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Perspective

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Molecular Determinants of AcrB-Mediated Bacterial Efflux Implications for Drug Discovery

Miniperspective

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Bacterial resistance to antibiotics is a serious threat to public health, with "superbugs" such as MRSA (methicillin-resistant *Staphylococcus aureus*) responsible for nearly as many deaths in the U.S. as AIDS, viral hepatitis, and tuberculosis combined.[1] More than half of bacterial strains isolated from patients in American intensive care units are resistant to at least one antibiotic,[2] and globally >80% of isolates are resistant.[3] In 2004, the Infectious Disease Society of America (IDSA) issued its oft-cited "Bad Bugs, No Drugs" report, highlighting an emerging public health crisis stemming from a decline in the development of new antibiotics while resistance increasingly renders existing antibiotics ineffective.[4] In 2009 IDSA published an update, observing that the situation had grown still worse.[5]

Of particular concern is the increased occurrence of resistant infections due to the Gram-negative pathogens *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*, both in the U.S. and globally. Like MRSA, resistant Gram-negative infections result in longer hospital stays, greater morbidity, and significantly higher mortality rates.[1,6] Strains resistant to more than one class of antibiotics are now emerging, culminating in pan-resistant Gram-negative strains causing serious and complicated infections for which no treatment is available.[7−9] Tragically, *Acinetobacter* and *Pseudomonas* infections have emerged particularly among immunocompromised patient populations, where they threaten the hard-fought gains made in areas such as HIV and cancer chemotherapy.

Resistance mechanisms specific to each class of antibiotics, for example, deactivating enzymes such as β-lactamases and aminoglycoside-modifying enzymes, have been described, but a mechanism common to all is that of reduced cell entry, by either reduced diffusion into the cell or efflux from it.[10] Increased expression of efflux pumps is also often involved in multidrug resistance, and in some cases strains have acquired resistance via new efflux pumps obtained through horizontal gene transfer.[11,12] Unlike Gram-positive bacteria, Gram-negative species have highly promiscuous efflux systems and pump a broad range of xenobiotics. Gram-negative pathogens are further protected by an outer cell membrane, which significantly limits the influx of solutes. This second membrane is asymmetric, with a phospholipid inner leaflet and a lipopolysaccharide outer leaflet punctuated by water-filled porin channels that allow small hydrophilic substrates required for bacterial growth to reach specific uptake systems in the inner membrane. Even in the absence of increased efflux pump expression, basal levels of pump activity are major contributors to primary resistance and largely responsible for the reduced antibiotic susceptibility observed, for instance, in *P. aeruginosa* relative to *Hemophilis influenzae*. Thus, primary resistance due to efflux plagues early stage drug discovery.[13−15]

AcrB is part of the most prevalent efflux transporter among Gram-negative species. It resides in the cytoplasmic membrane and complexes with AcrA and the TolC outer-membrane channel to pump substrates from the periplasmic space to the exterior of the cell[16] (Figure 1). Substrates bind in the AcrB subunit and are extruded through conformational changes driven by a proton gradient maintained across the cytoplasmic membrane.[17] AcrB has been implicated in the clinical resistance of *Escherichia coli*,[18,19] *P. aeruginosa*,[20,21] and *Klebsiella pneumoniae*[22] to antibiotics representing numerous classes, including tetracyclines, aminoglycosides, (fluoro)quinolones, cephalosporins, and carbapenems. It is a ubiquitous and extremely promiscuous transporter and is, in our experience, possibly the single most significant hurdle to achieving therapeutic levels of antibacterials within Gram-negative cells.

Numerous investigators have noted differences in physicochemical characteristics between antibiotics and other drugs. However, the exact set of properties that make small molecules effective against Gram-negative cells remains poorly understood. Among the handful of studies seeking systematic trends between Gram-negative activity and molecular properties, a common theme is that bacteria (and Gram-negatives in particular) tend to be more susceptible to hydrophilic compounds.[23−31] O'Shea and Moser[28] come closest to a set of guidelines for distinguishing compounds with probable Gram-negative activity based on simple physicochemical properties amenable to change through medicinal chemistry. They compared a set of 147 marketed and late-stage compounds with antibacterial activity to a background of drugs and druglike compounds and found that those with Gram-negative activity are profoundly more polar than other classes of drugs (as much as 4 orders of magnitude in terms of water/octanol partitioning) and that they possess a skewed distribution of molecular weight tending toward but not exceeding 600 Da, consistent with the limits imposed by porins.[32] It followed that antibacterial discovery programs should focus their efforts on relatively small and polar compounds.

Interestingly, these recommendations were somewhat inconsistent with our individual experiences within various lead optimization efforts, in which polarity seemed detrimental to Gram-negative activity, particularly by increasing the apparent susceptibility of compounds to efflux. To better understand this

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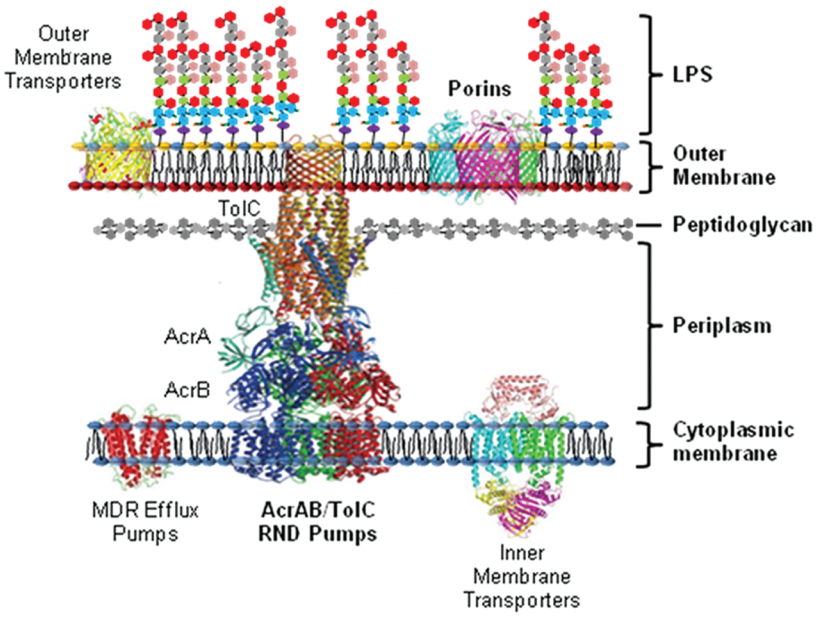

Figure 1. Cartoon representation of the Gram-negative cell envelope highlighting some of the many barriers to small molecule penetration. The lipopolysaccharide (LPS) chains emanating from the outer membrane present an initial and effective barrier to all but generally polar compounds small enough to pass through porin proteins.[32] In the periplasm, compounds too polar to diffuse rapidly through the cytoplasmic inner membrane are effluxed primarily by RND-type pumps (AcrAB/TolC system or equivalent),[16] and compounds in the cytoplasm are susceptible to efflux by a multitude of other pumps (e.g., multidrug resistant (MDR) pumps). Uptake of nutrients is facilitated by specific transporters in the inner and outer membranes. In addition to the more generalized mechanisms represented here, there are multiple species-specific and compound-specific mechanisms of acquired and innate resistance. Protein structures used in this figure were obtained from the Orientations of Proteins in Membranes database.[49]

discrepancy, we conducted a retrospective analysis on antibacterial compounds from discovery programs targeting Gram-negative pathogens at AstraZeneca. We used antimicrobial activities, measured as minimal inhibitory concentrations (MICs),[33] against a strain of *H. influenzae* lacking AcrB (and thus extrusion via its major AcrABTolC efflux pump) and its isogenic parental strain. *H. influenzae*, though not a serious Gram-negative pathogen, is a convenient model for this study. In contrast to *E. coli* and *P. aeruginosa*, it possesses only one orthologue each of AcrA, AcrB, and TolC, making inactivation of this major efflux system straightforward. In addition, the diameter of outer membrane porins in *H. influenzae* is larger than those from other Gram-negative species,[34−36] providing for a richer data set because a larger number and variety of compounds are active against this species. In order to normalize for differences in biochemical potency among compounds, our key parameter was an efflux ratio, defined as $MIC_{parent}/MIC_{mutant}$. The impact of other factors, including additional efflux systems that might show differential activity toward particular classes of compounds, is also minimized or canceled altogether by using the ratio of the AcrB knockout to wild-type MICs. Allowing for 2-fold variability in individual MIC values, compounds that exhibited ratios of >4 were considered as significantly effluxed via AcrB. Thus, only compounds with activity against *H. influenzae acrB* at least two dilutions below the maximal screening concentration could be included in the analysis. In an attempt to exclude compounds acting through nonspecific modes of action, we omitted compounds that showed activity against *Candida albicans* at, or below, the maximal screening concentration (typically 200 μM). This resulted in a total of 3066 compounds included in the analysis. These compounds represent multiple programs and at least 50 different scaffolds, generally identified in enzyme-based HTS campaigns against genetically validated bacterial targets. A number of physicochemical descriptors were calculated, among which molecular weight (MW) and fractional polar surface area (FPSA)[37] provided the most illuminating trends. FPSA was chosen as a measure of polarity because it is normalized roughly to overall molecular size and is thus less correlated with molecular weight than total polar surface area or other hydrophobicity measures such as clogP. Figure 2 shows a scatter plot of FPSA vs MW for all compounds in the analysis, color-coded by efflux ratio (red represents compounds with an efflux ratio of

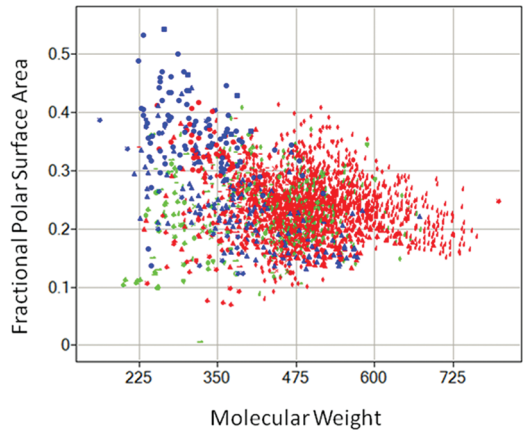

Figure 2. Scatter plot showing the relationship between fractional polar surface area (FPSA) and molecular weight for all compounds analyzed. Red represents ratios of >4. Green represents ratios of ≤4, and blue represents compounds with efflux ratios of ≤4 that possess polar functional groups implicated in circumventing efflux (see text and Figure 5). Marker shape represents chemical class.

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>4; green and blue represent compounds with efflux ratio of ≤4). The majority of compounds, which are significantly effluxed, are distributed around a mean MW of 500 and FPSA of 25% (or a clogP of 2 for the compounds in this set). Compounds with low efflux ratios are significantly smaller, with a mean MW of 400.

The monotonic relationship between efflux ratio and MW can be seen more clearly in Figure 3. As size increases, so does

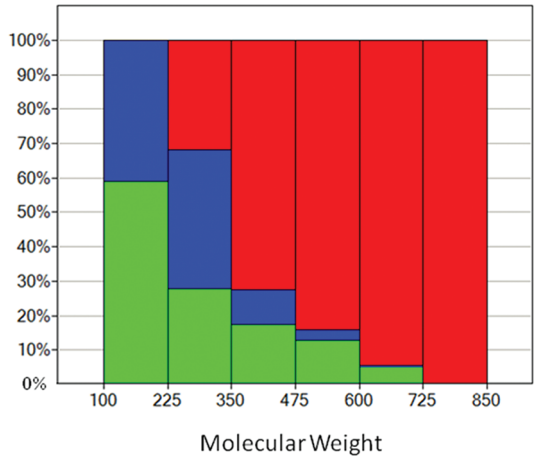

Figure 3. Normalized distribution of efflux ratios according to molecular weight, color-coded as in Figure 2. Bar height is shown normalized by the number of compounds in each bin so that 100% of the compounds in the 100−225 molecular weight range exhibit low efflux ratios, whereas 100% of the compounds in the 725−850 range are significantly effluxed.

the fraction of compounds with efflux ratios above 4. It is possible that compounds exhibit size-dependent affinity for AcrB and that larger compounds have greater rates of efflux. It is also possible that the larger compounds penetrate more slowly through the outer membrane and suffer from lower influx rates. A weakness of the efflux ratio is the inability to distinguish between these possibilities. However, the observation that nearly all compounds with efflux ratios less than 4 have molecular weights below 600 Da is consistent with the notion of size exclusion by porins[32] and hints at a model in which the rate of influx dominates the efflux ratio.

The trend with polar surface area is more complex. Figure 2 shows that compounds with low efflux ratios are distributed over a wider range of FPSA than those that are significantly effluxed. Strikingly, the most polar compounds in this group belong to established classes of antibiotics with Gram-negative activity and incorporate functional groups implicated in circumventing efflux. What we refer to as these "privileged" compounds are represented in blue in Figures 2−4. Figure 4 more clearly shows the relationship between efflux ratio and FPSA. Initially, the efflux ratio increases with the relative amount of polar surface area, consistent with our anecdotal experiences in proprietary chemical series. However, above about 25% FPSA, efflux begins to drop until the point where compounds with about equal amounts of polar and nonpolar surface area exhibit no efflux at all.

"Privilege" among compounds with low efflux ratios seems to be coupled more strongly to the presence of particular functional groups than it is with overall physical properties. The prevalence of discovery programs targeting orally available medications complicates the analysis, as there are many fewer

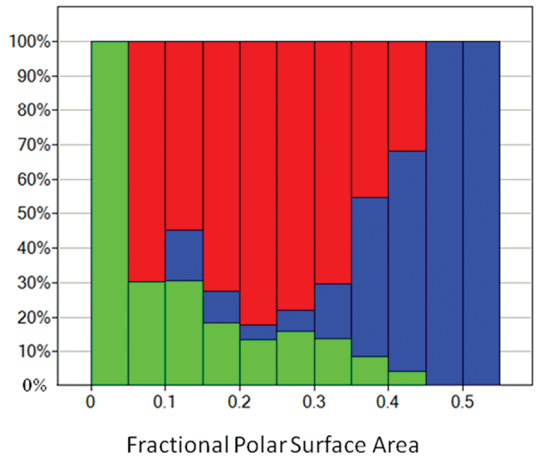

Figure 4. Normalized distribution of efflux ratios according to FPSA, color-coded as in Figure 2. Efflux exhibits a parabolic dependence on polarity, where moderately polar compounds (about 25% FPSA) show the highest propensity for efflux. Interestingly, nearly all compounds with low efflux ratios exhibit polar functional groups implicated in mechanisms that enhance potency and/or permeability, effectively circumventing efflux (see text and Figure 5).

small and polar compounds than there are in the moderately hydrophobic, 500 MW range. However, privileged compounds do appear relatively uniformly over much of the physical property space defined in Figure 2, demonstrating that a wide range of physical properties can result in low efflux ratios, so long as the presence of key functional groups is maintained. Notably, although the proprietary nature of the compounds in the analysis precludes a detailed breakdown, it can be seen that privilege spans numerous chemical classes, as shown by the different marker shapes in Figure 2.

Some examples of privileged compounds and the functional groups they display are shown in Figure 5. They exploit the following mechanisms:

(1) Periplasmic target. Compounds in this group (primarily β-lactams) must cross only the outer membrane to exert their mode of action for which the conventional rules of being small and polar seem to prevail.

(2) Irreversible inhibition. The oxaboroles, β-lactams (for example, carbapenems and cephalosporins), and hydroxamates[38] bind irreversibly (or with very low off-rates) to the target, sequestering them from efflux and reducing their need for high permeation rates.

(3) Self-promoted uptake. Compounds in this group, such as the aminoglycosides, exhibit basic functionalities that are thought to facilitate permeation through association with the outer membrane of Gram-negative cells.[39] It is thought that the basic centers of these compounds displace divalent metal cations bound among the anionic lipopolysaccharide chains of the outer leaflet. This results in destabilization of the LPS framework, effectively permeabilizing the outer membrane.

(4) Ion trapping. Compounds with weakly acidic functional groups (the β-keto acids in Figure 5b exhibit pK_a around 6.2[40]) take advantage of the pH gradient across the Gram-negative inner membrane[41] and ionize more fully in the cytosol than in the medium. Thus, the net permeability of these compounds into the cell exceeds the net rate out, effectively "trapping" them in the cytosol.[42,43]

Of course, in practice compounds can exploit more than one of these avenues. For example, the β-lactams combine action in

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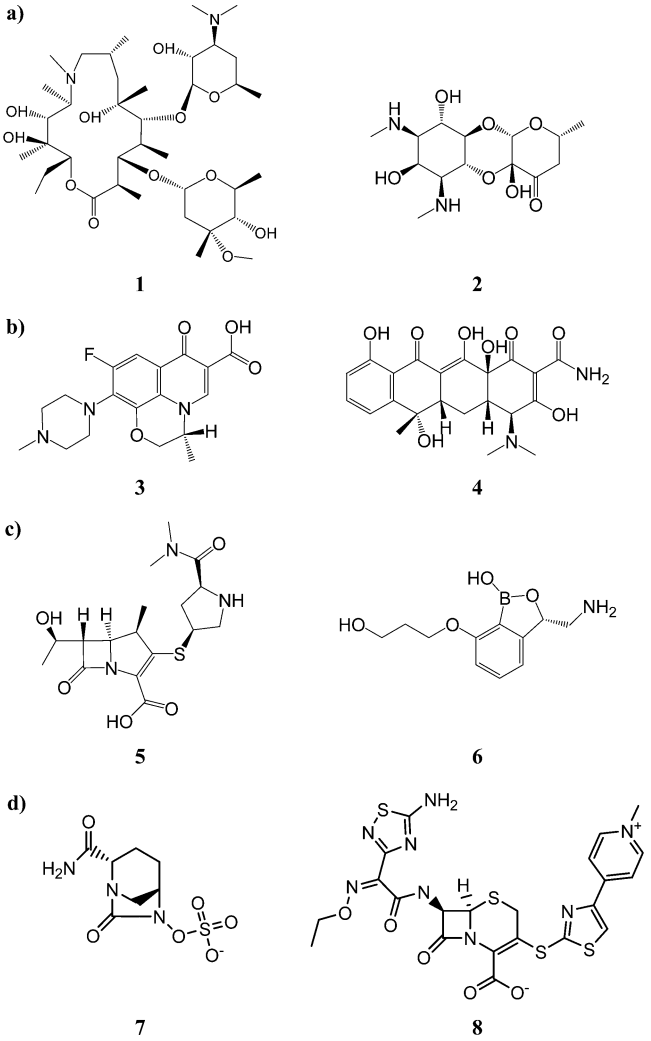

Figure 5. Examples of compounds with polar functional groups implicated in circumventing efflux. (a) Aminoglycosides azithromycin (**1**) and spectinomycin (**2**) utilize basic amines to exploit the self-promoted uptake pathway for improved permeability.[39] (b) Fluoroquinolones such as levofloxacin (**3**) and tetracycline(s) (**4**) exhibit acidic groups with finely tuned protonation states that effectively trap them in the cytosol.[44,45] (c) β-Lactams such as the carbapenem meropenem (**5**) and the oxaborole AN3365[50] (**6**) bind irreversibly or with slow off-rates via a chemical warhead, protecting them from efflux once they have reached the site of action. (d) Shown are compounds acting in the periplasm, in which polarity improves transit through porins and reduces affinity for AcrB and/or the cytoplasmic membrane (the β-lactamase inhibitor avibactam[51] (**7**) and the cephalosporin ceftaroline (**8**) are shown here).

the periplasm with effectively irreversible inhibition. The fluoroquinolones are known to bind Mg^{2+}, associated with the outer membrane, and it has been postulated that this enhances their permeability through self-promoted uptake,[44] although this model has been disputed.[45,46] It remains unclear whether the improved permeability observed among β-keto acids is due primarily to metal ion chelation or to ion trapping. A further boost may come from the incorporation of basic substituents at the 7-position on many fluoroquinolones, allowing them to exploit the self-promoted uptake pathway.

Even though the present analysis was confined to efflux mediated by a single pump in *H. influenzae*, the fact that it highlighted a number of compounds belonging to established classes with Gram-negative activity suggests that its implications may be more far-reaching. One reason to think so is that since the efflux ratio depends on influx rate, it provides information about the requirements for penetrating the outer membrane, which is morphologically similar across pathogenic species of Gram-negative bacteria targeted by drug discovery efforts. Another reason is the very prevalence of the AcrAB/TolC system and its homologues, which share broad but overlapping substrate specificities.[47,48] It is not unreasonable to expect similarities in physical property determinants of substrate binding affinity among these systems. Indeed, the specific examples in Figure 5 are all noted for clinical use in other more virulent species such as *E. coli* and *P. aeruginosa*.

One important implication for drug discovery is that targeting specific ranges of physical properties is not enough to guarantee antibacterial activity. Investigators should incorporate structural motifs that exploit one or more of the above avenues for circumventing efflux in the design and/or selection of novel compounds. Because of the apparent restriction on size (<600 Da), these combined observations may imply that the most promising approach to developing novel Gram-negative agents is to work within established classes.

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Biographies

John I. Manchester obtained his Ph.D. in Biophysics from SUNY Buffalo, NY, and was the recipient of a NORCUS Fellowship at Battelle Pacific Northwest National Laboratory in Richland, WA, modeling catalysis in cytochrome P450. He studied drug metabolism during a postdoc stay at the University of Rochester, NY, and worked in ADME property prediction at Camitro and ArQule prior to joining AstraZeneca in 2001, where he supports the discovery of novel anti-infectives using computational approaches.

Ed T. Buurman received his Ph.D. degree in Chemistry from the University of Amsterdam in 1991, studying the effect of cations on microbial metabolism and growth energetics. This was followed by postdoctoral research at the University of Chicago (IL) and University of Aberdeen (U.K.), working on various aspects of potassium ion transport in *Escherichia coli* and the importance of mannosyl transferases for virulence of *Candida albicans*, respectively. He then joined the collaborative antifungal drug discovery effort of Scriptgen Pharmaceuticals with Hoechst Marion Roussel, currently part of Sanofi. Three years later he moved to AstraZeneca R&D Boston since which he worked on multiple aspects of antimicrobial lead identification and characterization, ranging from target validation and hit deconvolution to mode of action studies.

Gregory S. Bisacchi is Associate Director, Infection Chemistry at AstraZeneca. Greg started his medicinal chemistry career at Squibb in New Jersey, in natural product based anti-infectives (including monobactams). Later, at Bristol-Myers Squibb, he worked in the areas of antivirals, antidiabetics, and pulmonary and cardiovascular agents. Greg received his B.S. and Ph.D. in Chemistry at UCLA, CA, and did a postdoc at Stanford University, CA.

Robert E. McLaughlin obtained his Ph.D. in Microbiology from Clemson University, SC, and studied lipo-oligosaccharide biology of *Hemophilus influenzae* and interactions of DNA binding proteins of *Streptococcus* sp. as a postdoctoral fellow at SUNY Buffalo, NY.

This was followed by research on sugar metabolism in *Streptococcus mutans* in the laboratory of Dr. Joseph Ferretti at the University of Oklahoma Health Science Center. He joined the faculty at OUHSC as a Research Assistant Professor in 1995, investigating virulence factors of *Streptococcus pyogenes*. With the introduction of microbial genome sequencing at OU his interests shifted to genomics and bioinformatics. He joined the Molecular Sciences group in Infection Discovery at AstraZeneca R&D Boston in 2001 as part of the Target Evaluation Team involved in bioinformatics for target analysis and selection.

■ ABBREVIATIONS USED

FPSA, fractional polar surface area; IDSA, Infectious Disease Society of America; LPS, lipopolysaccharide; MDR, multidrug resistant; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; RND, resistance nodulation cell division

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