 in the N-terminal half, and domains PC1 and PC2 correspond to the protrusion between helices 7 and 8 in the C-terminal half. The

Figure 2 Projection maps for OxlT and EmrE

A 7 Å projection map for the EmrE dimer (A) [45] is compared with the 6 Å projection map for the OxlT monomer (B) [27]. The projection maps for EmrE and OxlT are reproduced from Heymann, Sarker, Hirai, Shi, Milne, Maloney and Subramaniam, *Projection structure and molecular architecture of OxlT, a bacterial membrane transporter*, EMBO J., 2001, **20**, 4408–4413 [27], and Tate, Kunji, Lebendiker and Schuldiner, *The projection structure of EmrE, a proton-linked multidrug transporter from *Escherichia coli*, at 7 Å resolution*, EMBO J., 2001, **20**, 77–81 [45], by permission of Oxford University Press.

β -strands are packed together so that one helix, from PN1, is used to line the pore between the protomers, while the remainder are positioned at the periphery of the lower headpiece. The upper, funnel-like, headpiece is composed of two four-stranded mixed β -sheets, that interrupt the β - α - β repeats of PN2 and PC2. The headpiece is held together by an ~ 35 Å-long hairpin structure that protrudes from the upper headpiece domain of each protomer and inserts into the next protomer. It has been proposed that the upper headpiece interacts with the OMP TolC, with six vertical hairpins from the AcrB trimer contacting the six α -helix-turn- α -helix structures of the TolC trimer to form a continuous path across the periplasmic space [5]. Recent work has also shown that the periplasmic headpiece plays a critical role in determining the substrate specificity of RND drug pumps; this is discussed in a later section.

Comparisons between the AcrB structure and that of OxlT described above show a number of similarities. The most obvious of these is the fact that AcrB and OxlT both possess helices that are tilted to form a central cavity. However, although helices 4 and 10 'gate' AcrB on the periplasmic side, the cytoplasmic side is more open than in OxlT. It is possible that this difference reflects the fact that OxlT was crystallized with substrate bound.

The periplasmic headpiece and drug recognition in RND pumps

The two large loops that protrude between helices 1 and 2 and helices 7 and 8, to form the periplasmic headpiece of RND transporters, have been shown to play an important role in drug recognition by RND pumps [28–32]. A recent paper by Elkins and Nikaido [30] showed that the drug specificities of RND pumps could be changed by swapping sections of the periplasmic loops between two different RND transporters. The RND pump AcrD, which normally provides only weak resistance to a limited range of antimicrobials, was readily transformed into a prolific multidrug transporter when its periplasmic loops were swapped with those from AcrB. The swap allowed AcrD to provide drug resis-

tance levels with a wide range of substrates in a similar fashion to the native AcrB protein. When the periplasmic loops of AcrB were replaced by those from AcrD, the reverse situation prevailed. This phenomenon is not only restricted to the Acr pumps of *E. coli*. Mutations that allow the MexD pump from *Pseudomonas aeruginosa* to efflux the previously non-transported antibiotic carbenicillin were all mapped to the periplasmic loops [28], and in a further study, where chimaeric AcrB/MexB transporters were produced, the specificity of the transported substrates was also found to reside within the periplasmic loops [32]. Taken together, these studies support the hypothesis that the periplasmic loops of RND transporters are critical in substrate determination, and that they are likely to contain multiple binding sites for structurally unrelated compounds.

The SMR family

SMR transporters are much smaller than those belonging to the MF and RND families (Figure 1). SMR pumps are normally composed of around 100 amino acids that are putatively arranged into four helices [18]. The best characterized SMR pump is EmrE from *E. coli*, a multidrug transporter that contributes resistance to EtBr (ethidium bromide) and Methyl Viologen [33–35]. Another SMR multidrug pump from *Ps. aeruginosa* with close identity with EmrE has recently been characterized and is shown to play an important role in the intrinsic resistance of *Ps. aeruginosa* to EtBr, acriflavine and aminoglycoside antibiotics [36].

EmrE, a model of SMR function

SMR transporters are by far the smallest of the known bacterial drug pumps, and it is hard to imagine how a single SMR protein could comprise a functional drug transporter unit. This becomes even more apparent when SMR pumps are compared with their relatively large and complex MF and RND drug pump counterparts. However, studies of the SMR transporter EmrE revealed that it functions as an oligomer, most likely a trimer, suggesting that the functional complex is similar in tertiary structure to the MF transporters [37,38]. EmrE can be extracted from the membrane in a form that retains the capacity to bind drugs, a feature that has enabled detailed equilibrium binding assays to be performed on the detergent-solubilized purified protein [39,40]. These revealed that EmrE can bind the drug TPP⁺ (tetraphenylphosphonium) with high affinity (i.e. K_d 10 nM) at a stoichiometry of approx. 1 mol of bound drug to 3 mol of EmrE, suggestive of a trimeric functional complex. The pH-dependence of the binding of TPP⁺ was determined for the wild-type transporter and for a mutant in which Glu¹⁴, located in TM1 (transmembrane domain 1) and the only membrane-embedded charged residue, was changed to Asp. The mutant, which had little transport activity [41], was able to bind TPP⁺, but with a decrease in pK from 7.5 for the wild-type protein to less than 5.0 for the mutant protein. This behaviour has been interpreted in terms of a model for the translocation cycle [42,43], in which two of the three Glu¹⁴ residues of the functional trimer deprotonate upon binding of the drug. Subsequently, the transporter undergoes a conformational change in which the binding site closes behind and opens up in front of the drug to expose it at the other face of the membrane. Release of the drug is catalysed by the approach of two protons that protonate the two Glu¹⁴ residues. This is an attractive model, because it couples drug export to H⁺ import that is catalysed by the trimer.

Cysteine scanning mutagenesis has given further insight into the importance of a cluster of amino acids surrounding Glu¹⁴ on the

same face of TM1. This cluster comprises a substrate- and proton-binding domain where mutations in most of the constituents result in a protein with decreased substrate affinity. Mutations in Ala¹⁰, which is on the same face of TM1 as Glu¹⁴ but one helix turn away, produced a protein with decreased affinity for protons. The occupancy by ligand and protons at this site appears to be mutually exclusive, because any impairment of the ability to bind either results in a decrease in substrate- and proton-coupled fluxes. These results strongly suggest that, in EmrE, the amino acid cluster on the same face of TM1 as Glu¹⁴ provides a common substrate- and proton-binding domain [44].

Structural information provides an insight into EmrE functional organization

Structural information on EmrE is available: EmrE has been crystallized using 2D crystallographic techniques, with the resulting 2D crystals yielding a projection map to a resolution of 7 Å. This resolution is sufficient to define the arrangement of the helices [45]. Somewhat at odds with the functional trimer model suggested by Schuldiner and co-workers [45], the projection map indicated that EmrE exists as a dimer, with overall dimensions of 31 Å × 40 Å (Figure 2). The dimer comprises an arc of probably four highly tilted helices, which bind a central cavity, separating two helices nearly perpendicular to the membrane from another two helices, one tilted and the other nearly perpendicular. Although the EmrE dimer would only have eight helices, it has similar dimensions to OxlT (i.e. 31 Å × 40 Å compared with 48 Å × 32 Å). Again, this seems to be at odds with the cysteine scanning mutagenesis studies carried out on EmrE (e.g. probing the exposure of cysteine residues to *N*-ethylmaleimide), which suggest that the helices are very tightly packed together, that there is no continuous aqueous channel and that drugs are translocated via a hydrophobic pathway [46]. This contrasts with the results of similar cysteine scanning mutagenesis studies performed on the MF transporters TetA(B) [25] and OxlT [27], which provide evidence for an aqueous channel permeating these transporters. In the case of OxlT this is not surprising, because it transports hydrophilic anionic substrates, whereas EmrA and TetA transport hydrophobic substrates, including cationic drugs (e.g. TPP⁺ and [tetracycline Mg]⁺). Furthermore, the projection maps for EmrE and OxlT both indicate the presence of a central cavity of similar dimensions, which is a candidate for the aqueous channel [45]. It is possible to speculate that the surface loops of EmrE, which are obscured in the 2D crystals, block access to this cavity. The EmrE structure also revealed the dimer to be asymmetrical; this is an intriguing observation and suggests that the monomers have slightly different conformations that may define different roles in the functional unit [45]. In this context it is interesting to note that some SMR genes are paired within an operon, e.g. the *ykkCD* and *ebrAB* genes of *Bacillus subtilis*. Expression of both YkkC and YkkD in *E. coli* was required to confer resistance to a range of cationic dyes, such as EtBr [47]; similarly, expression of both EbrA and EbrB in *E. coli* was necessary to confer EtBr efflux [48].

Na⁺- AND ATP-DRIVEN TRANSPORT

It is important to stress that in prokaryotic systems, even though proton-driven antiport is the major mechanism of drug efflux, other important mechanisms have been described. A novel group of transport proteins described recently are the MATE (multidrug and toxic compound extrusion) family; the common theme shared by this group of proteins is that drug efflux is coupled to Na⁺ influx [49–51]. Another group of transporters that are present

in prokaryotic cells, but provide the predominant mechanism of efflux in eukaryotes, are the ABC transporters. This superfamily of proteins catalyses the efflux of drugs by utilizing the energy released upon the hydrolysis of ATP.

MATE transporters

MATE transporters are similar in size to the MF transporters, and are typically composed of approx. 450 amino acids which are putatively arranged into 12 helices; however, they do not have any sequence similarity to members of the MF superfamily [23]. This novel family was identified only quite recently, with the characterization of NorM, a multidrug Na⁺-antiporter from *Vibrio parahaemolyticus*, which confers resistance to dyes, fluoroquinolones and aminoglycosides (Table 1) [49]. NorM homologues have recently been found and characterized in *Neisseria gonorrhoeae* and *Neisseria meningitidis* [50]. Deletions of these pumps resulted in an increased susceptibility to cationic compounds. Another MATE transporter, YdhE from *E. coli*, has also been characterized and shown to confer resistance to cationic antimicrobials [52]. The MATE transporters are a relatively new family among bacterial drug transporters and thus, of all the of drug efflux families currently known, MATE transporters are the least well characterized. Little is known about their structure, regulation or mechanism, with most studies thus far describing their presence and resistance provided in a limited number of bacteria. This situation is likely to change rapidly, especially as MATE transporters appear to play an important role in drug resistance to clinically relevant antibiotics in pathogenic organisms. Clearly, MATE transporters merit further investigation.

ABC transporters

In contrast with prokaryotes, the major mechanism of efflux in eukaryotes is dependent on proteins that derive their transport energy from the hydrolysis of ATP. Many of these transporters belong to the ABC superfamily of membrane transporters. Members of this family include the clinically significant multidrug resistance pump P-gp (P-glycoprotein) [53–58] and MRP (multidrug resistance protein) [59], both of which confer resistance to anticancer drugs. Related transporters are also found in a number of pathogenic fungi and parasitic protozoa, where they confer resistance to antimicrobial drugs; for example, P-gpA, a MRP homologue, is an arsenic/antimony pump that is responsible for resistance to the antimonial drug Pentostam in *Leishmania* [60]. Additionally, a few bacterial homologues have also been identified; these include the LmrA [61] multidrug transporter from *Lactococcus lactis*, the DrrAB [62] doxorubicin/daunorubicin transporter from the anthracycline-producing actinomycete *Streptomyces peucetius* and, most recently, the MacB transporter from *E. coli* that is involved in the efflux of macrolide antibiotics [63]. Although most ABC transporters were discovered as drug transporters, they frequently transport a wide range of substrates, including dyes, ionophoric peptides, lipids and steroids (Table 1).

Genetic and functional organization of ABC transporters

In terms of structure, ABC transporter proteins generally have two similar halves, each containing two parts: a TMD that is putatively arranged into six α -helices, and a NBD (nucleotide-binding domain) (Figure 3) [64–68]. In most cases a single gene encodes the four domains; for example, a single gene encodes P-gp, which has a TMD–NBD–TMD–NBD structure [53]. Although

Figure 3 Diagrammatic representation of the membrane topology of drug transporters belonging to the ABC superfamily

The archetypal ABC transporter has two NBSs and 12 membrane-spanning α -helices arranged in two groups of six. A single gene can encode all four domains, as for P-gp [3], or one membrane domain and one NBD, as for LmrA [3]; alternatively, separate genes can encode the membrane domains and NBDs, as for DrrAB from *Streptomyces peucetius* [62]. Intermediate genetic organization of ABC transporters can also exist; for example, a single gene encodes both membrane domains (i.e. 12-helices) of ArsB [231]. This situation can also occur with the NBDs: both of the NBDs of ArsA [232] and RbsA [233] are encoded by a single gene. The ribose transporter, RbsAC, works in conjunction with a periplasmic binding protein, RbsB, which sequesters ribose in the periplasm and docks with the transporter, delivering the sugar for transport [233].

Figure 4 2D projection maps for P-gp

Projection map for P-gp determined to a resolution of ~ 10 Å. Reproduced from Rosenberg, Callaghan, Ford and Higgins (1997) Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. *J. Biol. Chem.* **272**, 10685–10694 [71], with permission from ASBMB; and Rosenberg, Velarde, Ford, Martin, Berridge, Kerr, Callaghan, Schmidlin, Wooding, Linton and Higgins, Repacking of the transmembrane domains of P-glycoprotein during the transport ATPase cycle, *EMBO J.*, 2001, **20**, 5615–5625 [72], by permission of Oxford University Press.

a single gene also encodes MRP, it differs from P-gp in having an additional five-helix membrane domain at the N-terminus [69]. On the other hand, the *lmrA* gene encodes a protein with a single combined TMD and NBD, and the *drrA* and *drrB* genes encode the NBD and membrane domain respectively as individual proteins [62]. However, LmrA is thought to function as a homodimer [70] and Drr as a tetramer [71], with two DrrA and two DrrB subunits, suggesting that overall their functional structure is similar to that of the full-size ABC transporters. With full-size ABC transporters, the two halves of the protein closely resemble one another, strongly suggesting that this organization has arisen by gene duplication [2].

ABC transporter structure

As is the case for all drug pumps, a full understanding of the mechanism by which ABC transporters pump drugs ultimately

requires detailed structural information. Crystallization of these ABC proteins has proved difficult, but progress has been made recently that has given an insight into their structure and mechanism. Electron microscopic studies of single particles and 2D crystals have provided low-resolution structural information, in the form of projection maps, for P-gp to a resolution of 10 Å [71,72] (Figure 4), and for MRP [73] and YvcC, a multidrug pump from *Bacillus subtilis* [74], to a resolution of 25 Å. For P-gp, these studies revealed a hexagonal ring of protein, of approximate dimensions $91 \text{ Å} \times 60 \text{ Å}$, with a central asymmetrical pore of approximate dimensions of $30 \text{ Å} \times 5\text{--}10 \text{ Å}$; within this structure, 12 major densities are apparent, presumably the helices, that are related by a pseudo-two-fold symmetry axis. To determine the spatial arrangements of the domains of P-gp, a 3D density map, to a resolution of approx. 25 Å, was calculated from negatively stained 2D crystals that were tilted with respect to the electron beam. This revealed that the protein ring is composed of two

Figure 5 Structure of MsbA

(A) View of the chamber opening of the MsbA dimer, showing the ICDs (dark blue) that connect the TMDs (red) to the NBDs. The α -helices are shown as cylinders connected by (green) loops. A model of lipid A is shown embedded into the lower bilayer, which is designated by solid and dotted green lines, of the membrane. Dotted cyan lines mark the disordered region of the NBDs. (B) A view of MsbA from the extracellular side of the membrane. The positions of the extracellular loops that probably gate the movement of lipid A through the transporter are marked EC1, EC2 and EC3. Reprinted with permission from Chang and Roth (2001) Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters, *Science* **293**, 1793–1800 [8], Copyright American Association for the Advancement of Science.

‘arcuate’ domains related to each other by pseudo-two-fold symmetry, each of which consists of three densities that presumably correspond to pairwise clustering of the six transmembrane helices of each TMD. These domains form a channel that appears open to the extracellular face of the membrane, but, interestingly, there are gaps between these domains that might permit access to the channel from the membrane. There are two further domains, one located beneath each putative TMD, extending outwards to form a lobe 30 Å in diameter, each of which is thought to be a NBD. Similarly, MRP1 is arranged as a pentameric ring of approximate dimensions 100 Å $\times$ 80 Å, and recent studies showed that the transporter associated with antigen processing, Tap, also forms a ring structure of approx. 100 Å in diameter [75], suggesting that ABC transporters have similar 3D structures.

Structure of the *E. coli* lipid transporter MsbA

A major advance in our understanding of the structure and function of ABC transporters has come from the crystallization and determination of the structure of EC-MsbA, a lipid transporter from *E. coli*, to a resolution of 4.5 Å (Figure 5) [8]. EC-MsbA is a half ABC transporter, like LmrA, that is thought to catalyse the transfer of lipids across the cytoplasmic membrane [68,76].

The crystallographic structure revealed a homodimer that adopts a cone shape, with base dimensions of 120 Å $\times$ 115 Å $\times$ 64 Å, due to the six α -helices of each monomer being tilted 30–40° from the plane of the membrane. The cone peak is formed by the α -helices from each monomer making contact on the extracellular side of the membrane, leading to the NBDs being separated by approx. 50 Å at the cone base. Although the structure of the NBDs is not well resolved, the ATP-binding sites are projected to face away from the dimer centre. Interestingly, while the six transmembrane helices are connected by loops on the extracellular side of the membrane, on the intracellular side they are essentially extended into the cytoplasm by connecting α -helical structure, with these cytoplasmic α -helices packing against the TMD and the NBD. The union of the transmembrane helices from each dimer provides entry and exit pores, with diameters of approx. 25 Å, at the intra- and extra-cellular sides of the membrane, which are too small for the passage of lipid A. However, the cone-shaped tunnel that is extended by the cytoplasmic helices is of sufficient diameter to allow lipid A entry to the protein from the cytoplasm. Comparisons of EC-MsbA structure and cryo-electron microscopy studies of YvcC suggest that, like YvcC, MsbA adopts a cone-shaped structure with protruding NBDs [74]. In contrast, the structures of P-gp and the Tap1Tap2 dimer are more compact, and the NBDs do not appear to protrude from the cytoplasmic surface [71,75].

The structure of EC-MsbA confirmed many of our expectations derived from previous and extensive biochemical analyses of other ABC transporters. These included such fundamental expectations showing that the TMDs are composed of six α -helices and that these form the translocation pathway. Additionally, it was also shown that the NBDs are located at the cytoplasmic face of the membrane, where they are unlikely to contribute to the translocation pathway, but are more likely to contribute to conformational changes in the TMDs during the hydrolysis of ATP. On the other hand, several features of the EC-MsbA structure were unexpected. Of these, the most intriguing was the discovery that the TMD and NBD are connected by an α -helical ICD (intracellular domain) corresponding to residues 97–139 (ICD1), which folds into three helices, and residues 193–252 (ICD2) and 302–327 (ICD3), both of which fold into two helices. ICD1 is positioned directly above the ABC signature sequence motif (LSGGQQ) of the NBD, where it is thought to transduce signals between the NBD and TMD. The structure also raises several interesting questions; most importantly, how would such a structure account for the observed interaction of the NBDs (which many biochemical studies of ABC transporters allude to) if they are held apart? For example, FRET (fluorescence resonance energy transfer) measurements [77,78] and cross-linking studies [79] indicate that the two NBDs of P-gp are in close proximity. Moreover, although the binding of fluorescent analogues of ATP to purified P-gp indicates that there is a single class of site [80–82] and therefore suggests that the two NBDs are equivalent, biochemical studies indicate that the substrate-stimulated ATPase activity of these sites requires interaction between the two halves of the molecule [83]. Inactivation by chemical modification or mutagenesis of either site causes a loss of activity, suggesting co-operativity between the sites [80,84].

Although the NBDs of P-gp resemble each other, this is not always the case for other mammalian ABC drug transporters, such as MRP1. Studies have shown that the NBDs of MRP1 are non-equivalent [85–87]. Additionally, several ATPase structures have been determined, including HisP [88], RbsA [89], MalK [90], MJ0796 [91], MJ1267 [92] and Rad50 [93]; these have all been shown to form dimers, suggesting that these subunits interact in the functional transporter. However, each crystal structure exhibits

Figure 6 Crystal structure of ArsA, the ATPase subunit of the arsenical pump

In view (A), two ADP molecules are shown, in red space-filling form, in the NBDs of ArsA, which face each other at the interface of the two domains of ArsA [94]. Three Sb(III) molecules are also shown, in blue space-filling form, bound to the metal-binding site [94]. The ArsA molecule was rotated about the *y*-axis, by 90° and 135°, to give views (B) and (C) respectively. As seen in (B), the three Sb(III) molecules sit between two protrusions that may attach to the membrane domain [94]. Consequently, they are ideally placed for transfer to ArsB, the membrane translocase, as proposed by Borges-Walmsley and Walmsley [1]. These sites might then be re-filled by drawing in more Sb(III) molecules through the central cavity shown in (C). Generated using Setor.

an apparently different dimeric structure, suggesting that most of the dimer structures may be crystallographic artifacts. For example, the HisP, RbsA, MJ0796 and MJ1267 crystallographic dimers suggest that the NBDs are distinct and point away from one another, and are very unlike the MalK crystallographic dimer, where there is an interface at the NBDs. In Rad50 there is also an interface, but the monomers contact each other in an upside-down arrangement relative to the MalK dimer. The structure of the Tap1 NBD has been crystallized as a monomer, but this transporter forms heterodimers with Tap2 [90]. Thus these studies have not provided a consensus on how the monomers contact each other. However, some insight into the arrangement of the NBDs in a functional dimer has come from the determination of the structure of ArsA, the ATPase subunit of the arsenical pump from *E. coli* (Figure 6) [94]. In contrast with the other ATPase subunits, ArsA contains two NBDs and a binding site for arsenic or antimony, occupancy of which stimulates ATPase activity. The crystal structure of ArsA reveals that both NBDs and the metal-binding site are located at the interface between two homologous domains, with a short stretch of residues connecting these sites, to provide a signal transduction pathway that conveys information on metal occupancy to the ATP hydrolysis sites. In common with the MalK dimer, the NBDs of ArsA are asymmetrically arranged. Since the two halves of ArsA are contained within a single polypeptide, the way in which they form an interface almost certainly reflects the real structure of the functional ATPase. Although apparently at odds with the structure determined for EC-MsbA, a rationale for the co-operative interactions that are apparent between NBDs is that the binding of ATP drives the association of the two NBDs, a proposal that is supported by the fact that the binding of ATP induces dimerization of both Rad50 [93] and MJ0796 [91].

Revealing the catalytic cycle of EC-MsbA

The recent structural determination of VC-MsbA (*Vibrio cholerae* MsbA), a close homologue of EC-MsbA, has provided ex-

planations for many of the unexpected features of EC-MsbA [9]. Structural comparison between VC-MsbA and EC-MsbA showed that the transporters share structurally conserved TMDs, but differ in the orientation of their respective NBDs [8,9]. In stark contrast with the open structure of EC-MsbA, the closed structure of VC-MsbA shows interaction between the two NBDs [9]. The closed conformation of VC-MsbA is likely to represent stage two of the EC-MsbA translocation cycle (Figure 7). In addition, the observed differing structures of VC-MsbA and EC-MsbA are likely to represent different snapshots in time of the conformational changes involved in a shared translocation mechanism. Indeed, the closed structure of VC-MsbA provides strong evidence of a 'power stroke' mechanism of substrate translocation. Most importantly, the closed structure revealed by VC-MsbA provides an elegant explanation of the conformational changes that allow the NBDs of EC-MsbA to interact. Both the NBDs of VC-MsbA and EC-MsbA can sample a large conformational space, suggesting that in the absence of nucleotide the NBDs have the ability to oscillate [9]. Upon substrate recognition, the MsbA transporters are proposed to undergo a conformational change that enhances the ATP-binding ability of the NBDs [9]. It is thought that ATP binding results in the rotation of the NBDs so that they face one another; this is supported by studies with P-gp in which cross-linking studies revealed rotation of the NBDs during the catalytic cycle [95]. Additionally, there is evidence to support the fact that NBDs can be driven together after nucleotide binding [91,93]. This seems to be the case for both EC-MsbA and VC-MsbA, and it is this step that represents the 'power stroke' of the transport cycle (Figure 7). These studies provide the first instance of closely related transporters being captured in different conformations. The importance of this cannot be underestimated. Comparing the two structures has revealed far more of the conformational changes that are associated with substrate translocation than each structure individually. A great deal of the function of EC-MsbA derived from its own structure, while accurate, was speculative and was only validated by the elucidation of VC-MsbA. These studies also highlight the static image that crystal

Figure 7 Schematic diagrams showing the contrasting models of EC-MsbA and BtuCD substrate translocation and conformational changes

(A) (1) Substrate recognition results in a conformational change that allows the NBDs to bind ATP. (2) Recruitment of ATP triggers rotation of the NBDs and drives NBD dimerization, causing closure of the chamber around lipid A; this represents the 'power stroke' phase of the translocation cycle, and at this point lipid A is flipped towards the outer leaflet of the membrane bilayer. The NBD dimer then co-operatively hydrolyses ATP to form the high-energy ADP · P_i intermediate [8]. (3) Conformational changes resulting from ATP hydrolysis result in the release of lipid A into the outer leaflet of the membrane. Relaxation of the high-energy intermediate allows the NBDs to dissociate and return the transporter to its resting conformation. The yellow rectangle and green circle represent the hydrophobic tails and sugar head groups respectively of lipid A [8]. The scissor-like mechanism of MsbA differs from that of BtuCD. For BtuCD both the NBDs and TMDs remain in contact throughout the translocation cycle (as shown in B). (B) (1) ATP hydrolysis is stimulated by the binding of the BtuH (periplasmic binding protein)–vitamin B₁₂ complex to the periplasmic domain of BtuC [6]. (2) Rearrangement of the domains (as indicated by the arrowheads) and release of vitamin B₁₂ from BtuH towards the site of entry at the large water-filled space between the BtuC and BtuD subunits at the cytoplasmic side of the membrane [6]. (3) Release of ADP allows the BtuCD transporter to return to its relaxed form [6].

Figure 8 Crystal structure of BtuCD

The vitamin B₁₂ transporter BtuCD is a tetramer, composed of two BtuC membrane-spanning subunits (yellow and blue) and two BtuD ATPase subunits (green and red) [6]. The diagram shows the arrangement of the α -helices that comprise the BtuC subunits from a periplasmic viewpoint (B), and of the ATPase subunits from a cytoplasmic viewpoint (C) [6]. The putative positioning of ATP (space-filling grey molecules) is shown positioned between the P-loop of one subunit and the ABC-signature motif of the other subunit (both of which are highlighted in grey) [6]. Generated using the pdb viewer and rendered with pov-ray.

structures provide of dynamic transport processes. The structural determination of VC-MsbA and EC-MsbA shows the importance of and requirement for structural comparisons between similar transporters; it would seem likely that these studies will provide a template for the future characterization of other transporters.

Structural determination of BtuCD

The structure of a second ABC transporter, BtuCD from *E. coli* that catalyses vitamin B₁₂ uptake, has recently been determined to a resolution of 3.2 Å (Figure 8) [6]. The functional and

crystallographic unit of this transporter consists of two copies of the membrane translocase BtuC and two copies of the ATPase BtuD. The complex resembles an inverted portal, with dimensions of $90 \text{ \AA} \times 60 \text{ \AA} \times 30 \text{ \AA}$, inserted into the membrane. Each subunit only contacts its immediate neighbours, creating a large, water-filled channel between the membrane and the ATPase subunits at the cytoplasmic face of the membrane. This structural arrangement differs significantly from that of EC-MsbA, in which there is no contact between the ATPase subunits, and the BtuD ATPase subunits sit directly below the membrane, rather than being connected by an ICD to the membrane subunits. Importantly, the NBSs (nucleotide-binding sites) are positioned at the interface between the subunits, with the residues of the ABC signature sequence of one subunit juxtapositioned on to the P-loop of the other subunit, which is involved in the binding of ATP. This arrangement of the two NBDs resembles that of the two ATPase subunits of Rad50, in which the ABC signature sequence motif completes the active site in the second subunit [93]. Moreover, each BtuC membrane subunit is composed of 10 α -helices, rather than the six in EC-MsbA, and these are packed together in a more intricate manner than those of EC-MsbA. Indeed, the helices within each BtuC subunit can be placed into two groups, helices 2–5 and 7–10, which are related by a pseudo-two-fold rotation axis orientated 20° to the plane of the membrane, whereas helices 1 and 6 are nearly perpendicular to the plane of the membrane. In contrast, all the helices of each EC-MsbA subunit are orientated in the same direction, $30\text{--}40^\circ$ relative to the plane of the membrane. In BtuC, the prominent cytoplasmic loop between helices 6 and 7 folds into two short helices, L1 and L2, which make extensive contacts with BtuD. There is a sequence within the L1–L2 region that is conserved in a number of ABC transporters, the mutation of which causes transporter malfunction, suggesting that it may represent a general interface between the membrane and NBDs. Interestingly, this region corresponds to the first cytoplasmic loop of drug exporters, suggesting that different ABC transporters might have different folds. Indeed, this sequence motif is found in ICD1 of EC-MsbA, which is in contact with the NBD. The interface between the BtuC subunits is formed by the antiparallel packing of helix 1 of one subunit and helix 10 of the other at a crossing angle of -143° , making a cavity between the four helices that spans two-thirds of the membrane and is open to the periplasm, and which probably acts as the translocation pathway. The cavity is closed to the cytoplasm by residues Thr¹⁴² and Ser¹⁴³ in the loops connecting helices 4 and 5, which probably act as the transporter gate, since they separate the translocation cavity from the large water-filled cavity at the centre of the transporter.

Transport mechanism of other ABC transporters

In contrast with our understanding of the structure–function relationship of the NBDs of ABC transporters, our knowledge of the drug-binding sites is still rudimentary. For example, we lack detailed knowledge of the architecture of the drug-binding site, nor do we know how many sites an ABC transporter possesses, or how the hydrolysis of ATP is coupled to the transport of drugs across the membrane. The structures of the ABC transporters EC-MsbA and BtuCD suggest that they possess a single site within the TMD, whereas the structure of the drug-binding repressor QacR reveals two overlapping drug-binding sites. Equilibrium binding and photoaffinity labelling studies with drug analogues suggest that P-gp has two or three drug-binding sites [96,97] that are most probably located within the TMDs, and helices 4–6 and 10–12 have been implicated [98,99]. However, some workers argue that P-gp has a common drug-binding site and that the

binding of unrelated substrates can be explained by a ‘substrate-induced fit’ in which the different P-gp substrates create their own binding sites by utilizing residues from different TMDs in an area circled by TMDs 4–6 and 9–12 [100]. It is also proposed that P-gp interacts with its substrates via the lipid bilayer, since many of them are hydrophobic and readily partition into the bilayer [101]. In addition, P-gp has also been shown to possess at least one regulatory site [102–104]. Differences between P-gp and EC-MsbA/BtuCD are likely to reflect the fact that the latter are dedicated transporters of large substrates (i.e. lipid A and vitamin B₁₂ respectively), whereas P-gp is a transporter of multiple hydrophobic compounds [105,106].

Advances in our understanding of the interactions of the drug-binding site and the NBS of an ABC transporter have been greatly aided by the construction of a dimeric LmrA. LmrA is a multidrug transporter with structural and functional identity with P-gp. It is responsible for the efflux of structurally unrelated antibiotics in the bacterium *Lactococcus lactis* [61,70,107,108]. Two copies of the *lmrA* gene were fused via a sequence corresponding to that of the linker region connecting the two halves of P-gp [109]. The LmrA fusion protein transported fluorescent dyes, but disruption of either NBD inhibited the ATPase activity of the protein and abolished transport, indicating that LmrA functions as a homodimer in which there is co-operativity between the two NBDs and between these sites and the drug-binding site(s). This is consistent with studies of P-gp, where disruption of either NBD blocks ATP hydrolysis by both sites [84], and it has been proposed that the NBDs function by an alternating-site mechanism in which the binding and hydrolysis of ATP by one site prevents the binding of ATP at the other site [110]. Moreover, the binding of the drug vinblastine to the LmrA fusion protein was co-operative, occurred with a stoichiometry of 2, and could stimulate the transport of fluorescent dyes, providing evidence for the existence of two drug-binding sites that co-operate in a positive manner. It was also possible to trap LmrA with ADP and vanadate, which form a complex that resembles the ADP · P_i transition-state complex produced during ATP hydrolysis, inducing a state for LmrA in which only the low-affinity drug-binding site was exposed on the extracellular surface of the membrane. These findings have led to the following proposal for the mechanism of operation of LmrA (Figure 9): ATP induces the interconversion of two conformational forms of the protein, one in which a high-affinity transport-competent site is exposed intracellularly and another in which a low-affinity drug-release site is exposed extracellularly, and interconversion of these conformers occurs via a transition state in which the drug transport site is occluded. The proposed mechanism of LmrA transport can be described as an alternating two-site transport mechanism.

P-gp behaves in a similar manner to LmrA, with ADP and vanadate trapping the protein in a state that has decreased affinity and binding capacity for vinblastine [111,112] and iodoary-lazidoprazosin, a photoactive derivative of the substrate prazosin [113], suggestive of a conformational change from a high- to a low-affinity drug-binding state. However, recent studies indicate that the coupling between ATP hydrolysis and drug transport is more complex than originally proposed (Figure 9) [114–118]. These studies provide evidence that ATP hydrolysis at one NBD causes a dramatic decrease in the affinity for both substrate and nucleotide. In other words, as one site undergoes catalysis, the affinity of the other site for ATP is decreased, which may mimic alternate-site catalysis. Following the spontaneous release of the hydrolysis products from the site undergoing catalysis, both sites regain a high affinity for ATP and either site can bind the next molecule of ATP. Alternatively, P-gp may not regain the state with high affinity for substrate upon release of the hydrolysis products

Figure 9 Contrasting models of P-gp transport

In both panels, the NBDs are represented in yellow and the membrane-spanning domains in green. Upper panel: alternating two-site transport model. Step 1: binding of ATP to the NBD of one half of the transporter triggers drug binding to a high-affinity intracellular drug-binding site. Steps 2–3: ATP is hydrolysed, occluding the drug-binding site. Steps 3–4: release of the generated P_i leads to the exposure of the drug at an extracellular-facing, low-affinity drug-binding site on the same domain. At this point the drug can be released. As a result of the remaining ADP and co-operative interactions between the two NBDs, ATP is likely to bind to the non-liganded NBD. Steps 4–5: further conformational changes are likely to result in the exposure of a high-affinity drug-binding site on the intracellular side of the membrane domain that is associated with the liganded NBD. Steps 5–6: drug binding at this site results in ATP hydrolysis and the drug-binding site becomes occluded. Steps 7–1: release of P_i exposes a low-affinity drug-binding site at the extracellular side and the drug is released. Release of the drug and ADP allows the return of the transporter to the original conformation, and the cycle can begin again. Lower panel: revised P-gp translocation cycle. Hexagonal TMDs and NBDs represent those with decreased affinity for drug and nucleotide respectively. Step 1: drug binds to the ON site of one of the TMDs. Step 2: ATP is hydrolysed and the drug is moved to a lower-affinity drug-binding site. Step 3: P_i is released and the drug is extruded into the extracellular environment. Step 3a: vanadate (V_i) replaces P_i , and a P-gp · ADP · V_i complex is generated. This complex has decreased affinity for both drug and nucleotide. Step 4: ADP and V_i dissociate from the complex and the ATP-binding sites return to a high-affinity state. However, the affinity of the drug-binding sites remains low. Step 5: dissociation of ADP in step 4 allows the hydrolysis of an additional ATP. Step 6: P_i is released. Step 6a: P_i is replaced by V_i , forming a P-gp · ADP · V_i complex, which continues to exhibit decreased drug-binding affinity. Steps 6b and 7: dissociation of ADP allows P-gp to return to its original conformation and its high-affinity drug-binding is restored. These models are adapted with permission from data published in Martin, Berridge, Mistry, Higgins, Charlton and Callaghan (2000) Drug binding sites on P-glycoprotein are altered by ATP binding prior to nucleotide hydrolysis. *Biochemistry* **39**, 11901–11906 [111], copyright (2000) American Chemical Society; and Sauna and Ambudkar (2000) Evidence for a requirement for ATP hydrolysis at two distinct steps during a single turnover of the catalytic cycle of human P-glycoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 2515–2520 [113], copyright (2000) National Academy of Sciences, U.S.A.

because a second ATP must be hydrolysed to reset P-gp to this high-affinity state [115,116]. Translocation of the drug is presumed to be concomitant with the decrease in affinity of P-gp for the substrate that is induced by the hydrolysis of ATP. Occupancy of the drug-binding site prevents trapping of ADP by vanadate at the NBS under non-hydrolysing conditions (i.e. trapping requires the addition of ATP, which must be hydrolysed to ADP for trapping to occur), suggesting that the translocation and ATP-hydrolysis steps are coupled [117]. Consistent with this finding, the binding of the non-hydrolysable ATP analogue p[NH]ppA

(adenosine 5'-[β,γ -imido]triphosphate) apparently has no effect on the affinity of P-gp for idoxylazidoprazosin [111]. On the other hand, both p[NH]ppA and adenosine 5'-[γ -thio]triphosphate have been reported to cause a decrease in the capacity and affinity of P-gp for vinblastine [2,72,111,118], suggesting that nucleotide binding is sufficient to trigger the conformational change from the high- to the low-affinity state associated with substrate translocation. Cryo-electron microscopy studies of P-gp have revealed major conformational changes in the presence of p[NH]ppA; however, the vanadate-trapped transition state has a

different conformation state, implying that further conformational changes accompany the hydrolysis of bound ATP [72]. If the binding of ATP to P-gp causes it to adopt a low affinity for the drug substrate, then this would imply that drug binding precedes ATP binding. Consistent with this, vinblastine [72] has been reported to increase, and verapamil, cyclosporin A and iodoarylazidoprazosin [72,114,117] to decrease, the affinity of P-gp for ATP. Furthermore, substrates can modulate the vanadate trapping of the photoaffinity label 8-azido-ADP, with verapamil and cyclosporin A respectively increasing and decreasing the amount of 8-azido-ADP trapped. Interestingly, this behaviour correlates with the effects of these drugs on the ATPase activity of P-gp, with verapamil and cyclosporin A respectively increasing and decreasing the ATPase activity of P-gp. Since P-gp has a very low affinity for P_i , such experiments suggest that ADP release is the rate-limiting step of the catalytic cycle and that substrates modulate the ATPase activity by controlling ADP release [72,114]. Although drug binding must precede nucleotide binding for the drug for it to assert these effects, verapamil and cyclosporin do not affect the binding of 8-azido-ATP under non-hydrolysing conditions (i.e. at 4 °C), indicating that they assert their effects on steps subsequent to the binding of the nucleotide. Clearly, then, whether binding or hydrolysis of the nucleotide is used to drive drug translocation is still controversial. The story becomes more intriguing when the discovery that drug binding to P-gp induces conformational changes in the NBDs is considered. In recent work, the conserved ABC signature sequence (LSGGQ) in each NBD was shown to be adjacent to the Walker A sequence in the opposite NBD [119]. Cross-linking between these opposing motifs was shown to be increased by substrates that induce P-gp ATPase activity and decreased by those substrates that lower ATPase activity [120]. It would therefore appear that the LSGGQ sequence can increase the rate of ATP hydrolysis the closer it comes to the Walker A motif. Drugs that increase ATPase activity must induce conformational changes that bring the two motifs closer together; the opposite effect will occur with those drugs that decrease ATPase activity. This finding provides strong evidence for substrate-induced conformational changes in the NBDs and strongly suggests that the interaction between the Walker A and LSGGQ sequences is vital in coupling ATP hydrolysis to drug translocation. This communication between the NBD and drug-binding site of P-gp has been described as 'cross-talk' [121], and may point to a more complex translocation cycle in P-gp than previously thought.

The above studies generally support a model in which the two NBSs are functionally equivalent, but where only one can undergo catalysis at any given time. However, some studies have reported that the NBSs function asymmetrically. For example, while many groups have reported that the ATPase activity of P-gp increases in a hyperbolic manner with ATP concentration, indicative of a lack of co-operativity, a recent study reported co-operative effects when ATPase activity was determined over an extended ATP concentration range [117]. Co-operativity has been reported for several bacterial ABC transporters [122–124]. In contrast with other reports [122,125–128], 8-azido-ATP has been reported to preferentially label the N-terminal NBD of P-gp [122,127]. There is clear evidence to indicate asymmetry of function of the NBDs of MRP1 and CFTR1 (cystic fibrosis transmembrane conductance regulator 1), which may reflect the much lower sequence similarity in the two NBDs of these transporters. For example, mutations in the consensus Walker motifs of the two NBDs of MRP1 have differential effects on solute transport, and there is differential labelling of the NBDs with 8-azido-ATP, with NBD1 labelled predominantly with 8-azido-ATP and NBD2 with the hydrolysis product 8-azido-ADP

[69,87,130,131]. The NBDs of CFTR also appear to behave differentially, with evidence to suggest that the binding of MgATP to NBD1 triggers channel opening, whereas hydrolysis of MgATP at NBD2 triggers channel closure [132,133].

How can the kinetic mechanism be reconciled with our knowledge of the biochemistry and structure of ABC transporters? One possibility is that the transport cycle is initiated by the binding of substrate to the transporter, which triggers conformational changes that modulate the interaction of the NBSs with ATP, possibly in a fashion similar to that seen for the substrate-induced interaction of the opposing LSGGQ and Walker A motifs of the P-gp NBDs. In the absence of substrate, the transporter can undergo a non-productive cycle that results in ATP hydrolysis. In the case of P-gp, vanadate-trapping experiments indicate that the ADP · P_i transition state has decreased capacity for substrates, which might be attributable to the occlusion of the substrate-binding site. In the presence of substrate, the binding and hydrolysis of ATP would then presumably result in occlusion of the substrate as the gate moves from in front of to behind the substrate, and the intracellular high-affinity binding site is converted into an extracellular low-affinity binding site, which facilitates the release of the substrate outside the cell. It is possible that ATP binding catalyses the initial occlusion event, but that ATP hydrolysis is required for the translocation event in which the transporter changes from a high- to a low-affinity state. The binding of ATP has been found to trigger the dimerization of some bacterial ATPase subunits that possess a single NBS (e.g. Rad50 [93], MJ0796 [91] and MJ1267 [92]). This is suggestive of a mechanism for the operation of ABC transporters in which the binding and hydrolysis of ATP drives the interaction of the two ATPase subunits or domains, with this movement being coupled to movements of the TMDs that lead to a repositioning of the translocation gate behind the substrate (Figure 7). In the case of EC-MsbA, this movement would bring the two NBDs together, closing the intracellular TM cavity in which lipid A would sit. Moreover, one can imagine that, as the two subunits interact, there is potential to bury the NBSs, leading to occlusion or trapping of ATP at one NBS, while preventing ATP binding to the other nucleotide-free NBS, thus leading to the apparent alternate-site kinetics. Interestingly, the binding, rather than the hydrolysis, of ATP drives dimerization of the ATPase subunits of MJ0796 and MJ1267 [91,92], suggesting that ATP hydrolysis is coupled to events subsequent to closure of the substrate-binding site. For example, ATP hydrolysis could be used to catalyse the subsequent opening of the substrate-binding site at the alternate face of the membrane. Alternatively, ATP hydrolysis could be used directly to reset the transporter by driving the NBDs apart, or indirectly by producing ADP that does not support the dimer. There is evidence to suggest that NBD1 and NBD2 of CFTR respectively stably bind and hydrolyse ATP [133]; perhaps one NBD controls dimerization and channel opening, while the other controls channel closure? Other ABC transporters, such as BtuCD (Figure 8) and P-gp, could operate by a similar mechanism, with the binding of ATP triggering further interaction of the ATPase subunits or NBDs, but presumably the conformational changes would be subtler than those occurring in EC-MsbA and VC-MsbA, perhaps reflecting a need for less dramatic rearrangements of the TMDs. The answer to these questions will lie in the determination of the structures of ABC transporters with bound substrate and at different stages of the ATPase reaction.

ACCESSORY PROTEINS REQUIRED FOR DRUG EFFLUX

More complex transport systems, which utilize a tripartite protein complex rather than a single transport protein, exist in

Gram-negative bacteria. These tripartite systems allow translocation of drugs across both the inner and outer membranes. Generally, these transport systems consists of an IMP (inner-membrane protein) transporter (such as AcrB; see previous sections), a MFP, which is anchored to the inner membrane by either a lipid moiety or a single α -helix, and a porin-like OMP (see Figure 1). Two well-studied examples are the AcrAB/TolC [134] and MexAB/OprM [135] transport systems that confer multidrug resistance in *E. coli* and *Ps. aeruginosa* respectively. In these transport systems the IMPs (e.g. AcrB and MexB respectively) belong to the RND family. The MFPs (e.g. AcrA and MexA respectively), which are linked to the inner membrane by a lipid moiety, are thought to couple the interaction of the IMPs with the structurally related porin-like proteins TolC [136] and OprM [112]. MF transporters also form tripartite systems; these include the EmrAB multidrug transport system [137] from *E. coli* that utilizes the MFP EmrA, which is anchored to the membrane by a single N-terminal α -helix. In a similar fashion to AcrA, EmrA is thought to facilitate the interaction of EmrB with TolC. *V. cholerae* possesses a homologue of the EmrAB system, VceAB [138]; the operon encoding these proteins also encodes an OMP, VceC, that resembles OprM [138]. The *E. coli* ABC transporter MacB has been shown to interact with TolC via the MFP MacA [63].

TolC plays a critical role in drug efflux

TolC is a multifunctional protein, being involved in the translocation of both small drugs and large polypeptide toxins, such as haemolysin. It is able to achieve these different roles by a process of substrate-induced transient interaction with the transporters of either drugs (e.g. AcrAB) or toxins (e.g. the haemolysin ABC transporter HlyB and the MFP HlyD) [139,140]. The structure of TolC has been determined by X-ray crystallography, revealing a trimeric arrangement in which the three TolC molecules are structured into a 140 Å cylindrical channel with an internal diameter of 35 Å (Figure 10) [141]. The outer-membrane end of the structure is open, providing solvent access, but the periplasmic end tapers to a virtual close, with the opening approx. 1.95 Å at its narrowest. The structure of TolC can be further subdivided into two major domains: an outer-membrane β -barrel and a periplasmic α -helical barrel. The β -barrel domain, which spans and provides an essentially open channel through the outer membrane, is composed of 12 β -strands, four donated by each TolC molecule, arranged into a right-twisted barrel. The α -helical domain is a left-twisted 12-helix barrel, which is constructed from two types of helices: long helices of 67 residues and short helices of 23 and 34 residues, with pairs of the shorter helices stacked to produce pseudo-continuous helices. The α -helices are further arranged into coiled-coils, and the mixed α/β structure connecting the shorter helices forms a belt around the helical barrel. The structure of TolC is clearly indicative of a protein that has the major function of channelling substrates across the periplasm and through the outer membrane. The diameter of TolC suggests that protein export probably requires the protein to be unfolded [142], possibly by being moved linearly via a number of contact sites that span the length of TolC. Indeed, knowledge of the structure of TolC has been used to drive experimental investigations of the mechanisms involved in the opening and closing of the periplasmic portal of TolC. Each monomer contributes two aspartate residues to form a group of six that surrounds the portal of the TolC trimer. Replacement of these aspartate residues, by mutagenesis, with alanine and subsequent electrophysical analyses revealed their importance in determining the size of the portal closure. The TolC mutants had higher

Figure 10 Crystal structure of TolC

TolC trimerizes to form a 140 Å protein cylinder that is open at the outer-membrane end, with an internal diameter of 35 Å, but comes to a narrow constriction at the periplasmic end [141]. The protomers are coloured individually. The diagram shows an exterior view of the TolC protein channel, normal to the plane of the membrane in (B), and looking down the channel from the outer-membrane (A) and periplasmic (C) ends of TolC [141]. There are two major domains: a β -barrel that spans the outer membrane, and an α -helical barrel, approx. 100 Å in length, that partially spans the periplasmic space [141]. Note the constriction of the helices, blocking the channel, at the periplasmic end. It is likely that the periplasmic end of TolC interacts transiently with a MFP, opening the protein channel to allow drug entry [141]. Generated using Setor.

levels of conductance when compared with the wild-type protein, strongly suggesting that the relatively smaller alanine molecules increased the size of the closure [143]. Additionally, in a related study, the opening and closing of TolC was shown to be a pH-dependent process, with wild-type TolC closing at low pH [144]. This pH dependence was abolished in the mutant. The TolC structure indicates that the periplasmic portal is constrained by a circular network of intra- and inter-monomer hydrogen bonds and salt bridges: in a series of mutagenesis studies these links were abolished. Simultaneous abolition of all of these connections resulted in a substantial increase in conductivity. When the TolC mutants and the pore-forming *S. aureus* α -toxin were compared, it was shown that they possess levels of conductance within the same range. The structure of α -toxin is known and the pore it forms has a diameter of 16 Å; thus it is possible to speculate that, in view of the similarities in conductance, the fully open TolC portal may share similar dimensions. A portal of this size would allow the passage of most drug pump substrates and unfolded proteins through TolC [145]. This study provided an excellent example of how structural information could be used to provide insight into the transport mechanism of a protein. However, several questions on the precise nature of the transport mechanism of TolC remain. For example, what is the actual *in vivo* trigger that induces the opening of TolC? Additionally, how are substrates passed from the IMP to TolC, and how do they traverse the periplasmic space? Addressing the latter of these questions, the predicted length of TolC cannot provide the answer. The TolC α -helical barrel is approx. 100 Å long, which is close to the lower estimates of the depth of the periplasmic space at 130 Å, but some estimates put

the depth of the periplasm at 250 Å [146,147] and TolC would be unable to span this depth. It is possible that a combination of TolC and a MFP might provide a longer tunnel and provide an escape route out of the periplasm.

There has been much speculation as to the precise functional role of the MFP. One possibility is that the MFP forms a channel between the inner and outer membranes; another suggestion is that it pulls the inner and outer membranes together, allowing direct ligand transfer between the inner- and outer-membrane translocases [148]. Interestingly, MFPs are predicted to have a structure that resembles that of TolC, and a database search using a sequence template for the TolC α -helical barrel revealed a match to several MFPs [149]. The MFP N- and C-termini are proposed to fold into a flattened β -barrel, with the intervening residues arranged into two long helices, each of approx. 60 or more residues, which fold back on one another, because of a hairpin, to form a coiled-coil. Consistent with this structure, both AcrA [148] and EmrA [151] have been shown, from hydrodynamic measurements, to have highly elongated structures, with a length in excess of 200 Å. The α -helix or lipid moiety of MFPs, which anchors them to the inner membrane, is at the N-terminus and would position the β -barrel at the inner membrane, with the α -helices radiating out across the periplasm to face the TolC α -helical barrel. Furthermore, the ability of MFPs to form stable trimers [139,140,151] suggests that their role is to form a connecting channel between the IMP and TolC. Indeed, the interaction of the MFP with TolC is most likely to be necessary to induce a conformational change in TolC that opens up its channel for substrate entry. The putative β -barrel of the MFP could act as the receiver domain for drugs released from the IMP, while the α -helical cylinder, produced by the six α -helices of the MFP trimer, could transiently overlap the closed end of the α -helical barrel of TolC, inducing it to open. On the other hand, the two β -strand domains, which flank the coiled-coil domain, each contain a motif that resembles the lipoyl domain of enzymes involved in the transfer of a covalently attached lipoyl or biotinyl moiety between proteins [149]. The formation of a similar domain would require the N- and C-terminal domains to interact, which might provide a mechanism for bringing the two membranes together. Perhaps the β -barrel domain functions in passing drugs between the IMP and TolC? Recent studies have shown that EmrA has the ability to bind substrates of the EmrB IMP at its periplasmic domain; this has provided the first evidence that a MFP may play a direct role in the translocation of drugs across the periplasmic space, rather than forming a passive tunnel between the IMP and OMP [151]. Considering this finding, one might speculate that the binding of drugs to EmrA provides the trigger that allows it to 'grab' the TolC trimer and then pass the drug into the channel formed by this trimer. However, the definitive role of the MFP is only likely to become apparent from detailed knowledge of its structure. In this area some progress has been made. The formation of 2D crystals of AcrA at the surface of a lipid layer has been reported, and this has provided a glimpse of the structure, but only to a resolution of 20 Å [152]. The unit cell, with a size of 157 Å $\times$ 95 Å and a thickness of approx. 100 Å, consisted of four asymmetrical units apparently arranged into two rings, each with a central opening of 30 Å in diameter. The density within each ring has been interpreted as following a pseudo-helical path of approx. 210 Å, which is similar to the length of AcrA that was determined from the hydrodynamic measurements. This 'spring-like' structure is suggestive of a structural role that pulls the IMP and OMP together, rather than being involved in forming a channel. Higher-resolution structural information is required in order to reach firm conclusions.

ARCHITECTURE OF THE MULTIDRUG-BINDING SITE

Considerable insight into drug-protein interactions has been provided by the determination of the structures of the repressors that control the expression of the drug pumps. One of the best studied repressors is TetR, which controls the expression of the TetA transporter involved in the efflux of tetracycline from *E. coli*. The structure of TetR has been determined with and without bound tetracycline analogues [153–156], and has been shown to be a ten- α -helical protein that functions as a homodimer. The N-terminal DNA-binding domain is composed of a pair of three-helix bundles, one donated from each protomer, which has an α -helix–turn– α -helix motif (helices 2 and 3); this domain is connected by helix 4 to the globular core domain that is composed of helices 5–10. The binding site for the substrate [tetracycline-Mg]⁺ is a tunnel-like pocket in each core domain, defined by helices 4–8 in one protomer and helices 8 and 9 from the other protomer, with movement of the latter helix closing the tunnel entrance after substrate entry. The A ring (of the four-ring carbocyclic structure) of tetracycline is stabilized in the tunnel by hydrogen bonds from His⁶⁴ (C-terminal to helix 5), Asn⁸² (helix 5), Phe⁸⁶ (helix 5) and Gln¹¹⁷ (helix 7). The B-, C- and D-rings are mainly stabilized by van der Waals interactions that are dependent upon the C- and D-ring substituents; this allows the rings freedom to move laterally about their plane. Mg²⁺ is coordinated by the side chains of His¹⁰⁰ (helix 6) and Glu¹⁴⁷ (helix 8 of the other protomer).

BmrR

The structure of another transcriptional regulator, BmrR, which functions as a dimer to control expression of the Bmr multidrug pump from *Bacillus subtilis*, has also been determined [157,158]. The drug-binding domain has a distorted eight-stranded β -barrel structure, while the DNA-binding domain, which is connected to the drug-binding domain by an α -helical linker, belongs to the superfamily of winged-helix proteins and contains a four-helix bundle and a three-stranded antiparallel β -sheet. The binding of the drug TPP⁺ to BmrR induces the unfolding and relocation of an α -helix to expose an internal drug-binding pocket. Drug binding is mediated via interactions with many hydrophobic residues (e.g. Phe²⁴⁴ and Tyr²⁶⁸) in this pocket and by an electrostatic interaction between the positively charged drug and a buried glutamate residue (e.g. Glu²⁵³). Modelling studies suggest that the binding pocket can accommodate a range of structurally dissimilar cationic drugs, but the affinity is dependent upon how close the positive charge of the drug comes to the carboxylate of Glu²⁵³. The MarR repressor, which regulates multiple antibiotic resistance in *E. coli*, has a similar structure to BmrR, with drugs binding to two pockets on the surface of the protein [159]. In each pocket the drug sits over a hydrophobic residue (e.g. proline and methionine respectively) and the interaction is stabilized by hydrogen bonds. Thus for both BmrR and MarR the drug-binding sites appear to be located at the protein surface, in contrast with the buried binding site of TetR. However, care must be taken in reaching such conclusions, since there is a possibility that the drugs could merely be binding to non-specific hydrophobic sites on the surface of the protein.

QacR and hPXR (human pregnane xenobiotic receptor)

The structures of three other multidrug-binding transcriptional regulators have recently been reported that more closely resemble TetR in having buried drug-binding sites: hPXR, which activates expression of a cytochrome P450 that metabolizes a variety of drugs [160], QacR, which represses transcription of the QacA

multidrug pump in *Staphylococcus aureus* [161], and MexR, which represses transcription of the MexAB/OprM multidrug pump in *Ps. aeruginosa*. In common with TetR, both hPXR and QacR have compact α -helical structures, although hPXR also possesses a five-stranded antiparallel β -sheet. Twenty hydrophobic residues, four polar residues and four charged residues line the drug-binding cavity of hPXR. However, a salt bridge between two of the charged residues effectively neutralizes their charge, and the drug-binding cavity is relatively hydrophobic, with the polar residues enabling specific interactions to be made with the drug. Indeed, determination of the structure of hPXR complexed with the drug SR12813 revealed that the protein utilizes different polar residues to bind the drug in three distinct orientations: one conformation is stabilized by van der Waals contacts with eight residues and a hydrogen bond with Ser²⁴⁷; the second by van der Waals contacts with seven residues and a hydrogen bond with His⁴⁰⁷; and the third by van der Waals contacts with six residues, hydrogen bonds with Ser²⁴⁷ and Gln²⁸⁵ and a water-mediated hydrogen bond with Ser²⁰⁸. This finding indicates that the binding site can accommodate a number of structures but still maintain specificity. The ability of hPXR to bind both small and large drugs has been attributed to a flexible loop (residues 309–321) that lies adjacent to the drug-binding cavity. Indeed, the loop contains four solvent-exposed hydrophobic residues, which could interact with hydrophobic drugs. Furthermore, in other nuclear receptors, which are highly specific for their cognate ligand, this loop is replaced by an α -helix in the proposed ligand access site of the binding cavity.

Structural determination of QacR with substrate bound

A better understanding of how the binding site can accept a number of structurally diverse drugs has come from studies of QacR, which has been crystallized with six different drugs: R6G (rhodamine 6G), EtBr, dequalinium, Crystal Violet, Malachite Green and berberine [161]. QacR is a nine-helix protein, with the first three helices forming the DNA-binding domain; this assumes a three-helix bundle that includes a helix–turn–helix motif, with the remaining helices comprising the drug-binding and dimerization domain. The binding of drugs to QacR triggers a coil-to-helix transition of residues Thr⁸⁹–Tyr⁹³, elongating helix 5 by one turn. This conformational transition is apparently triggered by the movement of Tyr⁹² from the hydrophobic core of the protein into the solvent, where it adopts a more favourable conformation. In addition, Tyr⁹³ is also displaced from the hydrophobic core to a more peripheral position, where it can stack against the drug. Thus Tyr⁹² and Tyr⁹³ act as drug surrogates that stabilize the binding site in the absence of drugs. The movement of these tyrosines triggers divergence of helices 6–8 in one protomer and helix 8 of the other protomer, revealing an alternate binding site, with helices 4–8 and helix 8 of the other protomer contributing to the site. Drug-induced conformational changes in one protomer are believed to preclude the binding of drugs to the other protomer, accounting for the fact that only one drug molecule is bound per dimer. Similarly, the multidrug-binding repressors BmrR [162] and EmrR [163] only appear to bind one drug molecule per dimer, but TetR dimers bind two drug molecules.

The binding site of QacR contains a number of hydrophobic residues, including a number of aromatic residues (e.g. Trp⁶¹, Tyr⁹³, Tyr¹⁰³, Tyr¹²³ and Phe¹⁶²) that can make hydrophobic stacking contacts with drugs, a number of polar residues, which can accept or donate hydrogen bonds and which are used selectively to stabilize different QacR–drug complexes, and four glutamate residues (Glu⁵⁷, Glu⁵⁸, Glu⁹⁰ and Glu¹²⁰) that are used selectively to complement the positive charge on cationic drugs.

Figure 11 Crystal structure of QacR, showing the positions of the R6G- and EtBr-binding sites

(A) Diagram showing the crystal structure of QacR with bound R6G (green); the position where EtBr (red) would bind is superimposed upon this structure [161]. This diagram was generated using the pdb viewer and rendered with pov-ray. (B) Chemical structures of the cationic lipophilic drugs R6G and EtBr.

The structures of the QacR–R6G and QacR–EtBr complexes indicate that QacR possesses two overlapping binding sites (Figure 11). The QacR structure reveals that the three-ringed system of R6G is wedged between Trp⁶¹ and Trp⁹³, in helices 4 and 5 respectively, while the phenanthridinium system of EtBr is sandwiched between Tyr¹⁰³ (in helix 6) of one protomer and Phe¹⁶² (in helix 8) of the other protomer (i.e. termed Phe^{162'}). In both complexes there is a hydrophobic interaction with Tyr¹²³. The interaction with R6G is further stabilized by hydrogen bonds with Gln⁶⁴ (in helix 5), Thr⁸⁹ (helix 5) and Gln⁹⁶ (helix 6), and an electrostatic interaction with Glu⁹⁰, while the interaction with EtBr is stabilized by just one hydrogen bond to Gln⁹⁶ and an electrostatic interaction with Glu¹²⁰ in helix 7. Residues Thr⁸⁹ and Glu⁹⁰ only enter and become part of the binding site as helix 5 is extended due to the R6G-induced coil-to-helix conformational change. Consequently, the drug-binding pocket, with dimensions of $\sim 10 \text{ \AA} \times 9 \text{ \AA} \times 23 \text{ \AA}$, is much larger than these drugs, and water molecules are used to fill the unoccupied space surrounding R6G and EtBr in the pocket. In contrast, dequalinium straddles both the R6G- and EtBr-binding sites; one quinolinium group is sandwiched by Trp⁶¹ and Tyr⁹³, as for R6G binding, but Glu⁵⁷ and Glu⁵⁸ (rather than Glu⁹⁰) complement the positive charge. The contacts in the EtBr-binding site are identical with those in the QacR–EtBr complex, with the second quinolinium group bound between Tyr¹⁰³ and Phe^{162'} and the positive charge complemented by Glu¹²⁰. The decamethylene linker adjusts to make an optimal fit, making van der Waals contacts with a number of residues

Figure 12 Crystal structure of AcrB with bound substrates

The AcrB inner-membrane pump is active as a trimeric assembly. AcrB is the inner-membrane component of a tripartite multidrug pump that consists of the OMP TolC and the periplasmic MFP AcrA [5]. The superficial structure has had its appearance described as 'jellyfish'-like [5]. Three distinct domains are formed by the trimer: the funnel, the pore and the large central cavity. The position of the three domains is further emphasized by the purple ribbon. All drugs were found to bind the central cavity, but at different positions [167]. The approximate positions of the drugs dequalinium (Dq), ciprofloxacin (Cip), EtBr (Et) and R6G and their interactions with AcrB trimer are indicated [167]. The positions of the inner and outer leaflets of the membrane are also shown. Reprinted with permission from Nature (www.nature.com/nature), Crystal structure of the bacterial multidrug transporter AcrB, S. Murakami, R. Nakashima, E. Yamashita and A. Yamaguchi, vol. **419**, pp. 587–593, copyright 2002, Macmillan Publishers Ltd.

(e.g. Leu⁵⁴, Ile⁹⁹, Met¹¹⁶ and Leu¹¹⁹). On the other hand, Crystal Violet does not have a planar geometry that would allow it to be sandwiched in the binding sites for either R6G or EtBr, and it binds in the overlapping region, with its aryl groups interacting with the hydrophobic residues at the edges of each site. The methyl groups of the first dimethylaminophenyl moiety form hydrophobic contacts with Trp⁶¹ and Tyr⁹³ of the R6G site, with the phenyl group stacking against Tyr¹²³; the methyl and phenyl groups of the second moiety make van der Waals contacts with Tyr¹⁰³ and Phe¹⁶² of the EtBr site; and the third moiety is anchored by contacts with Ala¹⁵³, Glu¹²⁰, Asn¹⁵⁴ and Asn¹⁵⁷. The delocalized positive charge of Crystal Violet is dissipated via contacts with Glu⁹⁰ and Glu¹²⁰.

Drug binding to efflux pumps

There is evidence to suggest that transporters of lipophilic cationic drugs may utilize similar mechanisms to select drugs. The *E. coli* multidrug transporter MdfA possesses a single acidic residue, Glu²⁶, in the first putative TMD of this protein, which provides drug selectivity [164]. The substitution of Glu²⁶ with a basic residue abolishes transport of positively charged EtBr, but not uncharged chloramphenicol. Many multidrug transporters belonging to the MF and SMR families possess acidic residues at a similar position in helix 1, suggesting that they utilize a similar mechanism of drug selectivity. Furthermore, using a direct drug-binding assay with the purified MdfA protein, it was possible to establish that chloramphenicol stimulates the binding of TPP⁺, indicating that MdfA, like QacR, has multiple binding sites. There is evidence that the two MF transporters LmrA [165] and QacA [166] also have at least two drug-binding sites.

AcrB has been co-crystallized with four of its known substrates (R6G, EtBr, dequalinium and ciprofloxacin) and the structures of these complexes determined (Figure 12) [167]. All four of these substrates were shown to bind to a large cavity in the centre of the AcrB trimer, but each drug interacts with a different range of

residues. R6G binds to a loop formed between helices 3 and 4, and by helices 5 and 6 within the large central cavity. Each subunit of the AcrB trimer was shown to bind one R6G molecule in an area between two neighbouring subunits, so that three R6G molecules are bound by the AcrB trimer. Each binding site is composed of five amino acid residues (Phe²⁸⁶, Ala³⁸⁵, Leu²⁵, Val³⁸² and Lys²⁹) contributed by one subunit and Phe²⁸⁶ from the neighbouring subunit. Furthermore, the bound R6G molecules appear to interact with one another, stabilizing the binding sites. With the exception of Lys²⁹, all of the other amino acids in the binding pocket are hydrophobic, indicative of the importance of hydrophobic and van der Waals interactions with the bound R6G. EtBr binds at a position approx. 6 Å above the R6G-binding site. In common with R6G, one EtBr molecule is bound per subunit via Phe²⁸⁶ and Ala³⁸⁵, and also Phe²⁸⁶ from the neighbouring subunit. The phenyl groups of EtBr and Phe²⁸⁶ are in close contact, sandwiching the EtBr molecule between the two subunits. The fact that the three EtBr molecules in the binding site are adjacent to each other suggests that they are also likely to interact strongly with one another. Ciprofloxacin is bound between two of the AcrB subunits at a position higher in the cavity than EtBr, via interactions with Phe⁴⁵⁸, Phe⁴⁵⁹ and Lys²⁹ from one subunit and Lys²⁹, Leu²⁵ and Ala³⁸⁵ from the neighbouring subunit. Dequalinium binds to the central cavity above the EtBr, R6G and ciprofloxacin sites, towards the periplasm, via two acidic residues (Asp⁹⁹ and Asp¹⁰¹), as well as Ile¹⁰² that interacts with the quinolinium moiety.

In addition to identifying the specific residues of AcrB that interact with each drug, the study of Yu et al. [167] revealed a number of unexpected observations. First, the binding cavity of AcrB was much larger than expected, and it had the ability, at least theoretically, to bind multiple drugs simultaneously. Secondly, the binding of drugs did not alter the conformation of the AcrB binding site. Finally, hydrophobic interactions predominated in the binding of all four drugs. Although AcrB resembles QacR in its ability to bind multiple drugs, in other respects it differs.

For example, the conformation of the QacR binding site changes upon binding of the drug, and interaction with EtBr and R6G is mediated largely by electrostatic interactions. However, there is still much to be learnt about how the substrates are bound and translocated by AcrB. An important question that needs to be addressed is whether there are additional binding sites, such as at the cytoplasmic face of the transporter. The binding of drugs to these sites could trigger conformational changes that lead to drug translocation across the membrane, delivering the drug to the large central cavity towards the periplasmic side of the transporter. If this is the case, then the present crystal structures may represent the end-point for the translocation cycle. Alternatively, the crystal structures may represent the first step of the translocation cycle. If the drug is to pass through the narrow central pore that links the central cavity to the periplasmic side, it is highly likely that some kind of conformational change would be required. The crystal structure may represent the initial drug-binding step. Subsequent conformational changes may result in the opening of the pore, therefore providing a route for the drug to the periplasmic funnel. However, neither of these potential mechanisms explain the role of the large periplasmic loops of AcrB. In an earlier section the large periplasmic loops and their role in defining the substrate specificity of AcrB was discussed [30,31]. It was concluded by these authors that it is highly probable that these loops contain drug-binding sites. What role, if any, would these potential binding sites have? One possibility is that the periplasmic loops may sequester drugs directly from the periplasm before passing them on to the AcrA–TolC complex. Alternatively, they may play a part in a relay mechanism where drugs are transported up through AcrB to the periplasm via a number of different drug-binding sites. Thus far the precise mechanism of AcrB-mediated drug efflux remains elusive. Determination of the mechanism of AcrB may be resolved by structural comparisons between different substrate-induced conformations in a manner similar to those for EC-MsbA and VC-MsbA discussed above. A much better understanding of the mechanism would be yielded from crystallization of the AcrA–AcrB–TolC complex; however, this represents a considerable challenge.

MEASURING TRANSPORT AND SUBSTRATE INTERACTIONS

Overexpression, purification and reconstitution

Multidrug transporters have been traditionally studied in whole cells or as components of membrane vesicles derived from disrupted cells. While these systems are undoubtedly useful, problems can arise due to the presence of other proteins or through interference from cellular processes [3]. In order to fully characterize efflux proteins both structurally and biochemically, a number of methods have been developed that allow the purification and functional reconstitution of membrane proteins into liposomes. A number of steps can make reconstitution problematic. First, the membrane protein has to be successfully overexpressed; secondly, the protein has to be extracted from the membrane, usually through the use of a suitable detergent or in some cases organic solvents; and thirdly, the purified protein–detergent complex has to be functionally reconstituted in an active form. A number of advances have been made in relation to these potential problems. Membrane proteins overexpressed in conjunction with tightly regulated expression systems and specialized expression strains can now result in yields of 5–30% of total cell protein [3]. The commercial availability of novel biological detergents has facilitated improvements in membrane protein extraction from membrane preparations. However, detergent choice still remains a critical factor; some detergents can inactivate proteins irreversibly,

and others can affect the level of purity and yield [3]. Furthermore, detergents in some cases act as substrates of efflux transporters [168]. After successful overexpression and detergent extraction, membrane proteins can now be routinely purified to a high degree (> 90%) using affinity chromatography [5,8,9,165,168–171]. This commonly involves using recombinant DNA techniques that introduce specific affinity tags to the protein of interest. IMAC (immobilized metal affinity chromatography) was the initial purification step used in the successful crystallization of the *E. coli* transporters AcrB, EC-MsbA and BtuCD, as well as VC-MsbA [5,6,8,9]. Using recent advances in membrane overexpression and purification, an increasing number of efflux transporters and accessory proteins have been successfully purified and reconstituted; these include P-gp, MdfA, HorA, AcrA, AcrB, LmrP, LmrA, NorA, EmrE and SMR [26,95,168–175]. In each case the imposition of an artificial proton or Na⁺ gradient or the addition of ATP has shown that these transporters are active and have the ability to transport multiple drugs [26,95,168–175]. Importantly, the transport function of these transporters was not dependent on the presence of accessory proteins or cytosolic components.

Fluorescent substrates

Once a suitable assay system for the transporter of choice has been determined, it allows a number of biochemical analyses to be undertaken that can measure transport function. Fluorescent probes are tools that are often used in such studies. The anti-tumour drug doxorubicin and the dyes R6G and EtBr are good examples of compounds that exhibit different levels of intracellular and extracellular fluorescence, which occurs due to their interaction with cellular components. In the case of R6G and doxorubicin, their fluorescence decreases in the intracellular environment, whereas the opposite is true of EtBr. This simple phenomenon can be used to directly monitor the efflux of these compounds out of treated cells. When de-energized *E. coli* strains that can efflux doxorubicin are loaded with the drug, active efflux upon energization can be monitored over time as an increase in fluorescence [176]. A similar phenomenon occurs with R6G [176]. In cells resistant to EtBr, efflux can be monitored as a decrease in fluorescence over time [177]. Fluorescence changes with these compounds that are associated with active efflux can be abolished when the preloaded cells are mutant strains lacking specific efflux pumps, such as the *acrB* mutant *E. coli* KAM3. The inability of KAM3 or other efflux mutants to expel doxorubicin, R6G or EtBr is shown by fluorescence changes that are associated with passive diffusion only [176,178].

The dye Hoechst 33342, a substrate of a number of multidrug resistance pumps, is another fluorescent probe that is particularly useful in membrane transport studies [172,177,179–182]. Hoechst 33342 is strongly fluorescent in the membrane environment, but has low fluorescence in aqueous solutions. This ability has been used to characterize a number of MDR transporters, including P-gp, LmrA, LmrP, NorA, YycC, MdfA and HorA [82,165,172,174,175,180,183,184]. Transport of Hoechst 33342 in everted vesicles containing the *Staphylococcus aureus* MDR transporter NorA was saturable. Additionally, Hoechst 33342 transport was shown to be inhibited competitively by the presence of the known MDR inhibitor verapamil [172]. Using similar methods, LmrP-mediated transport of Hoechst 33342 was inhibited competitively by quinine and verapamil, non-competitively with nifedipine and vinblastine, and uncompetitively with TPP⁺ [180]. This finding strongly suggests that LmrP can interact with substrates via a number of different sites [180]. Many of the drugs that LmrP has been shown to transport are highly hydrophobic and will readily partition into the lipid bilayer.

Fluorescent substrates can also be used to indicate the site of interaction between efflux pumps and their substrates. Using the non-fluorescent compound BCECF-AM [2',7'-bis-(2-carboxy-ethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester], which partitions into the membrane bilayer, LmrP-overexpressing cells have been shown to efflux BCECF-AM from the cell before it is hydrolysed by cytoplasmic esterases into the fluorescent compound BCECF [185]. This provides evidence that LmrP transports drugs from the membrane and not from the cytoplasm [185]. The point of interaction between LmrP and transported substrate was localized to the inner-membrane leaflet of the bilayer using the fluorescent probe TMA-DPH [1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene iodide] [185]. This probe shares similar characteristics with Hoescht 33342, i.e. it is fluorescent in the membrane but not in aqueous environments. TMA-DPH partitioning into the membrane of *Lactococcus lactis* was shown to be a biphasic process. This behaviour was shown to be a reflection of the fast entry of TMA-DPH into the outer leaflet, followed by a slower transbilayer movement to the inner leaflet of the membrane [185]. The initial rate of TMA-DPH extrusion correlates with the amount of probe associated with the inner leaflet, providing direct evidence that the ability of amphiphilic substrates to partition into the inner leaflet of the membrane is a prerequisite for recognition by multidrug transporters [185].

Fluorescent probes

Fluorescent probes do not need to be transported substrates. Site-directed labelling with fluorescent probes directly on to the efflux protein can provide alternative strategies. These probes are particularly useful tools when used to monitor conformational changes. Extensive studies by Liu and Sharom [186] have used the thiol-specific probe MIANS [1-(4'-maleimidyl-anilino)napthalene-6-sulphonic acid] to label P-gp via the two cysteine residues within the Walker A motifs of the NBDs. The MIANS group displays saturable quenching in the presence of ATP, ADP and non-hydrolysable analogues such as p[NH]ppA [187]. Quenching of the MIANS label is most probably due to local conformational changes caused by nucleotide binding. MIANS-dependent fluorescence changes also occur in the presence of P-gp substrates, suggesting 'cross-talk' between the drug-binding domains and the NBDs [82]. Further evidence of cross-talk between the domains of P-gp was discussed in an earlier section [121]. MIANS-labelled P-gp also exhibits saturable quenching in the presence of P-gp substrates, and provides a useful tool that allows the determination of P-gp substrate affinities [82].

Intrinsic fluorescence

Proteins contain natural fluorophores, most notably the aromatic amino acid residues tryptophan and tyrosine. This phenomenon is known as intrinsic fluorescence (see [188] for a practical review of this topic). Tryptophan residues are specifically excited maximally by light with a wavelength of 295 nm, and emit light maximally at 340 nm. The excitation and emission maxima of tyrosine are 275 nm and 310 nm respectively. In most cases tryptophan fluorescence predominates. Intrinsic fluorescence can be used to monitor protein-ligand interactions when the fluorescence of the protein-ligand complex differs from that of the free protein or ligand. The binding of ligand to the protein often results in a conformational change within the protein, and fluorescence changes occur due to movements of the aromatic residues. Changes in the intrinsic fluorescence of proteins in the presence of ligands, if saturable, can provide a useful means

by which the dissociation constants of particular ligands can be calculated. In this respect, intrinsic fluorescence has been used successfully to monitor the interactions between P-gp, transported substrates and nucleotides such as ADP and ATP [80,82,189]. A major advantage of intrinsic fluorescence is that the native protein can be used; for example, MIANS-labelled P-gp, which has seemingly unaltered affinity for substrates and cofactors, is catalytically inactive [82]. Using intrinsic fluorescence and MIANS labelling techniques, Sharom and co-workers [82] have determined the K_d values for over 80 P-gp substrates, finding a range of values from 25 nM to 250 μ M. The ability of P-gp to bind such a wide range of substrates with differential binding activities over four orders of magnitude is a stark demonstration of its substrate promiscuity. Intrinsic fluorescence has also been used to measure the affinities of glycerol 2-phosphate, glycerol 3-phosphate and P_i towards the *E. coli* MF transporter GlpT [190]. As yet, the use of intrinsic fluorescence has not been applied to bacterial efflux transporters; this probably reflects difficulties in overexpression and purification, the unsuitability of the substrates for these assays, or a lack of suitable intrinsic fluorophores.

CIRCUMVENTING DRUG EXTRUSION

Efflux-mediated MDR is a serious and increasing problem. Clearly, the development of clinically useful inhibitors that decrease the effectiveness of efflux pumps would represent a significant advance in our ability to provide successful treatments of MDR-related conditions. The ability of certain compounds to modulate the activity of P-gp has been known for a number of years; this is a well studied field and has been reviewed extensively elsewhere [82,191–195]. As yet, only preliminary advances have been made towards the development of clinically useful compounds that reverse bacterial MDR.

Reversing P-gp-mediated MDR

Chemical modulators that inhibit the action of P-gp can be divided into three broad categories: (a) high-affinity substrates of the pump, (b) efficient inhibitors of transport coupled to ATP hydrolysis, and (c) partial substrates or inhibitors. These modulators can be further divided into seven classes related to their structural or functional features: (a) calcium or sodium channel blockers, (b) calmodulin agonists, (c) flavonoid or steroidal compounds, (d) protein kinase C inhibitors, (e) indole alkaloids, (f) cyclic peptides, (g) quinolines, (h) surfactants and (i) antibodies [192–194]. Generally, most modulators have some common features, e.g. a high level of hydrophobicity, or the presence of aromatic ring structures or amino side chains that are protonated at physiological pH [192]. However, some inhibitors of P-gp possess none of these characteristics [192]. Certain P-gp modulators have been put to clinical use due to their ability to chemosensitize cells to the presence of anticancer drugs [191]. The chemosensitization process involves the co-administration of a P-gp inhibitor (modulator) along with a substrate (in the clinical setting, this describes anticancer drugs) of P-gp. Impairment of P-gp function in the presence of the modulator results in increased accumulation of the anticancer drug and can reverse P-gp-mediated MDR [191].

The calcium channel blocker verapamil was among the first generation of MDR reversal agents to be characterized [196–201]. Verapamil was shown to enhance the accumulation of anticancer drugs in a wide range of cell lines. This property is shared with a number of clinically available calcium antagonists [191]. One of the most effective first-generation modulators is

the immunosuppressant cyclosporin, which, like verapamil, can effectively reverse MDR in a wide range of cell lines [202]. Commonly, first-generation modulators are extremely effective at reversal of MDR *in vitro*, but can pose problems when administered clinically. Both verapamil and cyclosporin A require high doses to become effective *in vivo* [191]. Unfortunately, the doses that provide effective P-gp modulation in humans are sufficiently high to cause life-threatening toxicities [191]. Typically, beneficial therapeutic effects on the cardiovascular system can be elicited by verapamil at blood plasma concentrations between 0.4 and 1.2 μM ; toxic effects are observed at higher concentrations that encompasses the concentrations of verapamil required to induce MDR modulation (2–6 μM) [203]. This has focused attention on the development of second-generation P-gp modulators that retain the MDR reversal properties of the initial modulators, but are significantly less toxic. Structural analogues of verapamil, including emopamil, gallopamil and Ro11-2933, were developed and shown to be effective MDR reversal agents with greatly decreased toxicity *in vivo* [204–206]. In a similar vein, the non-immunosuppressant analogue of cyclosporin A, PSC 833, exhibits superior levels of MDR reversal [207]. Even though these second-generation modulators possess greatly decreased toxicity when compared with their first-generation counterparts, they can produce deleterious effects *in vivo*. Second-generation inhibitors have been shown to alter the pharmacokinetic and distribution properties of co-administered anticancer drugs [191,208]. Typically, this has resulted in the toxic accumulation of anticancer drugs in blood plasma or in organs such as liver, kidney or intestine [191]. Often as a result of this phenomenon, there is a need to decrease the dose of the anticancer drug, and it is thought that in some cases this may adversely affect the outcome of therapy [209].

Clearly, modulators that are non-toxic, do not affect the pharmacokinetics/distribution of the co-administered drugs and can be targeted specifically towards P-gp are needed. In this respect, some advances using combinatorial chemistry methods have been achieved. These third-generation modulators have effective MDR reversal concentrations that are low (i.e. 20–100 nM). However, they are yet to be rigorously tested clinically, and whether they will encounter the problems of the first- and second-generation modulators remains to be seen [191]. Other chemicals from sources as diverse as green tea extracts to commercially available chemical libraries have been screened against cell lines expressing P-gp, yielding potential inhibitors [210,211], but none of these have been fully evaluated in the clinical setting. Thus far, co-administration of P-gp modulators and anticancer drugs has had limited clinical value [212,213].

Reversal of bacterial MDR

Initial progress towards the development of specific modulators of bacterial efflux has been made, but as yet the development of clinically useful compounds remains in its infancy. This emerging subject has been reviewed elsewhere [214,215]. Thus far, a number of potentially useful inhibitors of bacterial efflux pumps have been discovered. The P-gp MDR reversal agent verapamil inhibits a number of bacterial MDR transporters, including NorA from *S. aureus* and the lactococcal transporters LmrP and LmrA. Verapamil has also been shown to work synergistically with tobramycin against *Burkholderia cepacia*, a chronic infection in cystic fibrosis sufferers [61,172,180,216,217]. Semi-synthetic analogues of tetracycline have been shown to inhibit tetracycline efflux in *E. coli* [218], and inhibitors of NorA in *S. aureus* have been shown to potentiate the effects of ciprofloxacin [219–221]. MC-207,110, a broad-range inhibitor (produced

by Microcide Biochemicals), inhibits RND multidrug pumps of *Ps. aeruginosa*, *Enterobacteriaceae*, *Haemophilus influenza* and *Stenotrophomonas maltophilia*, potentiating the effects of fluoroquinolone antibiotics [222–224]. Plant-derived compounds have been shown to act synergistically with ciprofloxacin in *S. aureus* in manner thought to involve inhibition of efflux [225]. The development of similar clinically useful compounds may represent a significant advance in combating antimicrobial resistance.

Rational drug design – targeting efflux pumps

What are we to learn from the slow progress towards the development of clinically effective MDR reversal agents? Most obviously, it is clear that new approaches towards the design of MDR reversal agents are required. One possibility is the use of structural information to rationally design novel efflux pump inhibitors. Current methods that rely on screening a wide range of chemicals often appear, superficially at least, to lack rationale. When they do succeed, it often appears to be through 'brute force'. Structure-based inhibitors would represent a significant advance. However, this opportunity is limited by the scarce amount of structural data pertaining to efflux transporters. Initial structural studies are already revealing new insights into the function of multidrug efflux pumps. Structural determination of transporters with substrate bound, such as the studies carried out with AcrB, will indicate residues that interact with drugs and may allow the design of MDR reversal agents that block these interactions. AcrB is, however, an exception [167]. The structures of the ABC transporters BtuCD, EC-MsbA and VC-MsbA have been determined and have already provided a wealth of valuable information [6,8,9]. Unfortunately, they are not drug transporters and their relevance to clinically important efflux pumps such as P-gp is as yet undetermined. It could also be argued that structural information derived from crystals, while a difficult technical achievement, might only provide a snapshot of the transport mechanism, as such structures are unable to show the dynamic conformational changes involved in substrate translocation. This has been further highlighted by the structure of VC-MsbA, which revealed far more on the potential transport mechanism of EC-MsbA than EC-MsbA on its own [8,9]. Undoubtedly, a key breakthrough will be supplied by the structural determination of a wide range of MDR transporters, with both bound substrate and in a variety of conformations that represent different phases of the transport process.

Other potential targets for MDR reversal agents exist that do not involve the transport aspect of efflux. Transcriptional regulators often modulate the expression of efflux systems by monitoring the intracellular levels of the transported substrate by direct binding of the substrate [226,227]. In relation to efflux transporters, there is a far greater understanding of the structural interactions between regulators and their bound substrates [153,157,158,161]. It is possible to envisage a situation where MDR is modulated indirectly by agents that inhibit efflux pump expression by interaction with a specific regulator. In Gram-negative organisms, inhibitors that prevent the formation of tripartite efflux transporters may provide another target. Structural determination of AcrB and TolC will greatly increase this potential [29,141]. However, no high-resolution structure of a MFP is available; clearly, elucidation of such a structure is an imperative. There are recent reports that the expression of bacterial multidrug pumps is controlled by two-component signal transduction systems [228,229]; components of such systems might represent novel drug targets for modulating the drug resistance response of bacterial infections undergoing antibiotic treatment. Indeed, such

an approach, using quorum sensing inhibitors, has been used to attenuate the virulence of *Ps. aeruginosa*, by increasing their susceptibility to tobramycin [230].

Novel alternatives to rational drug design

Although rational drug design may lead to the development of new drugs, we must also look at new methods of empirical high-throughput screening to identify compounds that modulate the activity of multidrug pumps. Such an approach might involve the development of drug-binding biosensors, based upon proteins (i.e. components of multidrug pumps or the transcription factors that regulate the expression of these proteins) that are optically responsive to the binding of drugs. These could be used to quickly and cheaply screen chemical libraries for new drugs.

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