Interaction of antibacterial compounds with RND efflux pumps in *Pseudomonas aeruginosa*

Jürg Dreier

Department of Physics, University of Cagliari – Cittadella University, Monserrato, Italy

Paolo Ruggerone

Basilea Pharmaceutica International Ltd., Basel, Switzerland

*Correspondence*

Jürg Dreier

Basilea Pharmaceutica International Ltd., Grenzacherstrasse 487, P.O. Box 4005, Basel, Switzerland

juerg.dreier@basilea.com

**Keywords:** efflux, multi-drug resistance, bacteria, RND, substrate recognition, *Pseudomonas aeruginosa*, antibiotic agents, efflux pump inhibitors

**Introduction**

Infectious diseases, including bacterial infections, are among the major causes of mortality worldwide (Mason et al., 2003). Infections by multi-drug resistant (MDR) pathogens are especially difficult to treat and are recognized as a major threat (CDC, 2013; Denys and Relich, 2014; Martinez and Baquero, 2014; ECDC, 2015).

The Gram-negative bacterium *Pseudomonas aeruginosa* is frequently involved in healthcare-associated infections like pneumonia, bloodstream infections, urinary tract infections, and surgical site infections (Hidron et al., 2008; Jones et al., 2009; Zhanel et al., 2010). About 8% of nosocomial infections reported to the Centers for Disease Control and Prevention in the US are ascribed to *P. aeruginosa*. 13% of the severe cases in the US are caused by MDR isolates and 14% of European isolates reported between 2005 and 2013 had combined resistance to several antibiotics (CDC, 2013; ECDC, 2015). MDR phenotypes have been described for clinical isolates from various places all over the world (Potron et al., 2015). MDR *P. aeruginosa* isolates are sometimes resistant to nearly all classes of antibiotics and have lost susceptibility toward fluoroquinolones, aminoglycosides,
cephalosporins, and carbapenems (Livermore, 2009; Riou et al., 2010; Poole, 2011; Castanheira et al., 2014).

The outer membrane (OM) of Gram-negative bacteria has an asymmetric structure including an outer leaflet made of lipopolysaccharides (LPS), an inner phospholipid leaflet, and porin channels (Kamio and Nikaido, 1976; Nikaido, 2003). Small, hydrophilic compounds can pass the OM by diffusion through the porin channels whereas large and/or hydrophobic compounds have to go through the lipid bilayer (Hancock, 1997; Delcour, 2009). In the Escherichia coli OM, there are trimeric porins like OmpF and OmpC present that allow a relatively rapid diffusion of small, hydrophilic substances (Nikaido and Rosenberg, 1983; Cowan et al., 1992; Schulz, 1993). P. aeruginosa does not make such trimeric porins but expresses the monomeric porin OprF at a low number with a small opening that only allows slow permeation (Angus et al., 1982; Yoshimura and Nikaido, 1982; Sugawara et al., 2006, 2010). P. aeruginosa has also specific channels such as OprD for basic amino acids and peptides, which is the main entry passage of carbapenem antibiotics (Nikaido, 2003). The structure of the OM can be adapted by which is the main entry passage of carbapenem antibiotics (Nikaido, 2003). The expression of RND pumps is regulated as a response to external stress factors such as reactive oxygen species (MexAB-OprM, MexXY-OprM), reactive nitrogen species (MexEF-OprN), and other agents imposing stress to the bacterial cell like membrane damaging agents (MexCD-OprJ) or ribosome blocking substances (MexXY-OprM), which affects fluoroquinolone export (Fukuda et al., 1990, 1995; Davin-Regli et al., 2008; Nishino et al., 2009; Castanheira et al., 2014).

Increased pump expression can be linked to decreased porin expression as for example in the nfxC mutants of P. aeruginosa. In these mutants OprD expression is downregulated, which impairs carbapenem uptake and MexEF-OprN expression is upregulated, which affects fluoroquinolone export (Fukuda et al., 1990, 1995; Davin-Regli et al., 2008; Nishino et al., 2009; Castanheira et al., 2014).

One possibility to circumvent efflux is the development of antibiotics that are not pump substrates, or are only poorly affected by pump activity (e.g., Hayashi et al., 2014). Alternatively, one may look for molecules that inhibit pumps and can be used as adjuvants in combination with antibiotics (e.g., Olivares et al., 2013). For both strategies it is crucial to understand the molecular properties that define pump substrates. This review describes experimental procedures that are sensitive to RND efflux pump activity and allow conclusions on substrate recognition by RND efflux pumps. The described methods include specifically designed efflux assays but also assays which were developed for other purposes than efflux studies but revealed substrates of efflux pumps (e.g., probes for membrane integrity have been found to be efflux pump substrates). Substrate recognition by MexB from P. aeruginosa can often not (or not yet) be investigated with the methods described for AcrB in E. coli but can be modeled with in silico methods based on experimental data. Specific data about substrate recognition by MexB from P. aeruginosa are still limited. Therefore we discuss in detail results obtained also for AcrB when they describe similarities between the two RND transporters. It is paradigmatic that to date and to our knowledge there is only a single computational study addressing the molecular aspects of MexB-substrate interactions.

Impact of Efflux on Antibiotic Activity

A direct impact of efflux on antibiotic activity on P. aeruginosa was shown for a core set of RND pumps by efflux-pump deletion mutants and could be confirmed by mutants that overexpress selected RND systems (Li et al., 1995; Lomovskaya et al., 1999; Masuda et al., 2000a; Poole, 2000, 2004, 2005, 2013; Schweizer, 2003). Susceptibility of P. aeruginosa towards many antibiotics has been restored when the four systems that are most relevant for antibiotic resistance (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM) have been deleted (Morita et al., 2001; Kumar et al., 2006). These P. aeruginosa RND pumps have overlapping but not identical substrate ranges as mentioned in the Section Introduction. RND efflux pumps have individual substrate specificities that include amphiphilic molecules (e.g., MexB) but also hydrophobic solutes (e.g., MexB, MexD) and the hydrophilic polycationic aminoglycosides (MexY), described to enter cells by a self-promoted mechanism (Hancock, 1997).
| Compound          | logP (o/w) | TPSA (Å²) | MW (Da) | PC+ | PC− | Net PC | Properties                                      |
|-------------------|-----------|-----------|---------|-----|-----|--------|------------------------------------------------|
| **Inhibitors**    |           |           |         |     |     |        |                                                 |
| D13-9001          | 0.85      | 210.8     | 693.8   | 9.96| −9.96| 0      | Not transported                                  |
| PAßN              | 2.65      | 149.5     | 448.6   | 9.99| −7.99| 2      | Transported                                      |
| Mefloquin         | 4.27      | 49.7      | 379.3   | 6.13| −5.13| 1      | Quinolone                                        |
| **Substrates acting as inhibitors** | | | | | | | |
| Minoycline        | −0.28     | 164.6     | 457.5   | 7.11| −7.11| 0      | Antibiotic                                       |
| Trimethoprim      | 2.65      | 106.8     | 291.3   | 5.27| −4.28| 1      | Antibiotic                                       |
| Taurocolatide     | 3.33      | 141.0     | 514.7   | 4.79| −5.79| −1     | Bile acid                                        |
| Erythromycin      | 2.76      | 156.1     | 734.9   | 9.73| −8.73| 1      | Antibiotic                                       |
| Glycolocystate    | 3.15      | 129.9     | 464.6   | 4.14| −5.14| −1     | Bile acid                                        |
| **Substrates**    |           |           |         |     |     |        |                                                 |
| Ceftobiprole      | −1.29     | 213.7     | 533.6   | 9.53| −10.5| −1     | Antibiotic                                       |
| Aztreonam         | −1.03     | 201.3     | 433.4   | 7.09| −9.10| −2     | Antibiotic                                       |
| Floxofor          | −0.96     | 171.8     | 495.5   | 7.34| −8.34| −1     | Antibiotic                                       |
| Cefazolin         | −0.73     | 158.9     | 453.5   | 5.65| −6.65| −1     | Antibiotic                                       |
| Meropenem         | −0.47     | 117.6     | 383.5   | 6.09| −6.09| 0      | Antibiotic                                       |
| FDG               | −0.40     | 234.3     | 656.6   | 10.38| −10.4| 0      | Intracellular conversion into a fluorescent product |
| Tigecycline       | −0.28     | 201.3     | 586.7   | 9.99| −9.99| 1      | Antibiotic                                       |
| Cefamandole       | −0.18     | 153.4     | 461.5   | 6.47| −7.47| −1     | Antibiotic                                       |
| (++)-Cerulenin    | 0.10      | 72.7      | 223.3   | 3.39| −3.39| 0      | Antibiotic                                       |
| Cefalotin         | 0.21      | 187.7     | 531.5   | 9.08| −10.1| −1     | Antibiotic                                       |
| Gemifloxacin      | 0.36      | 125.8     | 389.4   | 6.80| −6.81| 0      | Antibiotic                                       |
| Doxorubicin       | 0.51      | 207.7     | 544.5   | 10.38| −10.4| 0      | Intracellular conversion into a fluorescent product |
| Cephaparin        | 0.58      | 115.8     | 395.4   | 5.48| −5.48| 0      | Antibiotic                                       |
| Sulbenicillin     | 0.59      | 129.9     | 395.4   | 5.48| −5.48| 0      | Antibiotic                                       |
| Dodecyl-α-D-maltoside | 0.70   | 178.5     | 510.6   | 7.09| −7.09| −2     | Antibiotic                                       |
| Levofloxacin      | 0.80      | 76.15     | 360.4   | 4.57| −5.58| −1     | Antibiotic                                       |
| Norfloxacin       | 0.85      | 80.3      | 319.3   | 5.46| −5.46| 0      | Antibiotic                                       |
| Sulbenicillin     | 0.59      | 129.9     | 395.4   | 5.48| −5.48| 0      | Antibiotic                                       |
| Triclosan         | 0.46      | 115.8     | 395.4   | 5.48| −5.48| 0      | Antibiotic                                       |
| Moxalactam        | 0.38      | 212.0     | 518.5   | 7.60| −7.60| 0      | Antibiotic                                       |
| Moxifloxacin      | 2.01      | 89.5      | 401.4   | 5.87| −5.87| 0      | Antibiotic                                       |
| Thiolactomycin    | 2.41      | 93.4      | 415.5   | 6.14| −6.14| 0      | Antibiotic                                       |
| Cloxacillin       | 2.67      | 115.6     | 434.9   | 4.96| −5.96| −1     | Antibiotic                                       |
| Proflavin         | 2.80      | 66.2      | 210.3   | 4.03| −3.03| 1      | Fluorescent probe                                |
| Pyronin Y         | 2.98      | 15.5      | 267.4   | 3.14| −2.14| 1      | Fluorescent Probe, quenched intracellular fluorescence |
| Nitrocyn          | 3.02      | 181.2     | 515.5   | 7.20| −8.20| −1     | Antibiotic                                       |
| Clarithromycin    | 3.12      | 184.1     | 749.0   | 9.61| −9.61| 1      | Antibiotic                                       |
| Acriflavine       | 3.19      | 55.9      | 224.3   | 4.06| −3.06| 1      | Fluorescent Probe, quenched intracellular fluorescence |
| Leu-NAP           | 3.29      | 56.7      | 257.4   | 4.02| −3.02| 1      | Intracellular conversion into a fluorescent product |
| Novobiocin        | 3.30      | 198.9     | 611.6   | 8.03| −9.04| −1     | Antibiotic                                       |
| ANS               | 3.35      | 63.2      | 298.3   | 3.71| −4.71| −1     | Fluorescent Probe, enhanced intracellular fluorescence |
| Nile red          | 3.71      | 41.9      | 318.4   | 3.38| −3.38| 0      | Fluorescent Probe, enhanced intracellular fluorescence |
| Trehalose         | 3.84      | 173.1     | 813.0   | 9.91| −8.91| 1      | Antibiotic                                       |
| Fluorescin        | 4.13      | 76.0      | 323.2   | 4.16| −4.16| 0      | Fluorescent probe                                |
| DASPEI            | 4.38      | 7.12      | 253.4   | 3.45| −2.45| 1      | Fluorescent Probe, enhanced intracellular fluorescence |

(Continued)
TABLE 1 | Continued

| Compound             | logP (o/w) | TPSA (Å²) | MW (Da) | PC<sup>+</sup> | PC<sup>−</sup> | Net PC | Properties                                |
|----------------------|------------|-----------|---------|----------------|--------------|--------|-------------------------------------------|
| Hoechst H33342       | 4.47       | 74.3      | 453.6   | 6.09           | −5.09        | 1      | Fluorescent Probe, enhanced intracellular fluorescence |
| NPN                  | 4.51       | 12.0      | 219.3   | 2.40           | −2.40        | 0      | Fluorescent Probe, enhanced intracellular fluorescence |
| Rifampicin           | 4.56       | 221.4     | 824.0   | 11.1           | −10.1        | 1      | Antibiotic                                   |
| Ethidium             | 5.20       | 55.9      | 314.4   | 4.66           | −3.66        | 1      | Fluorescent Probe, enhanced intracellular fluorescence |
| TMA-DPH              | 5.29       | 0.0       | 290.4   | 4.18           | −3.18        | 1      | Fluorescent Probe, enhanced intracellular fluorescence |
| Rhodamine 6G         | 5.76       | 59.9      | 442.6   | 4.23           | −4.24        | 0      | Fluorescent probe                           |
| 1,2′-dinaphthylamine | 5.77       | 12.0      | 269.3   | 2.70           | −2.70        | 0      | Fluorescent Probe, enhanced intracellular fluorescence |
| DiOC<sub>2</sub>(3)  | 6.06       | 29.5      | 333.4   | 3.79           | −2.79        | 1      | Fluorescent Probe, enhanced intracellular fluorescence |

Other

| Imipenem             | −0.69      | 118.3     | 299.4   | 5.50           | −5.50        | 0      | Antibiotic, not a substrate                |
| Resorufin            | 1.49       | 58.9      | 213.2   | 2.75           | −2.75        | 0      | Fluorescent probe                           |
| Optochin             | 3.75       | 46.8      | 341.5   | 4.36           | −3.37        | 1      | Quinolone                                  |

Descriptors were calculated for pH 7 using Molecular Operating Environment (MOE), 2013.08; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2014.

<sup>a</sup>Logarithm of partition coefficient between n-octanol and water (o/w); <sup>b</sup>Total Polar Surface Area; <sup>c</sup>Molecular Weight; <sup>d</sup>Partial positive charge; <sup>e</sup>Partial negative Charge; <sup>f</sup>Net Partial Charge.

<sup>g</sup>Examples for which efflux inhibition was shown are listed. Other substrates may act as inhibitors too.

Note that most antibiotics are amphiphilic compounds with hydrophobic parts that are required to partition into a membrane but that there are considerable differences with regard to the impact of efflux on activity even within classes of antibiotics (Hancock, 1997; Nikaido, 2003; Delcour, 2009; Brown et al., 2010).

An effort to make tetracyclines that are not substrates of tetracycline-specific pumps of the major facilitator superfamily (MFS), led to the development of tigecycline (Chopra, 2002). However, tigecycline was still a substrate of the RND pumps MexY, MexB, and MexD in <i>P. aeruginosa</i> (Dean et al., 2003; Visalli et al., 2003; Chollet et al., 2004). This observation illustrates the flexibility of RND efflux pumps but it does not mean that a given RND transporter would accept all antibiotics of the same class. The macrolides erythromycin and clarithromycin mean that a given RND transporter would accept all antibiotics of the same class. The macrolides erythromycin and clarithromycin have been found to be better substrates of AcrB of <i>E. coli</i> and of <i>Enterobacter aerogenes</i> than the ketolide telithromycin (Dean et al., 2003; Visalli et al., 2003; Chollet et al., 2004). RND pumps in <i>P. aeruginosa</i> have been shown by in vitro susceptibility studies to transport substrates of the quinoline family with differential preference (Li et al., 1995; Kohler et al., 1997; Lomovskaya et al., 1999; Masuda et al., 2000a; Griffith et al., 2006; Dunham et al., 2010; Morita et al., 2015). Fluoroquinolones like levofloxacin, ciprofloxacin, norfloxacin, and others, have been reported to be most efficiently exported by MexEF-OprN and MexCD-OprJ and with less efficiency by MexAB-OprM and MexXY-OprM (or OprA). Non-fluorinated quinolones (e.g., nalidixic acid, piromidic acid, pipemidic acid, cinoxacin, oxolinic acid, and flumequine) have been shown to be preferentially transported by MexEF-OprN, less efficiently by MexCD-OprJ and MexAB-OprM, and least efficiently by MexXY-OprM (Kohler et al., 1997; Masuda et al., 2000b). It has been concluded from these results that fluoroquinolones with a positive charge and an electronegative fluorine atom are predominantly recognized by MexCD-OprJ, whereas quinolones without the fluorine atom are preferred substrates of MexEF-OprN. This result was in agreement with earlier work, suggesting that MexCD-OprJ expels amphiphilic substrates with a positive charge that can partition into a membrane (Masuda et al., 1996; Nikaido, 1996).

Studies on β-lactam efflux by AcrB in <i>Salmonella enterica</i> serovar Typhimurium have indicated that RND efflux pumps somehow recognize the hydrophobicity of a substrate (Nikaido et al., 1998; Ferreira and Kiralj, 2004). The studies showed that efflux efficiency varied with the lipophilicity of the β-lactam side chain and that molecules with more hydrophobic side chains were preferred substrates. Further work with β-lactams shed light on substrate specificity among RND pumps in <i>P. aeruginosa</i>. MexB transported a broad spectrum of β-lactams including penicillins, cephems, and carbapenems but not imipenem (Masuda et al., 2000b, 2001; Baum et al., 2009). MexD had a slightly narrower spectrum, which excluded the MexB substrates carbenicillin, sulbenicillin, ceftazidime, and moxalactam (Li et al., 1994; Masuda et al., 2000b). MexY had a yet narrower spectrum, which further excluded cefsulodin, flomoxef, and aztreonam (Masuda et al., 2000b; Hocquet et al., 2006). As carbenicillin and sulbenicillin have a negative charge that the other β-lactams in theses studies do not have, it seems that MexB accepts substrates with negative charges that MexD and MexY do not recognize. The finding that certain variants of MexD had altered substrate specificity has suggested that the recognition likely takes place via electrostatic interactions. MexD Q45K and MexD E89K with additional positively charged lysine residues have conferred resistance to the negatively charged carbenicillin, aztreonam, and ceftazidime (Mao et al., 2002). A role of charged residues in substrate recognition has been shown for AcrD too based on the fact that the binding pocket of AcrD contains an arginine residue (R625) not found in the mostly hydrophobic pocket of AcrB. Resistance to negatively charged β-lactams increased significantly when an arginine residue was introduced at the corresponding position in AcrB (I626R). Removal of a glutamic acid residue from the AcrB pocket (E673G), a residue not present at this position in AcrD, further enhanced the protective
effect and helped to explain the specificity of AcrD for small hydrophilic substrates such as the anionic β-lactams carbenicillin, aztreonam, and sulbenicillin (Wehmeier et al., 2009; Kobayashi et al., 2014).

Carbapenem activity was not uniformly affected by efflux pumps either. Meropenem was sensitive to overexpression of MexAB-OprM, MexCD-OprJ, or MexXY-OprM, whereas imipenem was not significantly affected (Masuda et al., 2000b; Ong et al., 2007; Riera et al., 2011; Castanheira et al., 2014). Efflux-pump expression levels in clinical isolates of *P. aeruginosa* indicated that MexAB-OprM and MexXY-OprM were most relevant for meropenem activity, followed by MexEF-OprN and with the lowest impact by MexCD-OprJ (Pai et al., 2001; Giske et al., 2005; Riera et al., 2011). A computer simulation of imipenem and meropenem transport by MexB suggested that the hydrophobicity and the flexibility of the side chains were probably responsible for efflux sensitivity (Collu et al., 2012b). The study indicated that the more rigid and hydrophobic tail of meropenem made strong interactions with the hydrophobic lining of the distal binding pocket in MexB (see Figure 1 for the structure of the transporter with two affinity sites) whereas the flexible and more hydrophilic tail of imipenem prevented this interaction (a detailed description of the extrusion pathway is given in the Section *Computational Study*). The computational results have further suggested that imipenem had no pronounced affinity for any particular site in MexB.

The anti-MRSA cephalosporin ceftobiprole is another β-lactam that is poorly recognized by MexB, MexD, and MexF in *P. aeruginosa* (Baum et al., 2009). Overexpression of these efflux pumps had only the marginal effect on the activity of ceftobiprole of a twofold increase of the minimum inhibitory concentration (MIC). Overexpression of MexXY caused a fourfold to eightfold increase of the MIC. The data have indicated that ceftobiprole was more efficiently transported by MexY than by MexB (Baum et al., 2009).

It should be pointed out that not all efflux substrates can be reliably detected by MIC. MIC may be a misleading indicator for efflux for any antibacterial that is effective at a concentration below the concentration required to reach maximal efflux velocity $V_{\text{max}}$. For example, AcrB had only a minor effect on the activity of cefaloridine (Nikaido et al., 1998; Mazzariol et al., 2000). Cefaloridine was later shown to be strongly effluxed by AcrB only at concentrations higher than those required to kill bacteria (Nagano and Nikaido, 2009). The discrepancy was explained by the finding that cefaloridine was effluxed with a high $V_{\text{max}}$ but also with a strong positive cooperativity. The cefaloridine concentration required to reach half-maximal velocity was much higher than the effective antibacterial dose (Nikaido and Normark, 1987; Nagano and Nikaido, 2009). Note that cefaloridine may not be an exception because other drugs (cephalotin, cefamandole) were also transported in a cooperative manner (Nagano and Nikaido, 2009). The situation may be

![FIGURE 1 | Binding pockets of MexB [PDB code: 2V50 (Sennhauser et al., 2009)]. The monomers L, T, and O are in tube representation and colored in blue, red, and green, respectively. In (A,B) side and top views of MexB with the distal binding pocket depicted as surface, in (C,D) the access binding pocket is highlighted as surface. In (E,F) the side and top views of L monomer are shown with access and distal binding pocket colored as blue and yellow surfaces, respectively. All the atomic-level figures were rendered using Visual Molecular Dynamics (VMD; Humphrey et al., 1996).](#)
different in *P. aeruginosa* because its OM has a lower permeability for β-lactams than the OM of *E. coli* (as explained before). This lower permeability leads to higher MIC and to a stronger impact of efflux than in *E. coli* and thus, less discrepancy between efflux studies and MIC data may be expected.

MIC tests in the presence of the model efflux-pump inhibitor (EPI) phenylalanine-arginine β-naphthylamide (PAβN) are often done as a quick check for efflux effects (e.g., Mesaros et al., 2007; Pages et al., 2009; Castanheira et al., 2014). Increased antimicrobial activity (i.e., lower MIC) of an antibiotic in the presence of PAβN is taken as an indication that the test compound is an efflux substrate. However, detailed studies with PAβN nicely illustrated the complexity of effects that can influence whole cell assays such as MIC determinations. It has already been mentioned in the original publication of PAβN that this agent does not only inhibit efflux pumps but also permeabilizes the OM of Gram-negative bacteria (Lomovskaya et al., 2001). This permeabilization property has been confirmed later in separate studies (Iino et al., 2012b; Lamers et al., 2013). Permeabilization of the OM may be mistaken as pump inhibition because facilitated entry of test compounds leads to increased intracellular levels. Pump inhibition and membrane permeabilization by PAβN have been shown to be dose-dependent, separable activities in *E. coli* because AcrB and AcrF have been specifically inhibited at low doses of PAβN, whereas membrane destabilization has been observed at higher concentrations of PAβN (Misra et al., 2015).

The protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) is often used in a similar way as PAβN to probe for efflux phenomena. RND pumps belong to the class of secondary transporters, which are driven by a transmembrane electrochemical potential of protons (reviewed in Borges-Walmsley et al., 2003; Poole, 2005; Dreier, 2007). CCCP indirectly inhibits secondary efflux pumps by dissipating the proton gradient across the inner membrane (Rosen and Kashket, 1978; Levy, 1992). It should be clear that whole cell assays require thorough controls to avoid confusion of pump inhibition with other effects. Susceptibility tests are useful to investigate general substrate recognition patterns whereas elucidation of detailed efflux mechanisms requires specifically designed assays.

**Efflux Assays**

Transport studies generally require at least two compartments and a method to measure concentration changes of a tracer substance. Entire RND efflux systems require three compartments (cytosol, periplasm, and cell exterior), which makes whole bacteria an obvious choice for efflux assays. In principle, cells can be incubated with any pump substrate and efflux can be measured by following the change of substrate concentration inside or outside of the bacteria (e.g., Kohler et al., 1997; Pumbwe and Piddock, 2000). Such a generic method may require the separation of intracellular from extracellular substrate, usually achieved by filtration or centrifugation. Cell lysis may be necessary too before a versatile detection method like liquid chromatography–mass spectrometry (LC–MS) or high performance liquid chromatography (HPLC) can be applied (e.g., Schumacher et al., 2007; Cai et al., 2009). Homogeneous assays use methods that allow substrate quantification without a separation step, which simplifies applications like time-course transport studies or high-throughput tests. Fluorescence was selected as the method of choice to assess fluoroquinolone accumulation in Gram-negative bacteria based on a comparison of various methods (Mortimer and Piddock, 1991). The study showed that steady-state concentrations and times to reach the steady-state condition varied in a substrate-specific manner within the quinolone family.

Many protocols use molecules whose fluorescent properties are sensitive to their environment and change upon entry into a cell (Figures 2A, B). A prominent example is ethidium bromide (EtBr) since the quantum yield of the fluorescence increases when ethidium intercalates into DNA (LePecq and Paoletti, 1967; Mine et al., 1999; Morita et al., 2001; Li et al., 2003). Active efflux causes reduced intracellular levels of EtBr and as a consequence a decrease of fluorescence whereas inhibition of efflux pumps is recorded as a signal increase (Figure 2A). The same assay principle (Figure 2A) applies to 1-anilinonaphthalene-8-sulphonate (ANS) accumulation because the fluorescence quantum yield increases when ANS binds to hydrophobic structures (e.g., proteins, membranes) in the cell. ANS is a substrate of MexD and has been used to study this transporter in *P. aeruginosa* (Walmsley et al., 1994; Mao et al., 2002; Kamal et al., 2013). Another example is 1,2-dinaphthylamine which has been used to measure AcrB efflux in *E. coli* because it is a substrate of AcrB and becomes strongly fluorescent when it partitions into a phospholipid bilayer (Bohnert et al., 2011a).

A widely used fluorophore with increased intracellular fluorescence is the bis-benzamidine dye Hoechst H33342 which becomes more fluorescent upon binding to DNA (Loontiens et al., 1990). H33342 was successfully used for accumulation studies with several Gram-positive bacteria, Enterobacteraeae, and Acinetobacter baumannii (van den Berg van Saparoea et al., 2005; Garvey and Piddock, 2008; Richmond et al., 2013). Transport of H33342 by intact *P. aeruginosa* has been difficult to measure because of the low OM permeability for such hydrophobic dyes (Loh et al., 1984; Ockatan et al., 1997; Germ et al., 1999). However, MexB readily transported H33342 when it was expressed in an *E. coli* host (Welch et al., 2010; Ohene-Agyei et al., 2012). This example shows that whole cell assays reflect a sum of events, which often need to be dissected to identify unambiguously substrates of a given efflux pump.

Another group of efflux probes allows efflux measurements based on a signal decrease upon intracellular accumulation (Figure 2B; e.g., Mao et al., 2002; Nakashima et al., 2013). Examples are the anthracycline cancer drug doxorubicin, whose fluorescence is quenched when the drug is internalized, Pyronin Y, whose intracellular fluorescence is quenched because of binding to RNA (Nishino and Yamaguchi, 2002; Nakashima et al., 2013), and acriflavine, whose fluorescence intensity decreases when it is bound to DNA (Chen et al., 2002). Pyronin Y has been successfully used for transport studies with MexD in *P. aeruginosa* (Mao et al., 2002).
Intracellular conversion of a substrate into a traceable entity is another elegant way to develop homogeneous efflux assays (Figure 2C). The non-fluorescent, blue-colored resazurin (sold under the trade name Alamar Blue®) is reduced to the fluorescent, pink-colored resorufin in live cells by oxidoreductases (Gonzalez and Tarloff, 2001; Rampersad, 2012). The conversion is an indicator for the presence of NADH but also for NADPH and FADH and is routinely used to detect living cells. Resazurin can be used to monitor bacterial growth because conversion rates depend on cell density. However, the method does not work equally well with all microorganisms and is not recommended for *P. aeruginosa*. It was shown that resazurin is a substrate of MexB in *P. aeruginosa* and of AcrB in *E. coli* and it was suggested that efflux pump activity in *P. aeruginosa* may account for the difficulties encountered with resazurin (Vidal-Aroca et al., 2009). Vidal-Aroca et al. (2009) showed that under conditions where *Pseudomonas* do not grow (i.e., in phosphate buffered saline), resazurin can be used to measure efflux pump activity. The assay was useful to identify meliophome as an inhibitor of MexB and AcrB and to show that optochin, another quinoline derivative, was not an inhibitor of these pumps.

The non-fluorescent alanine-β-naphthylamide ( Ala-NAP) and leucine-β-naphthylamide (Leu-NAP) are enzymatically converted to the highly fluorescent β-naphthylamide by cellular aminopeptidases (Marks et al., 1981; Butler et al., 1993). Assays for RND efflux were developed with Ala-NAP and Leu-NAP and used for the development of peptidomimetic EPI including the model EPI PAβN (Lomovskaya et al., 2001; Mao et al., 2002).

Fluorescein-di-β-D-galactopyranoside (FDG) is hydrolyzed in the cytoplasm of *E. coli* by β-D-galactosidase to produce fluorescein (Russo-Marie et al., 1993; Fieldler and Hinz, 1994; Yang and Hu, 2004). Both FDG and fluorescein were shown to be efflux-pump substrates and could be used to develop transport assays with *E. coli* cells expressing the pseudomonal MexAB-OprM and MexXY-OprM efflux systems (Matsumoto et al., 2011). Intracellular conversion of FDG into fluorescein could even be followed in single-cell mode with a microfluidic device using *E. coli* (Matsumoto et al., 2011; Iino et al., 2012b). FDG cannot be hydrolyzed by *P. aeruginosa* but FDG was useful to study MexB and MexY when they were expressed in an *E. coli* host (Iino et al., 2013). Another approach made use of femtoliter droplet arrays to monitor FDG based efflux in single *E. coli* cells (Iino et al., 2012a, 2013).

The following paragraphs describe fluorescent dyes, which were used as probes for membrane integrity or membrane potential and were later found to be substrates of efflux pumps. The physicochemical properties of these fluorescent dyes provide information on substrate recognition by efflux pumps irrespective of the original use of the probe.

The lipophilic membrane partitioning dye *N*-phenyl-1-naphthylamine (NPN) is useful as a probe for membrane integrity because the amount of NPN that can partition into the phospholipid membrane and hence the fluorescence signal increases when the structure of the OM is disturbed (Loh et al., 1984; Vaara, 1992; Lomovskaya et al., 2001). NPN was shown to be a substrate of AcrB in *E. coli* and of MexB in *P. aeruginosa* (Sedgwick and Bragg, 1996; Ocaktan et al., 1997; Lomovskaya et al., 2001; Murakami et al., 2004; Cai et al., 2009).

Fluorescent potentiometric probes such as the anionic oxonols and the cationic carbocyanines and rhodamines are useful to measure changes of bacterial membrane potentials (Shapiro, 2000). The overall fluorescence signal of these so-called slow-response dyes depends on the transmembrane distribution, which is sensitive to changes of the electrochemical potential across the membrane. Rhodamine measurements in *P. aeruginosa* were sensitive to the expression of the RND pumps MexAB-OprM, MexCD-OprJ, and MexHI-OpmD (Morita et al., 2001).
impressive turnover rate of 500 s$^{-1}$ by MexB has been shown in whole cells. Efflux rates decreased when AcrB was deleted (Xu et al., 2003; Viveiros et al., 2008; Paixao et al., 2009). EtBr transport in E. coli was estimated by immunoblotting methods (Ocaktan et al., 2004; Cai et al., 2009). The properties of the dyes that have been identified as substrates of MexB are listed in Table 1.

Substrate export can be directly followed from pre-loaded cells. Alternatively, pump activity can be deduced from intracellular accumulation of externally added substrates, which requires less preparation but reflects efflux in an indirect manner only and includes the hurdle of cellular uptake. Direct efflux measurements require pre-loading of cells with substrate. This extra complication pays off because efflux-related effects can be distinguished from permeation phenomena. The lipophilic dye Nile Red is basically non-fluorescent in aqueous solutions but becomes fluorescent in intracellular, non-polar environments (Greenspan and Fowler, 1985). Nile Red was used for efflux measurements of AcrAB-ToIC in E. coli (Greenspan and Fowler, 1985; Bohnert et al., 2010, 2011b). When E. coli cells were concomitantly loaded with substrate and inhibitor for competition studies, the strongest inhibition was seen with doxorubicin and minocycline, comparable to the effect of PAβN. Many other antibiotics caused slower Nile Red efflux, which is indicative of competitive transport. These results provided further evidence for specific substrate-pump interactions and corroborated the notion of substrate-specific inhibitor efficacy. Adaptation of the Nile Red assay for the use with P. aeruginosa would most likely not be straightforward because Nile Red was shown to stain extracellular rhamnolipids made by P. aeruginosa (Morris et al., 2011). Strains with deletions of rhamnolipid synthesis genes (rhlAB) may be helpful even if the swarming motility patterns influenced by rhamnolipids would be changed (Deziel et al., 2003; Caiazza et al., 2005). However, pre-loading of P. aeruginosa has been achieved with TMA-DPH (Ocaktan et al., 1997).

Several other dyes (doxorubicin, rhodamin for YhiUV, NPN, 1,2′-dinaphthylamine and DASPEI for AcrB) have been used to load E. coli for efflux assays (Lomovskaya et al., 2001; Nishino and Yamaguchi, 2002; Bohnert et al., 2011a).

Quantitative measurement of substrate transport is crucial for the characterization of efflux pumps. Kinetic analysis of EtBr transport in E. coli has shown that uptake rates increased and efflux rates decreased when AcrB was deleted (Xu et al., 2003; Viveiros et al., 2008; Paixao et al., 2009). EtBr transport by MexB has been shown in whole P. aeruginosa and an impressive turnover rate of 500 s$^{-1}$ has been determined for MexB in EtBr efflux assays when the number of pumps was estimated by immunoblotting methods (Ocaktan et al., 1997; Narita et al., 2003). A link of MexCD-OprJ expression with EtBr efflux from P. aeruginosa has been demonstrated by Morita et al. (2001, 2003) when they showed that significant EtBr efflux was only measurable after induction of MexCD-OprJ. MexCD-OprJ expression was inducible with tetrathenyl phosphonium, EtBr, rhodamine 6G, acriflavine, benzalkonium chloride, and chlorhexidine gluconate but not with various antibiotics including substrates of MexD (i.e., norfloxacin, tetracycline, chloramphenicol, streptomycin, or erythromycin).

A thorough quantitative analysis of efflux was achieved with β-lactams and intact E. coli cells (Nagano and Nikaido, 2009). The method relies on the hydrolysis of β-lactams by periplasmic β-lactamases and would probably not be directly applicable to P. aeruginosa because of the about 10–100 times lower permeability of the OM for β-lactams (Angus et al., 1982; Yoshimura and Nikaido, 1982; Parr et al., 1987). Under these circumstances it would be very difficult to saturate the efflux pumps and the method may be restricted to a few suitable β-lactams. Hydrolysis rates of β-lactams were measured in intact cells to calculate the periplasmic antibiotic concentration based on known kinetic parameters of β-lactamases. Maximal velocity and dose–response curves depended on the nature of the substrate. Positive cooperativity was detected with cefamandole, cephalotin, and cefaloridin but not with nitrocefin (Nagano and Nikaido, 2009). Cooperativity agrees with the current model of the functional complex of AcrAB-ToIC where multiple substrate binding sites reside within three AcrB monomers that adopt different structural conformations in a concerted way during substrate transport (Seeger et al., 2006; Murakami et al., 2006; Sennhauser et al., 2007). Nitrocefin transport was the slowest in the series, which could be explained by the rather strong binding of nitrocefin with its two aromatic rings to AcrB ($K_m$ of 5 μM). No significant efflux could be measured with cefazolin, which has two hydrophilic substituents. Previous work has shown that MICs of cloxacillin, which has a lipophilic side chain, dropped from 256 to 2 mg/L in S. enterica serovar Typhimurium and from 512 to 2 mg/L in E. coli upon inactivation of AcrB whereas no change was observed for the MIC of the hydrophilic cefazolin (Nikaido et al., 1998; Mazzariol et al., 2000). MIC values and efflux studies strongly suggested that AcrB from E. coli and S. enterica serovar Typhimurium have a preference for hydrophobic β-lactams.

Specific mode-of-action studies ask for less complex assay systems than entire bacterial cells. The activity of different bacterial membrane transporters could be studied with membrane vesicles. Vesicles proved to be very valuable tools for the study of tetracycline-specific pumps (e.g., Yamaguchi et al., 1990). Tetracycline-specific pumps belong to the MFS class of transporters spanning a single cell membrane (Chopra and Roberts, 2001), whereas RND pumps are multi-subunit complexes made of pump subunits (e.g., MexB), an OM channel (e.g., OprM), and an adaptor subunit (e.g., MexA). The pump is formed by three monomers placed in the inner membrane and protrudes into the periplasm (Murakami et al., 2002, 2006; Seeger et al., 2006; Sennhauser et al., 2007, 2009). The OM channel, formed by three monomers, passes the OM linking the periplasmic space with the outside of the cell (Koronakis et al., 2000; Akama et al., 2004a; Phan et al., 2010). The third subunit is an adaptor protein that is required for the assembly of the functional complex which spans the inner membrane, the periplasmic space, and the OM. The stoichiometry of the
subunits (MexB:MexA:OprM or AcrB:AcrA:TolC) is currently discussed with evidence for 3:6:3 (Du et al., 2014, 2015; Kim et al., 2015) or for 3:3:3 (Symmons et al., 2009; Trepout et al., 2010). The models are based on electron microscopy data at resolutions around 20 Å and on docking calculations. A higher-resolution crystal structure of the assembled efflux systems will likely provide a clear-cut response to the controversy regarding the stoichiometry and the construction of the tripartite complex.

The isolated pump subunits AcrB and AcrD from E. coli and MexB from P. aeruginosa were reconstituted in vesicles (Zgurskaya and Nikaido, 1999; Aires and Nikaido, 2005; Verchère et al., 2014, 2015) but reconstitution of a functional tripartite RND pump complex for in vitro transport studies is a truly difficult task that has been achieved only recently (Ntsgo-Enguene et al., 2015; Verchère et al., 2015). Competition studies with AcrB-containing vesicles have indicated that the bile salts taurocholate and glycocholate inhibited transport of a fluorescent phospholipid more efficiently than erythromycin or cloxacillin did (Zgurskaya and Nikaido, 1999). Chloramphenicol was identified in the same study as a substrate that did not inhibit phospholipid efflux. These results suggested that known AcrB substrates were transported with different efficiency reflecting specific substrate recognition patterns. Functional MexAB-OprM complexes have been assembled in vitro in a liposome system combining proteoliposomes with OprM and proteoliposomes with MexAB in a way that MexAB-OprM was able to transport EtBr driven by a proton gradient (Ntsgo-Enguene et al., 2015). A related approach used vesicles with MexB and bacteriorhodopsin, a light activated proton pump from Halobacterium salinarium (Verchère et al., 2014). Light activation caused bacteriorhodopsin to build up a proton gradient as the energy source for MexB. A change of pH was then taken as indication for substrate transport. The system confirmed that H33342 is a substrate of MexB and suggested that MexA was required for proton-coupled transport.

**Efflux Inhibitors**

Many chemically diverse EPI have been reported and their selectivity for pumps can provide insight into specific inhibitor–pump interactions (reviewed in Zechini and Versace, 2009; Van Bambeke et al., 2010). Inhibitors have been derived from natural products, antibiotics, drugs originating from other therapeutic areas or have been newly developed. PAβN and more advanced peptidomimetics of the same series are among the best studied examples (Lomovskaya et al., 2001; Mao et al., 2001; Renau and Lemoine, 2001; Renau et al., 2002; Watkins et al., 2003; Kriengkauskait et al., 2005). Selectivity for pumps indicated specific inhibitor–pump interactions. For example, PAβN was broadly active against MexAB-OprM, MexEF-OprN, MexCD-OprF, and MexXY-OprM in P. aeruginosa as well as against AcrAB-ToLC in several species of the Enterobacteriaceae family while the pyridopyrimidine efflux inhibitor D13-9001 had a much narrower spectrum with specificity for AcrB and MexB (Nakashima et al., 2013). PAβN has been shown to have synergistic activity with levofloxacin against wild-type P. aeruginosa or against P. aeruginosa strains overexpressing any of MexAB-OprM, MexCD-OprF, MexEF-OprN, or MexXY (Lomovskaya et al., 2001). On the other hand, PAβN had no significant effect on carbencillin or EtBr, which are substrates of MexB too, suggesting multiple binding sites for different substrates. An antagonistic effect of PAβN with aminoglycosides has been observed in the presence of MexXY-OprM, which was explained by the induction of this efflux pump by PAβN (Mao et al., 2001). PAβN was shown to be an efflux substrate likely to act by competition with other substrates for transport (Pages et al., 2005; Lomovskaya and Bostian, 2006; Mahamoud et al., 2007). D13-9001 was hardly transported but was shown to inhibit efflux by high affinity binding to a specific site (Nakashima et al., 2013). Many substances cannot be unambiguously classified as substrates or as inhibitors because there seems to be a gradual transition of properties and many molecules have both characteristics. Minocycline is a substrate of several RND pumps such as AcrB from E. coli, Proteus mirabilis, Morganella morganii, MexB, MexD, and MexY in P. aeruginosa, or AdeB and AdeJ in A. baumannii (Dean et al., 2003; Visalli et al., 2003; Hirata et al., 2004; Ruzin et al., 2005; Damier-Piolle et al., 2008) but it also acted as an inhibitor of nitrocefin efflux by AcrB (Takatsuka et al., 2010). Trimethoprim, an antibiotic that has been shown to be transported by several RND efflux pumps (i.e., MexB, MexD, and MexP in P. aeruginosa, AdeB, AdeJ, AdeG in A. baumannii, and BpeF in Burkholderia pseudomallei), inhibited the efflux of H33342 by AcrB in S. enterica serovar Typhimurium (Kohler et al., 1996; Maseda et al., 2000; Magnet et al., 2001; Coyne et al., 2010; Piddock et al., 2010; Amin et al., 2013; Podnecky et al., 2013). In fact, antibiotics could be modified to become efflux inhibitors as described in the case of fluoroquinolones, tetracyclines, or aminoglycosides (reviewed in Van Bambeke et al., 2010). In general, EPI have aromatic moieties and contain ionizable groups, which are structural features that are reminiscent of substrate characteristics deduced from antibiotic susceptibility tests (Poole, 2004, 2005).

Transport measurements are useful to identify molecules that interact with efflux pumps but investigation of interactions at a molecular level and precise mode-of-action studies require more refined methods.

**Binding Studies and Crystal Structures**

Binding of fluorescent substrates to purified AcrB in detergent solution could be measured by fluorescence polarization (Su and Yu, 2007). AcrB bound rhodamine 6G, ethidium, and proflavin, with similar strength (K_D of 5.5, 8.7, and 14.5 μM, respectively). Binding of ciprofloxacin was significantly weaker with a K_D of 74.1 μM. Competition studies have indicated that different binding sites may exist for different antibiotics. Experiments with purified AcrB which was immobilized to a surface, yielded K_D values of 530 μM for novobiocin and of 110 μM for the EPI MC-207,110 (PAβN; Tikhonova et al., 2011). Binding studies confirmed specific interaction of substrates or inhibitors with the pump subunit and revealed substrate-specific variation.
of binding strength (Vargiu and Nikaido, 2012; Vargiu et al., 2014).

Many molecules have been described to be efflux substrates or inhibitors but only a small subset thereof could be crystallized in complex with RND pumps (Ruggerone et al., 2013a). Nevertheless, X-ray structures have provided detailed information on substrate-pump interactions (Murakami et al., 2006; Seeger et al., 2006; Sennhauser et al., 2007, 2009; Nakashima et al., 2011; Eicher et al., 2012). A detailed description of the structures is provided in the next section where computational simulation approaches are discussed. The porter domain of the inner membrane transporter protrudes into the periplasmic space (Figure 1) and mediates substrate specificity as predicted from genetic studies with AcrB, AcrD, MexB, MexD and MexY (Nikaido, 2011). Gene segments coding for the periplasmic loops of the pump subunit were swapped between AcrB and AcrD of E. coli, between MexB and MexY of P. aeruginosa, and between AcrB of E. coli and MexB of P. aeruginosa (Elkins and Nikaido, 2002; Tikhonova et al., 2002; Eda et al., 2003). Altered substrate specificities of the new constructs suggested that substrate recognition was predominantly determined by the periplasmic region of the pump. Site directed mutagenesis in P. aeruginosa confirmed substrate recognition sites in the periplasmic loops of MexB and MexD (Mao et al., 2002; Middlemiss and Poole, 2004; Wehmeier et al., 2009). Q34K, E89K, and N67K mutations in MexD have led to the transport of negatively charged β-lactams that are not recognized by the wild type transporter (as discussed in the Section Impact of Efflux on Antibiotic Activity; Mao et al., 2002). A direct substrate contact for macrolide recognition was proposed for an asparagine residue in a phenylalanine-rich distal binding pocket in MexB (Wehmeier et al., 2009).

The high molecular weight substrates rifampicin, erythromycin, and doxorubicin dimers bound to an access (or proximal) binding pocket (AP) shown in Figures 1A,B, which is located close to the protein/periplasm interface (Nakashima et al., 2011; Eicher et al., 2012). The low molecular weight substrates minocycline, dodecyl-α-D-maltoside and doxorubicin have been found in a distal (or deep) binding pocket (DP) displayed in Figures 1C,D. AP and DP have also been proposed as two successive locations visited by a substrate during the translocation cycle. The two pockets are separated by a flexible loop (G-loop or switch loop) with two phenylalanine residues (Nakashima et al., 2011; Eicher et al., 2012). Substrate recognition is governed by a phenylalanine-rich hydrophobic region in the distal pocket. Minocycline, rifampicin, and erythromycin, made direct contact with phenylalanine residues (Murakami et al., 2006; Nakashima et al., 2011; Eicher et al., 2012). The MexB-specific pyridopyrimidine efflux inhibitor D13-9001 bound to a narrow pit in the phenylalanine cluster of the distal pocket making π-π-stacking interactions (Nakashima et al., 2013). The hydrophilic part of the inhibitor interacted with hydrophilic and ionic residues close to the binding site of minocycline and doxorubicin (Nakashima et al., 2013). Binding to MexY was sterically hindered by a tryptophan in agreement with the phenotypic specificity for MexB over MexY (Nakayama et al., 2003; Yoshida et al., 2007). The binding pocket of AcrD contains several oxygens in contrast to the mostly hydrophobic pocket of AcrB, which could explain the specificity of AcrD for small basic, hydrophilic substrates such as the anionic β-lactams carbenicillin, aztreonam, and sulbenicillin (Kobayashi et al., 2014). Domain swapping experiments have shown that the specificity of MexB for anionic β-lactams, which are not recognized by MexY, is determined by the periplasmatic domain of the pump subunit (Eda et al., 2003). Although, the complexity of substrate recognition by RND efflux systems has hindered the establishment of a straightforward criterion for the definition of good and poor substrates, it is possible that MexY, as shown for MexD and described before (Mao et al., 2002), does not have the required binding places (i.e., positively charged amino acid residues) in the substrate binding sites to accept negatively charged β-lactams. It cannot be excluded that other, subtle differences in the RND transporter-compound interaction patterns might affect the recognition and the transport at different extrusion stages and positions in the transporters (see Figure 1 where the different affinity sites are shown). Examples for such subtle but crucial interactions are imipenem and meropenem interacting with MexB (see Section Computational Study).

Mutagenesis of D133 in MexY of P. aeruginosa (D113A and D133S) has compromised resistance to several aminoglycosides but not to spectinomycin (Lau et al., 2014). The mutation of Y613A in the same protein affected resistance to aminoglycosides but not to erythromycin. This finding supported the view that substrate recognition is governed by binding to specific sites on the extrusion pathway because D133 and Y613 are located in a region of MexY that structurally corresponds to the proximal binding pocket of AcrB.

The experimental data discussed in this review, have provided evidence that hydrophobic moieties are a key property recognized by RND pumps and that hydrophilic parts of a substrate fit into mainly hydrophilic cavities adjacent to a phenylalanine-rich, hydrophobic pocket. Substrates of MexB, which are discussed in this review, are listed in Table 1.

Computational Study

Simulations have reached an impressive maturity as reflected by the increasing number of publications in the field. In particular, a range of simulation techniques have been developed and successfully applied to describe diverse biological systems. Highly precise quantum mechanical techniques (for example see the reviews of Dal Peraro et al., 2007; Zhou et al., 2010; Sgrignani and Magistrato, 2013; Steinbrecher and Elstner, 2013), the classical force-field-based approaches (Kollman et al., 2000), hybrid methods combining appropriate quantum mechanical and classical descriptions (Senn and Thiel, 2009; Steinbrecher et al., 2012) and multi-scale and statistical mechanical methodologies (Kamerlin et al., 2011; Karplus, 2014) are examples of such computational methods.

Although they have been successfully applied to several systems and used to tackle biology-inspired questions, these in silico techniques have been rarely used to investigate bacterial
efflux systems, in particular members of the RND family. Probably hampered by the lack of crystal structures of the whole systems (the single components have been crystallized) and by the complexity of the machineries (they are tripartite systems), computational studies have addressed RND efflux systems only recently (Collu and Cascella, 2013; Ruggerone et al., 2013a,b). Classical molecular dynamics (MD) simulations represent a particularly promising technique to cast a glimpse on the dynamic behavior of a protein and its immediate micro-environment. MD simulations offer insight into molecular behavior at a temporal and structural accuracy not reached by any other experimental technique today. Continuous improvement of the techniques is pushing the limits of the simulation processes toward longer simulations and thus to a description of increasingly larger biological systems. Extension of the simulation times has improved the quality of the predictions and allowed more robust evaluation of key features such as free energy of binding, interaction patterns, solvent interactions, and interaction lifetimes. Surely, limitations are still present due to the size of many systems and the length of processes of interest, but specific techniques to overcome these drawbacks are under continuous development. As an example of methods of conformational changes between two known states, and metadynamics (Schlitter et al., 1993), which allows induction of free energy profiles associated with possible processes. Advantages and drawbacks of these biased techniques have been extensively discussed in several publications, to which the interested reader is referred (e.g., Chipot, 2008; Laio and Gervasio, 2008; Biarnés et al., 2011), which is used to simulate rare events and provides free energy profiles associated with possible processes. The RND inner-membrane transporter subunit is integral to the function of the system. The transporter subunit of the RND-type tripartite complex functions as a proton/drug homotrimeric antiporter and is key for energy transduction and substrate specificity of the entire three-component complex (Murakami, 2008). Structural data have mainly been collected for AcrB, of E. coli, and to a lesser extent for MexB, the homolog of AcrB in P. aeruginosa (for a recent review, see Ruggerone et al., 2013a). According to crystallographic results, the shape of the protein resembles that of a jellyfish. Viewed orthogonally to the membrane plane, each protomer elongates for ∼120 Å, comprising a TM region of ∼50 Å composed of 12 α-helices (TM1 to TM12), and a periplasmic headpiece of about 70 Å, the latter being divided into a pore (porter) region and an upper region close to the lower part of the OMF (Murakami, 2008; Eicher et al., 2009).

After the first symmetric structure was solved (Murakami et al., 2002) other crystal structures of AcrB revealed asymmetric conformations of the three monomers in the trimer (Murakami et al., 2006; Seeger et al., 2006; Sennhauser et al., 2007). The three conformations were proposed to represent three consecutive states in a three-step peristaltic mechanism of the substrate translocation [called functional rotation (Murakami et al., 2006) or peristaltic motion (Seeger et al., 2006)]. The postulated functional rotation starts with recognition of substrate at a low affinity site on the L monomer, namely the AP. Global conformational transition converts the L to a T conformation, accompanied by tight binding of the substrate in a designated high-affinity binding pocket, i.e., the DP. Successively, a second peristaltic motion leads to a switch from the T to an O conformation, resulting in the release of the substrate toward the OMF. After substrate release, the O conformation relaxes back to the L conformation restarting the cyclic event. The conversion from the T to the O conformation is suggested to be the major energy-requiring step and should be accompanied by proton binding at the proton translocation site in the TM region. Proton release may occur during conversion from O to L. Computational studies based on targeted MD simulations supported this mechanism and the zipper-like closure of the DP (Schulz et al., 2010).

A 3.0 Å crystal structure of MexB showed the same overall fold as its close homolog AcrB did (Sennhauser et al., 2009). The three monomers constituting MexB assumed an asymmetric conformation supporting the general transport model for this family of multi-drug transporters derived from the AcrB structures (Murakami et al., 2006; Seeger et al., 2006; Sennhauser et al., 2007). However, the conformation of monomer L in MexB...
showed significant differences at the periplasmic portal to AcrB. In particular, no access to the binding cavity was observed in this subunit. A clear rationale for this structural difference is missing to date. Sennhauser et al. (2009) proposed, among other things, that the differences might be attributed to the L monomer being trapped in an intermediate conformational state between the extrusion and the binding of the substrates. Alternatively, specific resting states of MexB and of AcrB may account for the observed differences. Recently, Nakashima et al. (2013) solved the structure of MexB in complex with a pyridopyrimidine derivative. This work provided the first structural information on MexB-inhibitor interactions. The authors also crystallized free MexB and found a structure largely similar to that described by Sennhauser et al. (2009). The binding geometry of the pyridopyrimidine derivative to AcrB was also resolved, and showed relevant variations in the conformation of the ligand. These findings highlighted that subtle differences in the mechanisms of drug binding and translocation are relevant for the two pumps.

Several key features of the MexB structure, in particular the AP and DP sites, could be mapped onto the structure of AcrB (Murakami et al., 2006; Nakashima et al., 2011; Eicher et al., 2012). The first and until now the only computational study of antibiotic-MexB interactions was based on these data (Collu et al., 2012b). Collu et al. (2012b) investigated the behavior of two carbapenems, meropenem and imipenem, in the AP and the DP. The two structurally related compounds are differently affected by the RND efflux pumps. The activity of meropenem is significantly reduced by MexAB-OprM efflux whereas the activity of imipenem is not (Masuda and Ohy, 1992; Pai et al., 2001; Pournaras et al., 2005; Walsh and Amyes, 2007). The different sensitivities to RND efflux make imipenem and meropenem attractive candidates for a comparative study of carbapenem-efflux-pump interactions.

Lacking a crystal structure of MexB in complex with the two molecules, the starting configurations for all-atom MD simulations were extracted from docking runs with the ATTRACT package (May and Zacharias, 2008; May et al., 2008; de Vries and Zacharias, 2012). The systems obtained after solvation and equilibration were simulated for 50 ns. A stronger preference of meropenem for the DP than for the AP resulted from the simulations (binding free energies of −8.1 and 2.4 kcal mol$^{-1}$, respectively). Imipenem had nearly the same low affinity for both pockets (0.6 and 0.4 kcal mol$^{-1}$, respectively). This result agreed with microbiological data showing a fourfold increase of the MIC of meropenem but no significant change of the imipenem MIC upon overexpression of MexB in P. aeruginosa (Eguchi et al., 2007; Ong et al., 2007; Livermore, 2009; Riera et al., 2011). The contacts between meropenem and the DP extracted from the trajectories were qualitatively consistent with the recently determined structure of a MexB-inhibitor complex (Nakashima et al., 2013), suggesting a reliable prediction of the binding structures by the computational protocol of Collu et al. (2012b).

The AP is probably the first internal site to be occupied by compounds during the binding process. At the AP, imipenem and meropenem pointed their β-lactam rings toward the periplasmic region and the entrance to the DP, respectively. These could be considered as fingerprints of the different behavior of the two compounds. Meropenem tended to move toward the DP, following the plausible extrusion route, while imipenem had a propensity to reach regions close to the periplasm. Interactions with solvent molecules can be extracted and quantified from the trajectories as a further interesting detail in support of this picture (Sterpone et al., 2001; Collu et al., 2012a). Collu et al. (2012a) found that imipenem, unlike meropenem, formed long-lifetime interactions with water molecules inside of MexB.

The docking poses of two compounds in the DP were similar but the 50 ns-long simulations led to different equilibrium binding modes. Meropenem moved in the DP of MexB toward the external channel, assuming a position suitable for extrusion. Imipenem slid away from the entrance to the channel connecting the DP to the extrusion mouth and into a position similar to that assumed by doxorubicin in MD simulations of mutated AcrB F610A (Vargiu et al., 2011). Indeed, Vargiu et al. (2011) found that doxorubicin moved deeper into the DP of a F610A mutant and was not extruded by the induced functional rotation. This was in accordance with reduced doxorubicin MIC (i.e., increased activity) against the F610A variant of AcrB (Bohnert et al., 2008).

The calculated pump interaction patterns have been associated with the different physicochemical properties of the ligand molecules. With its bulky and hydrophobic tail, meropenem established a strong interaction pattern to the aromatic-hydrophobic environment of the DP. Conversely, the more flexible and hydrophilic tail of imipenem had a lower affinity for the DP. Solvent interactions played a major role in the different transport properties of the two carbapenems as well. In fact, the compounds remained highly solvated at all explored binding sites (Collu et al., 2012b). Nonetheless, the water dynamics around meropenem were significantly different in the DP than in the bulk solvent. On the other hand, imipenem showed the same solvent interactions in the DP as in the bulk solvent.

**Paths for Substrate Entry**

Substrate uptake by RND pumps is still largely unexplored and to our knowledge, only one computational study has addressed this issue (Yao et al., 2013). The entry of substrates into AcrB was studied by a combination of *in silico* tools and site-directed mutagenesis. The study indicated that uptake pathways of minocycline, acriflavine, and novobiocin differed significantly. Novobiocin is the largest and acriflavine the smallest among the three compounds while minocycline is more hydrophilic than the nearly equally hydrophobic novobiocin and acriflavine. All three molecules are typical substrates of AcrB but they vary in molecular size and hydrophobicity. A ligand-dependent drug uptake mechanism was proposed based on the analysis of the free energy associated with drug movement along AcrB tunnels. Strongly hydrophobic and lipophilic drugs of similar size were preferentially taken up via the vestibule path (the entrance of the vestibule path is shown in Figures 3A,B). This path starts close to the membrane surface at a region between two protomers and goes via the AP to the DP. Other drugs were translocated...
through the cleft path starting at a large external indentation of the periplasmic domain, formed by the subdomains PC1 and PC2 of a single AcrB protomer. The cleft path, whose entrance is of the periplasmic domain, formed by the subdomains PC1 and through the cleft path starting at a large external indentation. Dreier and Ruggerone Substrate recognition by RND efflux pumps

Figures 3C,D shown in PC2 of a single AcrB protomer. The cleft path, whose entrance is of the periplasmic domain, formed by the subdomains PC1 and through the cleft path starting at a large external indentation. The direct simulations identified a novel alternative uptake path, which is not visible in the crystal structure. This third path goes along the bottom of the porter domain toward PC1 and could be validated by site-directed mutagenesis of AcrB in E. coli. Mutations of residues located along the path significantly impaired the efflux efficiency. The work of Yao et al. (2013) is of great interest because it combined different techniques to gain insight into an important step of the efflux process. However, it has to be noted that the authors made several approximations. As in all the computational studies described in this review, the partners of the RND transporter (i.e., AcrA and TolC in the present case) were missing. It was also assumed that the drug uptake was mainly driven by hydrophobic interactions. Finally, some of the physicochemical properties such as the ordering of amphiphilic drugs in the lipid bilayer and the conformational flexibility of drugs with rotatable bonds were neglected. Despite of the necessary approximations, the work of Yao et al. (2013) was an important contribution to the understanding of drug RND efflux. The structures of the L monomers and ligand conformations in co-crystals suggest interesting mechanistic differences between MexB and AcrB (Sennhauser et al., 2009; Nakashima et al., 2013). Thus, a similar study on MexB would be of great interest.

Conclusion

Much progress has been made in the understanding of the transport mechanism of RND efflux pumps since they were discovered. Genetic and biochemical investigations have provided a good survey of the substrate specificity of different pump complexes in various bacteria. It has become accepted that RND pumps evolved as a first line of bacterial defense against exogenous substances, allowing the development of additional defense strategies (Olivares et al., 2013). Molecules that are able to penetrate the OM are potentially problematic for Gram-negative bacteria. Many sophisticated studies have supported this view as they have indicated that amphiphilic molecules (such as many antibiotics), which are able to pass the OM, are good substrates for export systems (Nikaido, 2011; Nikaido and Pages, 2012). X-ray crystallography has provided highly detailed structural information on binding interactions between pump subunits and substrates (Ruggerone et al., 2013a). It is intriguing that only a handful of a large number of known efflux substrates could be co-crystallized with efflux pumps. Although X-ray structures provide snapshots of a complex transport mechanism, they have been crucial for the setup of a transport model. Concerning specific experiments aiming at a more direct investigation of efflux dynamics, studies with whole bacteria have been performed. Whole cell efflux assays require thorough controls to rule out non-specific effects that interfere with transport measurements. Such control measurements include the use of membrane-interacting probes to monitor effects on membrane integrity or on the electrochemical potential across the inner membrane. The finding that many of the membrane-interacting probes are efflux substrates too, has confirmed the concept of recognition and export of membrane-active compounds. Based on experimental data, new in silico tools have been developed and brought to a point where they can reliably describe or even predict the dynamics of compound extrusion by RND pump subunits.

Substrate recognition by RND efflux pumps is a multi-factorial process that can be measured by different methods as described in this article. MexB, for example, has a broad substrate spectrum including large (e.g., erythromycin) and charged (e.g., aztreonam, PAβN) molecules (see Table 1). MexB transports hydrophobic dyes, many of which have been primarily used to study membrane properties, which indicates a preference of this pump for hydrophobic substrates. This is in agreement with the finding that the activity of the hydrophilic antibiotic cefotiboprole is not significantly affected by MexB. Crystal structures and MD simulations of MexB strongly suggested that the DP, lined with
hydrophobic amino acid residues, is the main structural element for the recognition of hydrophobic elements (Collu et al., 2012b; Nakashima et al., 2013). However, hydrophobicity alone is not sufficient to explain substrate recognition byMexB. Imipenem and meropenem have a similar size and hydrophobicity (Table 1) but only meropenem is transported byMexB. A computational study revealed that imipenem, unlike meropenem, made no high affinity contacts to the AP or to the DP but instead made long-range interactions with solvent molecules inside MexB and eventually did not enter MexB (Collu et al., 2012b).

The challenge for future studies will be to combine the precision of X-ray structures with the functional relevance of whole cell studies. MD simulations based on experimental data provide a promising tool to complement, integrate and rationalize these data and to shed some light onto this problem.

Despite the advances outlined in the review, dissecting the molecular and conformational steps that regulate transport of substrates by RND pumps is still a very challenging and intriguing task and requires a very efficient interplay between techniques and approaches coming from different fields. The fate of a compound, governed by the action of an RND transporter (i.e., efficiently or poorly transported), is determined by the subtle balance of different molecular contributions that are not necessary large. The interaction of meropenem and imipenem with MexB described in the Section Computational Study is a good example: small differences in flexibility and hydrophobicity are suggested to make the former a good substrate and the latter a poor one. Computational methods can offer insight at a level of accuracy that is not reachable by a single experimental technique but they need a continuous feedback from experiments. Further crystallographic studies of RND pumps in complex with substrates and more accurate modeling techniques with extended simulation times for large proteins are needed in order to achieve full atomic pictures of the entire tripartite complexes. Efflux kinetics might be affected by several factors that have to be included in the simulations but are not known a priori (Kinana et al., 2013). Computer simulations can integrate data from experiments on the molecular level and help to interpret data from whole cell assays (i.e., functional efflux pump ensembles).

The development of novel antibiotics that can bypass the effects of MDR pumps or the development of clinically useful EPI is still a challenging task. Understanding the mechanistic details and the structure-function relationship of bacterial efflux systems, as well as their regulation and the synergistic interactions between the pumps and other resistance mechanisms, is not only scientifically rewarding but can also stimulate applied research for effective new antibacterial drugs.

Addendum in Proof

While our manuscript was under review two comprehensive review articles by Li et al. (2015) and by Yamaguchi et al. (2015) were published that cover many aspects discussed in our article.

Acknowledgments

PR has received support from the Innovative Medicines Initiative Joint Undertaking under Grant Agreement n°115525, resources, which are composed of financial contribution from the European Union’s seventh framework program (FP7/2007–2013) and from EFPIA companies in kind contribution, as part of the Project TRANSLOCATION (http://www.imi.europa.eu/content/translocation).

We want to thank Dr. Stefan Reinelt for help with the calculation of the data in Table 1 and Dr. Jonathan Thwaite for critically reading the document.

References

Aires, J. R., and Nikaïdo, H. (2005). Aminoglycosides are captured from both periplasm and cytoplasm by the AcrD multidrug efflux transporter ofEscherichia coli, J. Bacteriol. 187, 1923–1929. doi: 10.1128/JB.187.6.1923-1929.2005

Akama, H., Kanemaki, M., Yoshimura, M., Tsukihara, T., Kashiwagi, T., Yoneyama, H., et al. (2004a). Crystal structure of the drug discharge outer membrane protein, OprM, of Pseudomonas aeruginosa: dual modes of membrane anchoring and occluded cavity end, J. Biol. Chem. 279, 52816–52819. doi: 10.1074/jbc.C400445200

Akama, H., Matsuura, T., Kashiwagi, S., Yoneyama, H., Narita, S.-I., Tsukihara, T., et al. (2004b). Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in Pseudomonas aeruginosa, J. Biol. Chem. 279, 25939–25942. doi: 10.1074/jbc.C400164200

Amin, I. M., Richmond, G. E., Sen, P., Koh, T. H., Piddock, L. J., and Chua, K. L. (2013). A method for generating marker-less gene deletions in multidrug-resistantAcinetobacter baumannii, BMC Microbiol. 13:158. doi: 10.1186/1471-2180-13-158

Angus, B. L., Carey, A. M., Caron, D. A., Kropinski, A. M., and Hancock, R. E. (1982). Outer membrane permeability in Pseudomonas aeruginosa: comparison of a wild-type with an antibiotic-supersusceptible mutant. Antimicrob. Agents Chemother. 21, 299–309. doi: 10.1128/AAC.21.2.299

Baum, E. Z., Crespo-Carbone, S. M., Morrow, B. J., Davies, T. A., Foleno, B. D., He, W., et al. (2009). Effect of MexXY overexpression on cefotaxime susceptibility in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 53, 2785–2790. doi: 10.1128/AAC.00018-09

Bavro, V. N., Pietras, Z., Furnham, N., Pérez-Cano, L., Fernández-Recio, J., Pei, X. Y., et al. (2008). Assembly and channel opening in a bacterial drug efflux machine, Mol. Cell. 30, 114–121. doi: 10.1016/j.molcel.2008.02.015

Biarnés, X., Bongarzone, S., Vargiu, A. V., Carloni, P., and Ruggerone, P. (2011). Molecular motions in drug design: the coming age of the metadynamics method. J. Comput. Aided Mol. Des. 25, 395–402. doi: 10.1007/s10822-011-9415-3

Blair, J. M., Bavro, V. N., Ricci, V., Modi, N., Cacciotto, P., Kleinekathöfer, U., et al. (2015a). AcrB drug-binding pocket substitution confers clinically relevant resistance and altered substrate specificity, Proc. Natl. Acad. Sci. U.S.A. 112, 3511–3516. doi: 10.1073/pnas.1419939112

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbofu, D. O., and Piddock, L. J. V. (2015b). Molecular mechanisms of antibiotic resistance. Nat. Rev. Microbiol. 13, 42–51. doi: 10.1038/nrmicro3380

Blair, J. M., Richmond, G. E., and Piddock, L. J. (2014). Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. Fust. Microbiol. 9, 1165–1177. doi: 10.2217/fmb.14.66

Bohnert, J. A., Karamian, B., and Nikaido, H. (2010). Optimized Nile Red efflux assay of AcrAB-TolC multidrug efflux system shows competition between the pumps and other resistance mechanisms.

Addendum in Proof

While our manuscript was under review two comprehensive review articles by Li et al. (2015) and by Yamaguchi et al. (2015) were published that cover many aspects discussed in our article.
and a deep binding pocket that are separated by a switch-loop. Proc. Natl. Acad. Sci. U.S.A. 109, 5687–5692. doi: 10.1073/pnas.1114944109

Eichler, T., Seeger, M. A., Anselmi, C., Zhou, W., Brandstätter, L., Verrey, F., et al. (2014). Coupling of remote alternating-access transport mechanisms for protons and substrates in the multidrug efflux pump AcrB. Elife 19, 3 doi: 10.7554/eLife.03145

Elkins, C. A., and Nikaido, H. (2002). Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrC of Escherichia coli is determined predominantly by two large periplasmic loops. J. Bacteriol. 184, 6490–6498. doi: 10.1128/JB.184.23.6490-6499.2002

Fernandez, L., and Hancock, R. E. (2012). Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin. Microbiol. Rev. 25, 661–681. doi: 10.1128/CMR.00043-12

Fernandez, Y., Monlezun, L., Phan, G., Benabdelhak, H., Benas, P., Ulyrnck, N., et al. (2012). Stoichiometry of the MexA-OprM binding, as investigated by blue native gel electrophoresis. Electrophoresis 33, 1282–1287. doi: 10.1002/elps.201100541

Ferreira, M. C. M., and Kiralj, R. (2004). QSAR study of β-lactam antibiotic efflux by the multidrug resistance pump AcrB. J. Chemom. 18, 242–252. doi: 10.1002/cem.867

Fieldler, F., and Hinz, H. (1994). No intermediate channelling in stepwise hydrolysis of fluorescein di-beta-D-galactoside by beta-galactosidase. Eur. J. Biochem. 222, 75–81. doi: 10.1111/j.1432-1323.1994.tb18834.x

Fischer, N., and Kandt, C. (2011). Three ways in, one way out: water dynamics in the trans-membrane domains of the inner membrane translocase AcrB. Proteins 79, 2871–2885. doi: 10.1002/prot.23122

Fischer, N., and Kandt, C. (2012). Porter domain opening and closing motions in the multi-drug efflux transporter AcrB. Biochim. Biophys. Acta 1828, 632–641. doi: 10.1016/j.bbamem.2012.10.016

Fischer, N., Raunest, M., Schmidt, T. H., Koch, D. C., and Kandt, C. (2014). Efflux pump-mediated antibiotics resistance: insights from computational structural biology. Interdiscip. Sci. 6, 1–12. doi: 10.1007/s12539-014-0191-3

Fukuda, H., Hosaka, M., Hirai, K., and Iyobe, S. (1990). New norfloxacin resistance gene in Pseudomonas aeruginosa Pao. Antimicrob. Agents Chemother. 34, 1757–1761. doi: 10.1128/AAC.34.9.1757

Fukuda, H., Hosaka, M., Iyobe, S., Gotoh, N., Nishino, T., and Hirai, K. (1995). nfxC-type quinolone resistance in a clinical isolate of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 39, 790–792. doi: 10.1128/AAC.39.3.790

Garvey, M. I., and Paddock, I. J. (2008). The efflux pump inhibitor reserpine selects multidrug-resistant Streptococcus pneumoniae strains that overexpress the ABC transporters PatA and PatB. Antimicrob. Agents Chemother. 52, 1677–1685. doi: 10.1128/AAC.01644-07

Germ, M., Yoshibara, E., Yoneyama, H., and Nakae, T. (1999). Interplay between the efflux pump and the outer membrane permeability barrier in fluoroscent dye accumulation in Pseudomonas aeruginosa. Biochim. Biophys. Res. Commun. 261, 452–455. doi: 10.1016/bbrc.1999.1045

Giske, C. G., Boren, C., Wretlind, B., and Kronvall, G. (2005). Meropenem effects of efflux transporter genes on susceptibility of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 50, 1347–1351. doi: 10.1128/AAC.50.4.1347-1351.2006

Humphrey, W., Dalke, A., and Schulten, K. (1996). Vmd: visual molecular dynamics. J. Mol. Graph. 14, 33–38. doi: 10.1016/0749-7252(96)00033-5

Husain, F., and Nikaido, H. (2010). Substrate path in the AcrB multidrug efflux pump of Escherichia coli. Mol. Microbiol. 78, 320–330. doi: 10.1111/j.1365-2958.2010.07330.x

Iino, R., Hayama, K., Amezawa, H., Sakakihara, S., Kim, S. H., Matsumoto, Y., et al. (2012a). A single-cell drug efflux assay in bacteria by using a directly accessible femtoliter droplet array. Lab. Chip. 12, 3923–3929. doi: 10.1039/c2lc40394c

Iino, R., Nishino, K., Noji, H., Yamaguchi, A., and Matsumoto, Y. (2012b). A microfluidic device for simple and rapid evaluation of multidrug efflux pump inhibitors. Front. Microbiol. 3:40. doi: 10.3389/fmicb.2012.00040

Iino, R., Matsumoto, Y., Nishino, K., Yamaguchi, A., and Noji, H. (2013). Design of a large-scale femtoliter droplet array for single-cell analysis of drug-tolerant and drug-resistant bacteria. Front. Microbiol. 4:360. doi: 10.3389/fmicb.2013.00360

Jones, R. N., Stilwell, M. G., Rhomberg, P. R., and Sader, H. S. (2009). Antipseudomonal activity of piperacillin/tazobactam: more than a decade of experience from the SENTRY Antimicrobial Surveillance Program (1997–2007). Diagn. Microbiol. Infect. Dis. 65, 331–334. doi: 10.1016/j.diagmicrobio.2009.06.022

Kamal, M. Z., Ali, J., and Rao, N. M. (2013). Binding of bis-ANS to Bacillus subtilis lipase: a combined computational and experimental investigation. Biochim. Biophys. Acta 1834, 1501–1509. doi: 10.1016/j.bbapap.2013.04.021

Kamerlin, S. C., Vicatos, S., Dryga, A., and Warshel, A. (2011). Coarse-grained (multiscale) simulations in studies of biophysical and chemical systems. Annu. Rev. Phys. Chem. 62, 41–64. doi: 10.1146/annurev-physchem-032210-133335

Kamio, Y., and Nikaido, H. (1976). Outer membrane of Salmonella typhimurium: accessibility of phospholipid head groups to phospholipase c and cytochrome c oxidase activated dextran in the external medium. Biochemistry 15, 2561–2570. doi: 10.1021/bi06057a012

Kaprus, M. (2014). Development of multiscale models for complex chemical systems: from H+H(2) to biomolecules (Nobel Lecture). Angew. Chem. Int. Ed. Engl. 53, 9992–10005. doi: 10.1002/anie.201403924

Kim, J. S., Jeong, H., Song, S., Kim, H.-Y., Lee, K., Hyun, J., et al. (2015). Structure of the tripartite multidrug efflux pump acrAB-ToLC suggests an alternative assembly mode. Mol. Cells 38, 180–186. doi: 10.14348/molcells.2015.2277

Kisada, S., Vargiu, A. V., and Nikaido, H. (2013). Some ligands enhance the efflux of other ligands by the Escherichia coli multidrug pump AcrB. Biochemistry 52, 8342–8351. doi: 10.1021/bi400330v

Kobayashi, N., Tamura, N., Van Veen, H. W., Yamaguchi, A., and Murakami, S. (2014). beta-Lactam selectivity of multidrug transporters AcrB and AcrD resides in the proximal binding pocket. J. Biol. Chem. 289, 10680–10690. doi: 10.1074/jbc.M114.577794

Kohler, T., Kok, M., Michals-Hamzeloup, M., Plesiat, P., Gotoh, N., Nishino, T., et al. (1996). Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 40, 2288–2290.
Krishnamoorthy, G., Tikhonova, E. B., and Zgurskaya, H. I. (2008). Fitting Li, X. Z., Plesiat, P., and Nikaido, H. (2015). The challenge of efflux-mediated Li, X. Z., Ma, D., Livermore, D. M., and Nikaido, H. (1994). Role of efflux pump(s) Frontiers in Microbiology | www.frontiersin.org 119, 68–77. doi: 10.3109/ 10.1016/j.jac.dkl493 Mao, W., Warren, M. S., Lee, A., Mistry, A., and Loontiens, F. G., Regenfuss, P., Zechez, A., Dumortier, L., and Clegg, R. M. (1990). Binding characteristics of Hoechst 33258 with calf thymus DNA, poly(d[A- T]), and d(CGCGAATTCCGCG): multiple stoichiometries and determination of tight binding with a wide spectrum of site affinities. Biochemistry 29, 9029–9039. doi:10.1021/bi00490a021 Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., and Hearst, J. E. (1993). Molecular cloning and characterization of acrA and acrE genes of Escherichia coli. J. Bacteriol. 175, 6299–6313. Magnet, S., Courvalin, P., and Lambert, T. (2001). Resistance-nodulation- cell division-type efflux pump involved in aminoglycoside resistance in Acinetobacter baumannii strain BM4545. Antimicrob. Agents Chemother. 45, 3375–3380. doi:10.1128/AAC.45.12.3375-3380.2001 Mahamoud, A., Chevalier, J., Alibert-Franco, S., Kern, W. V., and Pages, J. M. (2007). Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. J. Antimicrob. Chemother. 59, 1223–1229. doi:10.1093/jac/dkl493 Marks, N., Suahar, A., and Benuck, M. (1981). Peptide processing in the central nervous system. Adv. Biochem. Psychopharmacol. 28, 49–60. Markwick, P. R., and McCammon, J. A. (2011). Studying functional dynamics in molecular mechanics and continuum models. Adv. Drug Deliv. Rev. 71, 126601. doi: 10.1088/0034-4885/71/12/ 126601 Mao, W., Warren, M. S., Lee, A., Mistry, A., and Loontiens, F. G. (2001). MexXY- OprF efflux pump is required for antagonism of aminoglycosides by divalent cations in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 45, 2001– 2007. doi:10.1128/AAC.45.7.2001-2007.2001 Marks, N., Suahar, A., and Benuck, M. (1981). Peptide processing in the central nervous system. Adv. Biochem. Psychopharmacol. 28, 49–60. Markwick, P. R., and McCammon, J. A. (2011). Studying functional dynamics in bio-molecules using accelerated molecular dynamics. Phys. Chem. Chem. Phys. 13, 20053–20065. doi:10.1039/c1cp21200k Martinez, J. L., and Baquer, F. (2014). Emergence and spread of antibiotic resistance: setting a parameter space. Ups. J. Med. Sci. 119, 68–77. doi: 10.3109/ 03009734.2014.901444 Maseda, H., Yoneyama, H., and Nakae, T. (2000). Use of the fluorescent probe 1-N- phenyl-naphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 44, 658–664. doi:10.1128/AAC.44.4.658-664.2000 Mason, E. O. Jr., Wald, E. R., Bradley, J. S., Barson, W. J., and Kaplan, S. L. (2003). Macrolide resistance among middle ear isolates of Streptococcus pneumoniae observed at eight United States pediatric centers: prevalence of M and MLSB phenotypes. Pediatr. Infect. Dis. J. 22, 623–627. doi:10.1097/01.inf.00001124.06451.93 Masuda, N., Gotoh, N., Ohya, S., and Nishino, T. (1996). Quantitative correlation between susceptibility and Oprl production in NfxB mutants of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 40, 909–913.
Masuda, N., and Ohya, S. (1992). Cross-resistance to meropenem, cephems, and quinolones in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 36, 1847–1851. doi: 10.1128/AAC.36.9.1847

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., and Nishino, T. (2001). Hypersusceptibility of the Pseudomonas aeruginosa mfxB mutant to beta-lactams due to reduced expression of the ampC beta-lactamase. Antimicrob. Agents Chemother. 45, 1284–1286. doi: 10.1128/AAC.45.4.1284-1286.2001

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., and Nishino, T. (2000a). Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 44, 2242–2246. doi: 10.1128/AAC.44.9.2242-2246.2000

Morita, Y., Murata, T., Mima, T., Shiota, S., Kuroda, T., Mizushima, T., et al. (2003). Morita, Y., Tomida, J., and Kawamura, Y. (2015). Efflux-mediated fluoroquinolone resistance in a multidrug-resistant Pseudomonas aeruginosa clinical isolate PA7: identification of a novel MexS variant involved in upregulation of the mexE-mexF-oprN multidrug efflux operon. Front. Microbiol. 6:8. doi: 10.3389/fmicb.2015.00008

Morris, J. D., Hewitt, J. L., Wolfe, L. G., Kamatkar, N. G., Chapman, S. M., Diener, J. M., et al. (2011). Imaging and analysis of Pseudomonas aeruginosa swimming and rhimnolipid production. Appl. Environ. Microbiol. 77, 8310–8317. doi: 10.1128/AEM.06644-11

Mortimer, P. G., and Piddock, L. J. (1991). A comparison of methods used for measuring the accumulation of quinolones by Enterobacteriaceae, Pseudomonas aeruginosa and Staphylococcus aureus. J. Antimicrob. Chemother. 28, 639–653. doi: 10.1093/jac/28.5.639

Murakami, S. (2008). Multidrug efflux transporter, AcrB—the pumping mechanism. Curr. Opin. Struct. Biol. 18, 459–465. doi: 10.1016/j.sbi.2008.06.007

Murakami, S., Nakashima, R., Yamashita, E., Matsumoto, T., and Yamaguchi, A. (2006). Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. Nature 443, 173–179. doi: 10.1038/nature05076

Murakami, S., Nakashima, R., Yamashita, E., and Yamaguchi, A. (2002). Crystal structure of bacterial multidrug efflux transporter AcrB. Nature 419, 587–593. doi: 10.1038/nature01050

Murakami, S., Tamura, N., Saito, A., Hirata, T., and Yamaguchi, A. (2004). Extramembrane central pore of multidrug exporter AcrB in Escherichia coli plays an important role in drug transport. J. Biol. Chem. 279, 3743–3748. doi: 10.1074/jbc.M308893200

Nagano, K., and Nikaido, H. (2009). Kinetic behavior of the major multidrug efflux pump AcrB of Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 106, 5854–5858. doi: 10.1073/pnas.0810595106

Nakashima, R., Sakurai, K., Yamasaki, S., Hayashi, K., Nagata, C., Hoshino, K., et al. (2013). Structural basis for the inhibition of bacterial multidrug exporters. Nature 500, 102–106. doi: 10.1038/nature12300

Nakashima, R., Sakurai, K., Yamasaki, S., Nishino, K., and Yamaguchi, A. (2011). The structure of the multidrug exporter AcrB reveal a proximal multisite drug binding pocket. Nature 480, 565–569. doi: 10.1038/nature10641

Nakayama, K., Ishida, Y., Ohtsuka, M., Kawato, H., Yoshida, K., Yokomizo, Y., et al. (2003). MexAB-OprM-specific efflux pump inhibitors in Pseudomonas aeruginosa. Part 1: discovery and early strategies for lead optimization. Bioorg. Med. Chem. Lett. 13, 4201–4204. doi: 10.1016/j.bmcl.2003.07.024

Narita, S., Eda, S., Yoshiihara, E., and Naka, T. (2003). Linkage of the efflux–pump expression level with substrate extrusion rate in the MexAB-OprM efflux pump of Pseudomonas aeruginosa. Biochim. Biophys. Res. Commun. 308, 922–926. doi: 10.1016/S0006-291X(03)01512-2

Nikaido, H. (1996). Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. 178, 5835–5839.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. 67, 593–656. doi: 10.1128/MMBR.67.4.593-656.2003

Nikaido, H. (2011). Structure and mechanism of RND-type multidrug efflux pumps. Adv. Enzymol. Relat. Areas Mol. Biol. 77, 1–60. doi: 10.1002/9780470920541.ch1

Nikaido, H., Basina, M., Nguyen, V., and Rosenberg, E. Y. (1998). Multidrug efflux pump AcrAB of Salmonella typhimurium excretes only those beta-lactam antibiotics containing lipophilic side chains. J. Bacteriol. 180, 4686–4692.

Nikaido, H., and Normark, S. (1987). Sensitivity of Escherichia coli to various beta-lactams is determined by the interplay of outer membrane permeability and degradation by periplasmic beta-lactamases: a quantitative predictive treatment. Mol. Microbiol. 1, 29–36. doi: 10.1111/j.1365-2958.1987.tb00523.x

Nikaido, H., and Pages, J. M., et al. (2011). Imaging and analysis of multidrug efflux pumps in Staphylococcus aureus. FEMS Microbiol. Rev. 36, 340–363. doi: 10.1111/j.1571-6976.2011.00290.x

Nikaido, H., and Rosenberg, E. Y. (1993). Porin channels in Escherichia coli: studies with liposomes reconstituted from purified proteins. J. Bacteriol. 153, 241–252.

Nishino, K., Nikaido, E., and Yamaguchi, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1794, 834–843. doi: 10.1016/j.bbadis.2009.02.002

Nishino, K., and Yamaguchi, A. (2002). EvgA of the two-component signal transduction system modulates production of the yhi multidrug transporter in Escherichia coli. J. Bacteriol. 184, 2319–2323. doi: 10.1128/JB.184.4.2319-2323.2002
ntsogo-Enguene, V. Y., Verchère, A., Phan, G., Broutin, I., and Picard, M. (2015). Catch me if you can: a biotinylated proteoliposome affinity assay for the investigation of assembly of the MexA-MexB-OprM efflux pump from Pseudomonas aeruginosa. Front. Microbiol. 6:541. doi: 10.3389/fmicb.2015.00541

Oaktan, A., Yoneyama, H., and Nakae, T. (1997). Use of fluorescence probes to monitor function of the subunit proteins of the MexA-MexB-OprG drug extrusion machinery in Pseudomonas aeruginosa. J. Biol. Chem. 272, 21964–21969. doi: 10.1074/jbc.272.35.21964

Ohene-Agyei, T., Lea, J. D., and Venter, H. (2012). Mutations in MexB that affect the efflux of antibiotics with cytoplasmic targets. FEMS Microbiol. Lett. 333, 20–27. doi: 10.1111/j.1574-6968.2012.02594.x

Olatan, A. O., Morand, S., and Rolain, J. M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front. Microbiol. 5:643. doi: 10.3389/fmicb.2014.00643

Olivares, J., Bernardini, A., Garcia-Leon, G., Corona, F. M. B. S., and Martínez, J. L. (2013). The intrinsic resistance of bacterial pathogens. Front. Microbiol. 4:103. doi: 10.3389/fmicb.2013.00103

Ong, C. T., Tessier, P. R., Li, C., Nightingale, C. H., and Nicolau, D. P. (2007). Antimicrob. Agents Chemother. 44, 2233–2241. doi: 10.1128/AAC.44.9.2233-2241.2000

Oo, K. (2004). Efflux-mediated multiresistance in Gram-negative bacteria. Clin. Microbiol. Infect. 10, 12–26. doi: 10.1111/j.1469-069X.2004.00763.x

Oo, K. (2005). Efflux-mediated antibacterial resistance. J. Antimicrob. Chemother. 56, 20–51. doi: 10.1093/jac/dki171

Oo, K. (2011). Pseudomonas aeruginosa: resistance to the max. Front. Microbiol. 2:65. doi: 10.3389/fmicb.2011.00065

Oo, K. (2013). "Pseudomonas aeruginosa efflux pumps," in: Microbial Efflux Pumps, eds E. W. Yu, Q. Zhang, and M. H. Brown (Norfolk: Caister Academic Press).

Oo, K. (2014). Stress responses as determinants of antimicrobial resistance in Pseudomonas aeruginosa: multidrug efflux and more. Can. J. Microbiol. 60, 783–791. doi: 10.1139/cjm-2014-0666

Potron, A., Poirel, L., and Nordmann, P. (2015). Emerging broad-spectrum resistance in Pseudomonas aeruginosa and Acinetobacter baumannii: mechanisms and epidemiology. Int. J. Antimicrob. Agents 45, 568–585. doi: 10.1016/j.ijantimicag.2015.03.001

Pourmaras, S., Maniati, M., Spanakis, N., Ikonomidou, A., Tsioss, P. T., Tsakris, A., et al. (2005). Spread of efflux pump-overexpressing, non-metallo-beta-lactamase-producing, meropenem-resistant but cefazidime-susceptible Pseudomonas aeruginosa in a region with BlaVIM endocytosis. J. Antimicrob. Chemother. 56, 761–764. doi: 10.1093/jac/dki296

Pumbwe, L., and Piddock, L. J. (2000). Two efflux systems expressed in multidrug-resistant Pseudomonas aeruginosa. J. Antimicrob. Chemother. 44, 2861–2864. doi: 10.1128/AAC.44.10.2861-2864.2000

Rampersad, S. N. (2012). Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability biosassays. Sensors (Basel) 12, 12347–12360. doi: 10.3390/s120912347

Raunest, M., and Kandt, C. (2012). Locked on one side only: ground state dynamics of the outer membrane efflux duct TolC. Biochemistry 51, 1719–1729. doi: 10.1021/bi201814s

Renau, T. E., Leger, R., Yen, R., She, M. W., Flamme, E. M., Sanglard, J., et al. (2002). Peptidomimetics of efflux pump inhibitors potentiate the activity of levofloxacin in Pseudomonas aeruginosa. Bioorg. Med. Chem. Lett. 12, 763–766. doi: 10.1016/S0960-894X(02)00066-9

Renau, T. E., and Lemoine, R. C. (2001). Efflux pump inhibitors to address bacterial and fungal resistance. Drugs Fut. 26, 1171–1178. doi: 10.1358/doi.2001.26.12.644122

Richmond, G. E., Chua, K. L., and Piddock, L. J. (2013). Efflux in Acinetobacter baumannii can be determined by measuring accumulation of H33342 (benzamide). J. Antimicrob. Chemother. 68, 1594–1600. doi: 10.1093/jac/dkt052

Riera, E., Cabot, G., Mulet, X., García-Castilló, M., Del Campo, R., Juan, C., et al. (2011). Pseudomonas aeruginosa carbapenem resistance mechanisms in Spain: impact on the activity of imipenem, meropenem and doripenem. J. Antimicrob. Chemother. 66, 2022–2027. doi: 10.1093/jac/dkr232

Riou, M., Carbonnelle, S., Arrain, L., Mesaros, N., Pinay, J. P., Bilocq, F., et al. (2010). In vivo development of antimicrobial resistance in Pseudomonas aeruginosa strains isolated from the lower respiratory tract of Intensive Care Unit patients with nosocomial pneumonia and receiving antipseudomonal therapy. Int. J. Antimicrob. Agents. 36, 513–522. doi: 10.1016/j.ijantimicag.2010.08.005

Rosen, B. P., and Kashket, E. R. (1978). "Energetics of active transport," in: Bacterial Transport, ed. B. P. Rosen (New York: Marcel Dekker Inc.).

Ruggerone, P., Murakami, S., Pos, K. M., and Vargiu, A. V. (2013a). RND efflux pumps: structural information translated into function and inhibition mechanisms. Curr. Top. Med. Chem. 13, 3079–3100. doi: 10.2174/1568026613136660220

Ruggerone, P., Vargiu, A. V., Collu, F., Fischer, N., and Kandt, C. (2013b). Molecular dynamics computer simulations of multidrug RND efflux pumps. Computat. Struct. Biotechnol. J. 5:e2013002008. doi: 10.1515/csbj-2013-00208

Russo-Marie, F., Roederer, M., Sager, B., Herzenberg, L. A., and Kaiser, D. (1993). Beta-galactosidase activity in single differentiating bacterial cells. Proc. Natl. Acad. Sci. U.S.A. 90, 8194–8198. doi: 10.1073/pnas.90.17.8194

Ruzin, A., Keeny, D., and Bradford, P. A. (2005). AcrAB efflux pump plays a role in decreased susceptibility to tigecycline in Morganella morganii. Antimicrob. Agents Chemother. 49, 791–793. doi: 10.1128/AAC.49.2.791-793.2005
Schulz, R., Vargiu, A. V., Ruggerone, P., and Kleinekathöfer, U. (2015). Computational study of correlated domain motions in the acrB efflux transporter. BioMed. Res. Int. 12:487298. doi:10.1155/2015/487298

Schumacher, A., Triller, T., Bohnert, J. A., Kummerer, K., Pages, J. M., and Shapiro, H. M. (2000). Membrane potential estimation by flow cytometry. Science 281, 16220–16229. doi:10.1126/science.281.4695-6655-66. 

Senn, H. M., and Thiel, W. (2009). QM/MM methods for biomolecular systems. Angew. Chem. Int. Ed. Engl. 48, 1198–1229. doi:10.1002/anie.200802019

Sennhuser, G., Amstutz, P., Briand, C., Storchenger, O., and Gutter, M. G. (2007). Drug export pathway of multidrug exporter AcrB revealed by Darpin inhibitors. PLoS Biol. 5:e7. doi:10.1371/journal.pbio.0050007

Sennhuser, G., Bukowska, M. A., Briand, C., and Gutter, M. G. (2009). Structural organization of the multidrug exporter MexB from Pseudomonas aeruginosa. J. Mol. Biol. 389, 134–145. doi:10.1016/j.jmb.2009.04.001

Sgrignani, J., and Magistrato, A. (2013). First-principles modeling of biological systems and structure-based drug-design. Curr. Comput. Aided Drug Des. 9, 15–34.

Shapiro, H. M. (2000). Membrane potential estimation by flow cytometry. Methods 21, 271–279. doi:10.1006/meth.2000.1007

Steinbrecher, T., and Elsner, M. (2013). QM and QM/MM simulations of proteins. Methods Mol. Biol. 924, 91–124. doi:10.1007/978-1-62703-017-5_5

Steinbrecher, T., Prock, S., Reichert, J., Wadhwan, P., Zimpfer, B., Bürck, I., et al. (2012). Peptide-lipid interactions of the stress-response peptide TisB that induces bacterial persistence. Biophys. J. 103, 1460–1469. doi:10.1016/biophysj.2012.07.060

Stepphane, F., Ceccarelli, M., and Marchi, M. (2001). Dynamics of hydration in hen egg white lysozyme. J. Mol. Biol. 311, 409–419. doi:10.1006/jmbi.2001.4860

Su, C. C., and Yu, E. W. (2007). Ligand-transporter interaction in the AcrB multidrug efflux pump determined by fluorescence polarization assay. FEBS Lett. 581, 4972–4976. doi:10.1016/j.febslet.2007.09.035

Sugawara, E., Nagano, K., and Nikiado, H. (2010). Factors affecting the folding of Pseudomonas aeruginosa OprF porin into the one-domain open conformer. MBio 1:e00228–10. doi:10.1128/MBio.00228-10

Sugawara, E., Nestorovich, E. M., Bezrukov, S. M., and Nikiado, H. (2006). Pseudomonas aeruginosa porin OprF exists in two different conformations. J. Biol. Chem. 281, 16220–16229. doi:10.1074/jbc.M600680200

Sun, J., Deng, Z., and Yan, A. (2014). Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochem. Biophys. Res. Commun. 453, 254–267. doi:10.1016/j.bbrc.2014.05.090

Symonov, M., Bokma, E., Koronakis, E., Hughes, C., and Koronakis, V. (2009). The assembled structure of a complete tripartite bacterial multidrug efflux pump. Proc. Natl. Acad. Sci. U.S.A. 106, 7173–7178. doi:10.1073/pnas.090066-9310

Takatsuka, Y., Chen, C., and Nikiado, H. (2010). Mechanism of recognition of compounds of diverse structures by the multidrug efflux pump AcrB of Escherichia coli. J. Bacteriol. 192, 5659–5665. doi:10.1128/JB.00416-10

Thannasi, D. G., Cheng, L. W., and Nikiado, H. (1997). Active efflux of bile salts by Escherichia coli. J. Bacteriol. 179, 2512–2518.

Tikhonova, E. B., Wang, Q., and Zgurskaya, H. I. (2002). Chimeric analysis of the multicomponent multidrug efflux transporters from gram-negative bacteria. J. Bacteriol. 184, 6499–6507. doi:10.1128/JB.184.23.6499-65.07.2002

Tikhonova, E. B., Yamada, Y., and Zgurskaya, H. I. (2011). Sequential mechanism of assembly of multidrug efflux pump AcrAB-TolC. Chem. Biol. 18, 454–463. doi:10.1016/j.chembiol.2011.02.011

Trepout, S., Taveau, J. C., Benabdellah, H., Granier, T., Ducruix, A., Franakis, A. S., et al. (2010). Structure of reconstituted bacterial membrane efflux pump by cryo-electron tomography. Biochim. Biophys. Acta 1798, 1953–1960. doi:10.1016/j.bbamem.2010.08.019

Varga, M. (1992). Agents that increase the permeability of the outer membrane. Microbiol. Rev. 56, 395–411.

Vaccaro, L., Koronakis, V., and Sansom, M. S. P. (2006). Flexibility in a drug transport accessory protein: molecular dynamics simulations of MexA. Biophys. J. 91, 558–564. doi:10.1529/biophysj.105.080010

Vaccaro, L., Scott, K. A., and Sansom, M. S. P. (2008). Gating at both ends and breaching in the middle: conformational dynamics of TolC. Biophys. J. 95, 5681–5691. doi:10.1016/biophysj.108.136028

Van Bembeke, F., Pagès, J.-M., and Lee, V. J. (2010). Inhibitors of bacterial efflux pumps as adjuvant in antibiotic therapy and diagnostic tools for detection of resistance by efflux. Front. Anti Infect. Drug Discov. 1:138–175.

van den Berg van Saparoea, H. B., Lubelski, J., Van Merkerk, R., Mazurkiewicz, P. S., and Driessen, A. I. (2005). Proton motive force-dependent Hoescht 33342 transport by the ABC transporter LmrA of Lactococcus lactis. Biochemistry 44, 16931–16938. doi:10.1021/bi515497Y

Vargiu, A. V., Collu, F., Schulz, R., Pos, K. M., Zacharias, M., Kleinekathöfer, U., et al. (2011). Effect of the F610A mutation on substrate extrusion in the AcrB transporter: explanation and rationale by molecular dynamics simulations. J. Am. Chem. Soc. 133, 10704–10707. doi:10.1021/ja201120q

Vargiu, A. V., and Nikiado, H. (2012). Multidrug binding properties of the AcrB efflux pump characterized by molecular dynamics simulations. Proc. Natl. Acad. Sci. U.S.A. 109, 20637–20642. doi:10.1073/pnas.1218348109

Vargiu, A. V., Ruggerone, P., Opperman, T. J., Nguyen, S. T., and Nikiado, H. (2014). Molecular mechanism of MexB2319 inhibition of Escherichia coli AcrB multidrug efflux pump and comparison with other inhibitors. Antimicrob. Agents Chemother. 58, 6224–6234. doi:10.1128/AAC.07283-14

Verchère, A., Dezi, M., Adrien, V., Brouin, L., and Picard, M. (2015). In vitro transport activity of the fully assembled MexAB-OprM efflux pump from Pseudomonas aeruginosa. Nat. Commun. 6, 6890. doi:10.1038/ncomms7890

Verchère, A., Dezi, M., Brouin, L., and Picard, M. (2014). In vitro investigation of the MexAB efflux pump from Pseudomonas aeruginosa. J. Vis. Exp. 84:e50894. doi:10.3791/50894

Vidal-Aroca, F., Meng, A., Minz, T., Page, M. G., and Dreier, J. (2009). Use of the F610A mutation on substrate extrusion in the AcrB transporter: explanation and rationale by molecular dynamics simulations. J. Am. Chem. Soc. 127, 6224–6234. doi:10.1021/ja908201k

Visalli, M. A., Murphy, E., Projan, S. J., and Bradford, P. A. (2003). AcrAB efflux pumps are associated with reduced levels of susceptibility to tigecycline (GAR-936) in Proteus mirabilis. Antimicrob. Agents Chemother. 47, 665–669. doi:10.1128/AAC.47.2.665-669.2003
Viveiros, M., Martins, A., Paixao, L., Rodrigues, L., Martins, M., Couto, I., et al. (2008). Demonstration of intrinsic efflux activity of Escherichia coli K-12 AG100 by an automated ethidium bromide method. *Int. J. Antimicrob. Agents* 31, 458–462. doi: 10.1016/j.ijantimicag.2007.12.015

Walsmey, A. R., Martin, G. E., and Henderson, P. J. (1994). 8-Anilino-1-naphthalenesulfonate is a fluorescent probe of conformational changes in the D-galactose-H+ symport protein of *Escherichia coli*. *J. Biol. Chem.* 269, 17099–17019.

Walsh, F., and Amyes, S. G. (2007). Carbapenem resistance in clinical isolates of *Pseudomonas aeruginosa*. *J. Chemother.* 19, 376–381. doi: 10.1179/joc.2007.19.4.376

Wang, B., Weng, J., Fan, K., and Wang, W. (2012). Interdomain flexibility and pH-Induced conformational changes of AcrA revealed by molecular dynamics simulations. *J. Phys. Chem. B* 116, 3411–3420. doi: 10.1021/jp212221v

Watkins, W. J., Landaverry, Y., Leget, R., Litman, R., Renau, T. E., Williams, N., et al. (2003). The relationship between physicochemical properties, in vitro activity and pharmacokinetic profiles of analogues of diamine-containing efflux pump inhibitors. *Bioorg. Med. Chem. Lett.* 13, 4241–4244. doi: 10.1016/j.bmcl.2003.07.030

Webber, M. A., and Piddock, L. J. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *J. Antimicrob. Chemother.* 51, 9–11. doi: 10.1093/jac/dkg050

Wehmeier, C., Schuster, S., Fahnrich, E., Kern, W. V., and Bohnert, J. A. (2009). Site-directed mutagenesis reveals amino acid residues in the *Escherichia coli* RND efflux pump AcrB that confer macrolide resistance. *Antimicrob. Agents Chemother.* 53, 329–330. doi: 10.1128/AAC.00921-08

Welch, A., Awah, C. U., Jing, S., Van Veen, H. W., and Venter, H. (2010). Promiscuous partnering and independent activity of MexB, the multidrug transporter protein from *Pseudomonas aeruginosa*. *Biochem. J.* 430, 355–364. doi: 10.1042/B20091860

Xu, X. H. N., Brownlow, W., Huang, S., and Chen, J. (2003). Single-molecule detection of efflux pump machinery in *Pseudomonas aeruginosa*. *Biochem. Biophys. Res. Commun.* 305, 79–86. doi: 10.1016/S0006-291X(03)00692-2

Xu, Y., Lee, M., Moeller, A., Song, S., Yoon, B.-Y., Kim, H.-M., et al. (2011). Funnel-like hexameric assembly of the periplasmic adapter protein in the tripartite multidrug efflux pump in gram-negative bacteria. *J. Biol. Chem.* 286, 17910–17920. doi: 10.1074/jbc.M111.238535

Yamaguchi, A., Nakashima, R., and Sakurai, K. (2015). Structural basis of RND-type multidrug exporters. *Front. Microbiol.* 6,327. doi: 10.3389/fmicb.2015.00327

Yamaguchi, A., Udagawa, T., and Sawai, T. (1990). Transport of divalent cations with tetracycline as mediated by the transposon Tn10-encoded tetracycline resistance protein. *J. Biol. Chem.* 265, 4809–4813.

Yang, N. C., and Hu, M. L. (2004). A fluorimetric method using fluorescein di-beta-D-galactopyranoside for quantifying the senescence-associated beta-galactosidase activity in human foreskin fibroblast Hs68 cells. *Anal. Biochem.* 325, 337–343. doi: 10.1016/j.ab.2003.11.012

Yao, X.-Q., Kimura, N., Murakami, S., and Takada, S. (2013). Drug uptake pathways of multidrug transporter AcrB studied by molecular simulations and site-directed mutagenesis experiments. *J. Am. Chem. Soc.* 135, 7474–7485. doi: 10.1021/ja310548h

Yoshida, K., Nakayama, K., Ohtsuka, M., Kuru, N., Yokomizo, Y., Sakamoto, A., et al. (2007). MexAB-OprM specific efflux pump inhibitors in *Pseudomonas aeruginosa*. Part 7: highly soluble and in vivo active quaternary ammonium analogue D13-9001, a potential preclinical candidate. *Bioorg. Med. Chem.*** 15, 7087–7097. doi: 10.1016/j.bmc.2007.07.039

Yoshimura, F., and Nikaido, H. (1982). Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. *J. Bacteriol.* 152, 636–642.

Zechini, B., and Versace, I. (2009). Inhibitors of multidrug resistant efflux systems in bacteria. *Recent Patents Anti Infect. Drug Discov.* 4, 37–50. doi: 10.2174/157489109787236256

Zgurskaya, H. I., and Nikaido, H. (1999). Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 96, 7190–7195. doi: 10.1073/pnas.96.13.7190

Zhanel, G. G., Decorby, M., Adam, H., Mulvey, M. R., Mcrracken, M., Lagace-Wiens, P., et al. (2010). Prevalence of antimicrobial-resistant pathogens in Canadian hospitals: results of the Canadian Ward Surveillance Study (Canward 2008). *Antimicrob. Agents Chemother.* 54, 4684–4693. doi: 10.1128/AAC.00469-10

Zhou, T., Huang, D., and Calfisch, A. (2010). Quantum mechanical methods for drug design. *Curr. Top. Med. Chem.* 10, 33–45. doi: 10.2174/156802610790232242

**Conflict of Interest Statement:** Jürg Dreier is employee of Basilea Pharmaceutica International Ltd. Paolo Ruggerone declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Dreier and Ruggerone. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.