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Defining New Chemical Space for Drug Penetration into Gram-Negative Bacteria

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Abstract

We live in the era of antibiotic resistance, and this problem will progressively worsen if no new solutions emerge. In particular, Gram-negative pathogens present both biological and chemical challenges that hinder the discovery of new antibacterial drugs. First, these bacteria are protected from a variety of structurally diverse drugs by a low-permeability barrier comprised of two membranes with distinct permeability properties, in addition to active drug efflux, making this cell envelope impermeable to most compounds. Second, chemical libraries currently used in drug discovery contain few compounds that can penetrate Gram-negative bacteria. As a result, intensive screening campaigns have led to few successes, highlighting the need for new approaches to identify regions of chemical space that are specifically relevant to antibacterial drug discovery. Herein, we provide an overview of emerging insights into this problem and outline a general approach to addressing it using prospective analysis of chemical libraries for the ability of compounds to accumulate in Gram-negative bacteria. The overall goal is to develop robust cheminformatic tools to predict Gram-negative permeation and efflux, which can then be used to guide medicinal chemistry campaigns and the design of antibacterial discovery libraries.

Introduction

Drug-resistant bacteria pose an increasing threat to public health[1-3]. In particular, Gram-negative pathogens pose a serious and urgent problem, comprising four out of the six

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Author Contributions

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Competing Interest Statement

Merck is a collaborating institution on the SPEAR-GN project and has provided in-kind support to the labs of D.S.T. and H.I.Z. D.S.T. serves on the External Advisory Board of the Institute for Research in Biomedicine, Barcelona; is a shareholder and has been a paid consultant and speaker for Merck; and has been a paid consultant or speaker for Eli Lilly, Elsevier, Emerson Collective, and Venenum Biosciences. H.I.Z. has been a paid speaker for Genentech and Novartis Research Institutes.

ESKAPE pathogens that have high intrinsic antibiotic resistance and can acquire additional resistance through mutations and horizontal gene transfer[4,5]. Infections caused by these pathogens are difficult to treat using existing drugs and, of the handful of new drugs in clinical development for these indications, none represents a novel structural class or mechanism of action[6]. Thus, a robust pipeline of new drugs must be developed to keep pace with the continuing emergence of drug-resistant pathogens.

Discovery of new antibiotics against drug-resistant Gram-negative pathogens is hindered by both biological and chemical challenges. The major biological challenge is the two-membrane cell envelope of Gram-negative bacteria, which presents a low-permeability barrier that protects the cells against a variety of structurally diverse drugs (Fig. 1)[7-12]. The outer membrane (OM) restricts penetration of large and hydrophobic compounds. Conversely, the cytoplasmic or inner membrane (IM) limits hydrophilic compounds from crossing[8,9]. Trans-envelope efflux pumps extrude molecules that have successfully penetrated through the membranes. This combination of orthogonal membranes and efflux pumps creates a barrier that is impermeable to most compounds[13-15]. This biological challenge is then the basis for the major chemical challenge, which is the identification of new molecules that can penetrate both membranes and avoid efflux. Existing screening collections have provided few such compounds[16,17] and our limited knowledge of the chemical properties required for Gram-negative penetration makes it difficult to design molecules with improved permeability[9,11,18]. To address this critical problem, we propose herein a comprehensive approach to understanding the biological and chemical factors that influence small-molecule permeation and efflux in Gram-negative bacteria, with the ultimate goal of developing robust, predictive cheminformatic models that can be used to design libraries and individual molecules with improved properties.

Recent advances in understanding the Gram-negative permeability barrier

Emerging hierarchy of rules of permeation.

Recent efforts have generated significant advances in rationalization of compound permeation across Gram-negative cell envelope, leading to emergence of a hierarchy of rules of permeation[12]. At the highest level, permeation into Gram-negative bacteria differs from that in Gram-positive bacteria because of the two-membrane cell envelope, with active efflux acting across both membranes (Fig. 1). The OM of Gram-negative bacteria is an asymmetric lipid bilayer consisting of an inner leaflet of phospholipids and an outer leaflet of glycolipids, primarily lipopolysaccharide (LPS)[19,20]. The glycolipids of different bacterial species vary in structure, resulting in membranes with differing permeability properties[21]. The OM also contains various porin proteins, which provide channels through which small, polar molecules can pass[7,22]. Furthermore, although multidrug efflux pumps are present in the cytoplasmic membranes of both bacterial phyla, active efflux across the low permeability barrier of the OM in Gram-negative cells is the most effective[14,23]. These efflux pumps establish non-linear, synergistic relationships between permeation across the OM and active efflux from both the periplasm and cytoplasm in Gram-negative bacteria, rendering the Gram-negative cell envelope virtually impermeable to certain classes of molecules[13,14].

At the next level, the OM and active efflux exclude compounds based on different molecular and physicochemical principles. Separation of the contributions of the OM barrier and active efflux to intracellular accumulation, and hence to the activities of antibiotics, can be achieved by breaching the OM barrier or changing the expression of efflux pumps. However, this is not a trivial task and a variety of approaches have been used. Permeabilization using cationic peptides such as polymyxins is effective against susceptible Gram-negatives but fails against intrinsically resistant species such as *Burkholderia* spp. and resistant clinical isolates that contain lipid A modifications[24]. Alternatively, the structure of the OM bilayer can be compromised by genetic mutations, but analysis of the results is complicated by pleiotropic effects of these mutations[25]. More recently, hyperporinated strains that produce an inducible large pore in the OM have been constructed, in which the OM is permeable to a variety of compounds[15,26]. On the other hand, efflux efficiency can be controlled by inactivation or overproduction of efflux pumps. The downside of this approach is that, in some species such as *Acinetobacter spp., Burkholderia spp.,* and others, efflux pumps are tightly integrated in cell physiology, and changes in their activities may affect multiple pathways[27,28]. Nevertheless, by modulating the OM barrier through hyperporination and deletion of efflux pumps, it has been shown that antibiotics cluster into different groups, depending on the main permeation barrier that they encounter while acting on cells, and that the OM barrier and efflux pumps recognize different properties in compounds[15]. Furthermore, at least in the case of *Escherichia coli,* these properties are orthogonal, with efflux pumps most efficiently recognizing compounds that can permeate the OM.

At the lower levels, bacterial species-specific differences in the composition and properties of the OM and efflux pumps also affect drug permeation. Although the basic architecture and composition of the cell envelope is conserved across different Gram-negative bacteria, different evolutionary paths and environmental conditions have led to significant variability in their properties[29]. A well-recognized example is the permeability barrier of *Pseudomonas aeruginosa*, which has only one-eighth the permeability of a typical *E. coli* cell[8]. In this case, the repertoires of efflux pumps (12 RND [resistance-nodulation-division] pumps in *P. aeruginosa* vs. four in *E. coli*) and OM porins (*P. aeruginosa* lacks general 600-Da cut-off porins that are abundant in *E. coli*) define the differences in permeability barriers. In addition, the structures of lipids, especially in the OM, define the differences in permeation in *Acinetobacter* spp. and *Burkholderia* spp.

Permeation through general porins of *E. coli*.

Predictive models based on physicochemical features of compounds and their interactions with *E. coli* porins have been reported recently[22,30] and success stories in using these models to optimize the activity of compounds against Gram-negative bacteria have also emerged[31-36]. Porin-mediated permeation is a multivariate, dynamic process that is governed by multi-step interactions and physicochemical properties of both the antibiotic and the channel. These interactions have been studied in detail using electrophysiological analyses in reconstituted lipid bilayers[37,38] Quantification of these intermolecular interactions is essential for revealing “rules” of permeation. Recently, a quantitative model was developed to predict the permeability of molecules through the OM based exclusively on structural features of *E. coli* general porins[39]. In general, antibiotic permeation through the

constriction zone (the narrowest part of the channel) of a porin requires the molecule to adopt a conformation that is compatible with both the shape and functional groups presented by the channel. The model integrates the size of the molecule and its electrostatic interactions with the porin through its charge and dipole moment. These properties have been identified as top predictors of the antibacterial activities of β-lactam and fluoroquinolone antibiotics and their dependence on permeation in *E. coli*[40], and in intracellular accumulation experiments discussed below[31,41].

New kinetic models of drug permeation against active efflux in the context of two membranes.

The synergy between the OM permeability barrier and active efflux was recognized in early studies based on multiplicative changes in the activities of antibiotics in bacterial strains with compromised OM barriers or efflux systems[42]. However, kinetic models that integrate both factors in the permeation across the Gram-negative cell envelopes have only recently been developed and validated[13,14] (Fig. 1). These models predict non-linear relationships between the extracellular concentration of a compound and its intracellular accumulation, based on the rate of compound permeation across the OM and the efficiency of its efflux. The system is controlled by two kinetic parameters: a barrier constant and an efflux constant that account for the efficiency of these two processes, respectively. Multiple transporters acting across either one or both membranes can be readily integrated into the model and their contributions to the intracellular accumulation can be predicted.

The quantitative kinetic models also revealed basic rules that define how the kinetic parameters are integrated in a multi-transporter system functioning in the context of two membranes[13,14]. The level of saturation and final accumulation of compounds depend on their mode of interaction with and passage across the permeability barriers. Amphiphilic compounds that permeate the membrane barriers by transmembrane diffusion are expected to diffuse slowly across the OM, whereas hydrophilic molecules that permeate through the water-filled porins are instead limited by the IM. The presence of multiple drug transporters located in same membrane (inner or outer) decide the kinetic outcome of drug translocation. Modeling and experimental data show that transporters located in the same membrane function in an additive manner. In contrast, a multiplicative response was observed between transporters acting across different membranes. A synergy could be predicted between efflux transporters and the transmembrane flux/diffusion operating at the same membrane. These kinetic models can now guide the development of experimental techniques to quantify the intracellular accumulation of compounds.

Despite these advances, only qualitative matches have been found between minimum inhibitory concentration (MIC) values of antibiotics predicted by mathematical models and those determined experimentally, and between antibacterial activity and experimentally determined intracellular concentrations across several series of antibiotics[13,43,44]. Further efforts are needed to develop kinetic models that can predict antibacterial activity based on intracellular accumulation of compounds[13].

The need to define new Gram-negative chemical space

Leading antibacterial drug discovery groups in industry have reported very low success rates in large screening campaigns for novel antibiotics, particularly for Gram-negative bacteria[16,17]. These studies also revealed the difficulties in converting hits identified in biochemical assays, and in whole-cell assays using permeability- and efflux-compromised strains, to lead compounds with antibacterial activity against wild-type bacteria, highlighting the need for a better understanding of permeability and efflux of small-molecule antibiotics.

Several studies have highlighted differences in physicochemical properties between known antibiotics and other classes of drugs[45,46], as well as hits from screening campaigns[47]. However, general and robust rules that can be used to predict Gram-negative permeation and efflux have not yet been developed[9,11,18]. Such rules would provide a critical means to design compounds for increased accumulation in Gram-negative bacteria, both in the context of medicinal chemistry optimization of screening hits as well as in the earlier-stage design of screening libraries tailored for antibacterial drug discovery.

Bacterial accumulation studies of antibiotics.

Toward this goal, numerous studies have sought to correlate physicochemical properties with bacterial compound accumulation (Table 1). Many of the earliest studies focused on OM permeability of β-lactam antibiotics in *E. coli*, as periplasmic cleavage by β-lactamase enzymes can be detected with an iodometric assay and used to infer the periplasmic concentration of the drug[48]. These studies identified inverse correlations between hydrophobicity and OM diffusion[49,50]. Similar correlations were also found for penetration through individual porins in *E. coli*[51] and when reconstituted in proteoliposomes[52]. For the OmpF and OmpC porins, increased penetration for zwitterionic analogues and decreased penetration for dianionic analogues was also noted. Analogues with especially bulky or protruding side chains penetrated less effectively than their hydrophobicity would predict, suggestive of inability to pass through the porin constriction zone.

Moving beyond β-lactam antibiotics, investigation of intracellular accumulation of tetracyclines in *E. coli* using a reporter gene system also revealed an inverse correlation with hydrophobicity[53]. Studies of quinolone antibiotics in *E. coli* using radiolabeling or intrinsic fluorescence provided some of the first detailed insights into accumulation kinetics, revealing an initial rapid accumulation phase followed by a second, slower phase[54,55].

In an early effort to correlate biochemical inhibition, compound accumulation, and antibacterial activity, further studies of fluoroquinolones identified moderate inverse correlations between hydrophobicity and accumulation in *E. coli* and *P. aeruginosa*, but a moderate positive correlation in the Gram-positive bacteria *Staphylococcus aureus*[56]. Notably, correlations between biochemical inhibition of DNA gyrase and MIC values could be improved by correcting for the accumulation level in *E. coli* and *P. aeruginosa*. This provided important proof of concept for integration of accumulation data with biochemical inhibition to predict antibacterial activity. A contemporaneous study found similar correlations for fluoroquinolone accumulation in these three bacteria and also tested the impact of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), an inhibitor of energy-

dependent efflux, finding increased accumulation in both of the Gram-negative bacteria[57]. However, a subsequent study found no correlations between hydrophobicity or molecular weight with accumulation or antibacterial activity of other quinolones in any of the three bacteria[58]. Moreover, later work with novel fluoroquinolones also showed no correlations in *E. coli*, nor any correlation between accumulation and antibacterial activity[59]. These divergent results highlight the challenges in identifying simple correlations between individual physicochemical parameters and bacterial accumulation and in extending those correlations to predict antibacterial activity.

Notably, later work highlighted two important approaches to probing the roles of other barrier components in compound accumulation studies, using *E. coli* porin mutants to dissect the effects of porins on OM passage[60], and *E. coli* spheroplasts to differentiate between OM and IM effects[61]. More recently, titratable induction of *P. aeruginosa* OprD porin expression in *E. coli*, in which the three major porins had been knocked out (*ompA*, *ompC*, *ompF*), was used to infer OprD-mediated OM permeability of a panel of carbapenems from the proportionality of antibacterial activity to the level of porin expression[41].

Bacterial accumulation studies of diverse compounds.

One of the main constraints of these early efforts may, ironically, have been their focus on antibiotics. This inherently limited the diversity of structures evaluated, thus potentially limiting the opportunity to identify general correlations between physicochemical properties and bacterial accumulation. In addition, studies that rely upon radiolabeling or intrinsic fluorescence are severely limited in the range of chemical structures that can be used. To address these limitations, two recent studies have used prospective, activity-independent analysis of diverse chemical libraries in bacterial compound accumulation assays. These compounds were not designed to have specific targets or antibacterial activity, but rather to cover a wide area of chemical space. This structure-agnostic analysis was enabled by the use of liquid chromatography–tandem mass spectrometry (LC-MS/MS) to quantitate intracellular compound concentrations in bacterial lysates[62].

In the first of these two studies, a general platform for prospective compound accumulation studies was reported[46]. Initially, concentration-, time-, and efflux-dependent accumulation of salicyl-AMS (5′-*O*-[*N*-salicylsulfamoyl]adenosine), a siderophore biosynthesis inhibitor[63], was assessed in *E. coli*. Accumulation at a single timepoint was then determined for a panel of 10 acyl-AMS analogues with diverse physicochemical properties in *E. coli*, *Bacillus subtilis* (Gram-positive), and *Mycobacterium smegmatis*, in the presence or absence of efflux pump inhibitors. Cheminformatic analysis using a set of 20 calculated physicochemical parameters identified significant positive correlations between accumulation in *E. coli* or *B. subtilis* and hydrophobicity, as well as inverse correlations with polarity and solubility. Additional positive correlations with *B. subtilis* accumulation were identified for surface area and rotatable bonds. For *M. smegmatis* accumulation, ring counts correlated positively while sp^3-hybridized carbon fraction and ring complexity correlated negatively. Several significant correlations were also identified between physicochemical properties and efflux sensitivity. Subsequently, based on correlations identified in *E. coli*,

two new salicyl-AMS analogues were designed and shown to exhibit higher accumulation as predicted. This provided proof-of-concept for the potential utility of this platform in developing predictive rules for use in antibiotic medicinal chemistry.

More recently, this approach was extended to a larger analysis of a diversity library of 100 compounds built primarily around five natural product-derived scaffolds[31,64]. Notably, only 12 positively-charged compounds accumulated significantly in *E. coli*, and within this subset, no correlation was observed with hydrophobicity or molecular weight, in contrast to some early studies of quinolones[53,57]. Subsequent synthesis and evaluation of designed analogues revealed a requirement for unhindered primary amines. Cheminformatic analysis using 297 calculated molecular descriptors led to a random forest classification model in which rotatable bonds and globularity were negative predictors of accumulation while amphiphilic moment (distance between hydrophobic and hydrophilic portions of a molecule) was a positive predictor. Evaluation of selected compounds in *E. coli* protoplasts, stripped of their OM and peptidoglycan layer, indicated limited differences in accumulation, suggesting that the OM represented the major selection filter for these compounds.

Notably, this study did not identify linear relationships between individual properties and accumulation, but rather established a set of threshold guidelines for accumulation, based on an unhindered primary amine, presence of some degree of hydrophobicity, rigidity, and low globularity. These were later codified into “eNTRy rules” (http://entry-way.org), representing presence of a primary amine (N), low three-dimensionality (T; globularity ≤0.25), and ≤5 rotatable bonds (R). These guidelines were then leveraged to design a new primary amine-containing analogue of deoxynybomycin, an antibiotic that is restricted to Gram-positive bacteria, which had increased accumulation and antibacterial activity against the Gram-negative bacteria *E. coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*. Notably, however, the analogue showed only modest activity against *P. aeruginosa*, indicating the need for further species-specific analyses. While correlations of antibacterial activity with positive charge are well-established in antibiotic medicinal chemistry[41,51,52] and the impact of molecular shape in porin passage through the outer membrane has also been suggested previously[52], this seminal study demonstrated the utility of large, prospective, activity-independent analyses of diversity libraries in the systematic identification of physicochemical properties that are predictive of accumulation and can be used in medicinal chemistry optimization. This dataset also served as the basis for the computational model for OM porin passage described above[39]. Moreover, the eNTRy rules have been successfully applied to develop Gram-negative active analogues of the Gram-positive-restricted antibiotics Debio-1452, an inhibitor of the enoyl-acyl carrier reductase enzyme FabI[32], and ribocil, an inhibitor of the *E. coli* riboflavin riboswitch[33].

In another recent study using LC-MS/MS detection, accumulation of >100 NAD-dependent DNA ligase (LigA) inhibitors was tested in *E. coli*[43]. Consistent with several earlier small studies[58,59], they found poor correlation between the overall level of bacteria-associated compound and antibacterial activity in compounds with matched biochemical activities. This suggested the importance of considering subcellular localization as well as accumulation kinetics in efforts to correlate biochemical inhibition, compound accumulation, and antibacterial activity.

A roadmap for future efforts

The studies highlighted above have provided useful insights into physicochemical properties that impact compound accumulation in Gram-negative bacteria and highlighted the challenges of correlating intracellular accumulation with antibacterial activity. This provides a roadmap for further efforts to develop robust, predictive tools for compound accumulation to support antibacterial drug discovery. However, several challenges remain:

1. Bespoke chemical libraries must be designed and synthesized to probe chemical space systematically.
2. Various bacterial species have considerable differences between their uptake and efflux pathways that must be assessed individually.
3. Combined effects of OM, IM, and efflux on overall accumulation and subcellular localization must be dissected.
4. Time and concentration dependence of accumulation kinetics may need to be considered to develop robust models.
5. Accumulation assays that are both high-throughput and structure agnostic are needed.
6. Cheminformatic models that accommodate multivariate and non-linear impacts of physicochemical properties on accumulation and kinetics must be developed.

We envision that future efforts should continue to involve large-scale, prospective analyses of designed chemical libraries for accumulation in multiple pathogenic Gram-negative bacteria, integrating assessments of OM, IM, and efflux contributions to accumulation, and considering both steady-state and kinetic accumulation data, as well as antibacterial activity where relevant, to develop robust cheminformatic models to predict accumulation.

With these considerations in mind, our labs at Memorial Sloan Kettering Cancer Center and the University of Oklahoma, with collaborators at Merck Research Labs, have launched one such multidisciplinary program called SPEAR-GN (Small-molecule Penetration and Efflux in Antibiotic-Resistant Gram-Negative bacteria) to pursue this approach (Fig. 2). The name draws analogy between the issues of permeation and efflux to equipment used in spearfishing, in which the spear must penetrate its target and is also barbed to prevent it from falling out. This work requires contributions from disparate disciplines, including diversity-oriented synthesis, medicinal chemistry, molecular microbiology, high-throughput assay technologies, analytical chemistry, biophysical modeling, and cheminformatics. Importantly, many key enabling technologies have been developed in recent years to make this feasible, and these are discussed in further detail below.

Evolution of enabling technologies

Bespoke chemical libraries.

Previous analyses have demonstrated the need to explore new regions of chemical space tailored specifically for antibacterial drug discovery[16,17,45-47]. Indeed, the “eNTRy” rules described above were formulated using a diversity library based on novel scaffolds[31,64].

Future efforts should continue to expand the range of scaffolds tested, as well as use *a priori* cheminformatic analysis to ensure that the libraries span a wide range of properties. Syntheses of such libraries at a scale sufficient for comprehensive analysis is now readily feasible following over two decades of work in the field of diversity-oriented synthesis[65,66].

In our work, we are also using a common set of substituents across different scaffolds and regioisomeric positions to assess the generality of models we derive from these libraries. In the limit, it may be possible to develop a universal model that is applicable to all chemotypes, but this is viewed as unlikely, given that penetration through OM porins and efflux by various pumps are protein-mediated processes that have some degree of substrate specificity. A more likely scenario is that models will have some aspects that are generally applicable across multiple chemotypes, while other aspects are chemotype-specific. In the other extreme, it may be necessary to develop a specific model for every chemotype. However, even if this is the case, generalizable platforms such as that in the SPEAR-GN project may enable relatively rapid development of such models for individual, high-value chemotypes.

Species-specific contributions of OM, IM, and efflux.

Previous large compound accumulation screens have given limited consideration to species-specific effects and subcellular localization[31,43,46]. As a consequence, the resulting models do not always translate between different bacterial species[31]. Inability of a drug to access the same subcellular compartment as its target may also contribute to poor correlations between accumulation and antibacterial activity[9,43]. Future efforts should incorporate such considerations directly into screening and modeling. Toward this end, we are conducting our studies in isogenic strains of *E. coli*, *P. aeruginosa*, and *A. baumannii* that allow cross-species comparisons and enable the relative roles of OM and IM passage and efflux to be differentiated, thus also allowing inference of subcellular localization. These four-strain sets are comprised of the wild-type strain, a hyperporinated strain engineered with an inducible large-bore porin that renders the OM freely permeable, a pan-efflux knockout strain, and a doubly compromised strain that is both hyperporinated and efflux deficient and provides direct assessment of IM permeability[14,15,26,40]. The hyperporinated strain provides advantages over spheroplasts or protoplasts, as it more closely approximates the normal cell envelope structure and is also readily grown in culture prior to induction of pore expression. Use of efflux mutants is preferred over small-molecule efflux inhibitors that can have pluripotent effects and alter cell envelope structure. This approach also provides practical advantages over subcellular fractionation, which is experimentally demanding and relatively low-throughput[67]. By comparing results across the four strains in each bacterial species, it is possible to determine the relative contributions of the OM, IM, and efflux to accumulation, thereby allowing identification the relevant barrier(s) and the development of predictive models for each barrier.

Consideration of accumulation kinetics.

Early studies of antibiotic accumulation kinetics generally showed a rapid initial uptake phase, followed by a slower second phase[54-57]. More recent large compound accumulation screens have used a single timepoint, assumed to approximate a steady-state concentration

while minimizing effects on bacterial growth[31,43,46]. However, we have reported detailed kinetic modeling of compound accumulation in Gram-negative bacteria indicating that differential effects of OM and IM passage, efflux, and target binding result in a complex kinetic landscape that is dependent upon both time and concentration[14]. Thus, in addition to these conventional steady-state analyses, which can be done for hundreds to thousands of compounds, we are also testing representative subsets of compounds at multiple timepoints and concentrations to enable determination of kinetic parameters. This may provide predictive models that are more robust and quantitative than the steady-state-based models developed to date.

High-throughput accumulation assays.

Recent large compound accumulation screens have relied upon manual, tube-based assays that limit throughput[31,43,46]. Analysis of larger libraries, across multiple strains and species, using multiple timepoints and concentrations will require a high-throughput assay that incorporates compound incubation, bacterial lysis, lysate extraction, and sample analysis, ideally in a structure-agnostic fashion. Recently, a 96-well plate assay was developed using a filter plate to separate cells from unbound compound, and leveraging automated solid-phase extraction (SPE) technology for rapid sample cleanup and MS analysis[44]. Use of the SPE-MS technology allowed each sample to be analyzed in as little as 9 sec. We are now using a similar approach in our own work. Of note, an alternative approach that quantitates the remaining extracellular compound has been reported[68]. While this approach avoids the lysis step, its dynamic range is likely limited by the small intracellular volume of the cells compared to the extracellular solution.

Importantly, a kaleidoscope of technologies has been developed to analyze bacterial compound accumulation (Table 2). Each of these approaches has advantages and limitations with respect to chemical structural requirements, sensitivity, throughput, and other considerations. Radiolabeing provides quantitative, sensitive measurements[54], but limits structural diversity and throughput for practical reasons. Numerous studies have used intrinsic fluorescence[55], allowing for more convenient and higher-throughput measurements, as well as increased resolution down to the single-cell or subcellular levels[69-75]. However, this also presents significant structural constraints and the alternative approach of labeling non-fluorescent compounds with a fluorophore introduces a large substituent that is likely to perturb accumulation. MS-based approaches in use in our project provide an attractive combination of structure independence, sensitivity, and throughput, although they require significant investments in instrumentation and extensive experimental optimization. LC-MS/MS with electrospray ionization (ESI) integrates chromatographic sample separation, is versatile in detecting a variety of chemotypes, and provides good limits of detection[62]. Matrix-assisted laser desorption/ionization (MALDI) has also been used to ‘image’ antibiotics in lungs of tuberculosis-infected animals[76,77], and it is possible that such an approach could be adapted to higher-throughput formats using cell culture samples. As noted above, the advent of automated SPE systems[44] has increased sample processing throughput considerably. Analysis speeds may be further increased to the ultrahigh-throughput regime through integration of microfluidic technologies[78,79] with mass spectrometric methods of compatible speed, such as desorption electrospray ionization

(DESI)[80,81] and acoustic droplet ejection (ADE)[82], although such direct sampling of biological matrices without sample cleanup also results in decreased sensitivity. Other approaches have also been reported recently for more specialized applications, including Raman spectroscopy[71,83], which allows label-free detection of antibiotics and metabolites inside intact bacterial cells; electrophysiology[84,85], which can be used to study antibiotic transport dynamics in whole cells and artificial membranes, and biomimetic membrane models[85,86], which allow detailed studies of the kinetics and mechanisms of passive diffusion.

Cheminformatic modeling approaches.

The ultimate goal of these studies is to develop predictive cheminformatic models that can be used to guide antibacterial drug discovery library design and medicinal chemistry efforts. Both regression and classification approaches have been used previously, including linear regression analysis, Pearson pairwise correlation coefficient analysis, principle component analysis, and random forest classification[31,46,53,56]. Notably, random forest classification can consider non-linear correlations and interactions between multiple parameters, which may be important for identifying useful relationships between physicochemical properties and bacterial accumulation. We envision that, as in QSAR (quantitative structure–activity relationship) modeling, the models are likely to be more useful to chemists if they are readily interpretable, rather than derived from a black box as in the case of neural networks. These models will be developed to predict overall accumulation as well as individual kinetic parameters where sufficient data is available. The models will then be tested by designing and testing new analogues predicted to have increased or decreased overall accumulation or kinetic rates for individual barriers, as demonstrated previously by us and others[31,46]. Validated models can then be used to inform subsequent medicinal chemistry efforts on individual scaffolds and broader efforts to design novel libraries for antibacterial drug discovery.

Conclusion

Recent efforts to screen collections of diverse compounds in a prospective, activity-independent fashion have demonstrated the utility of these approaches in identifying physicochemical properties that correlate with accumulation and can be used in the design of antibacterial drug discovery libraries as well as in medicinal chemistry campaigns[31,46]. We envision that future efforts in this area should continue to address the six challenges highlighted above. Studies should continue to expand the use of diverse, fit-for-purpose chemical libraries that probe broad regions of chemical space. Such libraries will certainly be distinct from existing screening collections that have evolved largely from campaigns against human protein targets, and may also be distinct from known classes of antibiotics. Analyses must be carried out across multiple bacterial species to differentiate between cheminformatic insights that are generally applicable and others that are species-specific. It will also be important to assess how broadly insights derived from laboratory model strains can be applied across various clinical isolates, including antibiotic-resistant strains. Subcellular localization must also be considered in assay designs to ensure that the derived models predict concentrations at the relevant site of action. Differentiation of impacts of the

individual barriers (OM, IM, efflux) may also allow separate models to be developed for each and may facilitate medicinal chemistry optimization efforts. Consideration of accumulation kinetics may also provide more robust models than steady-state measurements, although this will need to be balanced judiciously with the necessary reduction in compound throughput to ensure that sufficient data is available for cheminformatic modeling. To accommodate the daunting number of experiments envisioned above, there remains a great need for development of accumulation assays and quantitative detection methods that are both high-throughput and structure-agnostic. Ideally, these technologies should be broadly accessible to the scientific community with respect to both technical complexity and cost to ensure widespread uptake and maximal utility. Finally, any cheminformatic models developed must be readily interpretable in terms of structural and physicochemical properties in order to be useful to medicinal chemists. Regression models that correlate accumulation or kinetic parameters with specific properties, and classification models that identify relevant structural features will be most useful in guiding the design of new compounds. Conversely, although complex, black box models may be more accurate and robust, they are difficult to implement effectively in the context of prospective medicinal chemistry design.

In our own SPEAR-GN program, these efforts are well underway, including cheminformatic design and synthesis of new libraries, engineering of isogenic four-strain sets, development of a 96-well plate accumulation assay, integration with SPE-MS analysis, development of a computational tool for kinetic modeling, and completion of pilot assays using a panel of known antibiotics. This sets the stage for forthcoming large-scale screening to enable the development and validation of predictive cheminformatic models to guide antibacterial drug discovery efforts in the future.

After more than 40 years of study, the problem of predicting small-molecule permeability in Gram-negative bacteria remains largely unsolved. However, while the complexity of the Gram-negative cell envelope poses a formidable challenge, recent advances suggest that this problem is becoming increasingly tractable. Solving it will require a concerted, sustained, and multidisciplinary effort across the scientific community, and would have widespread and lasting impacts on public health.

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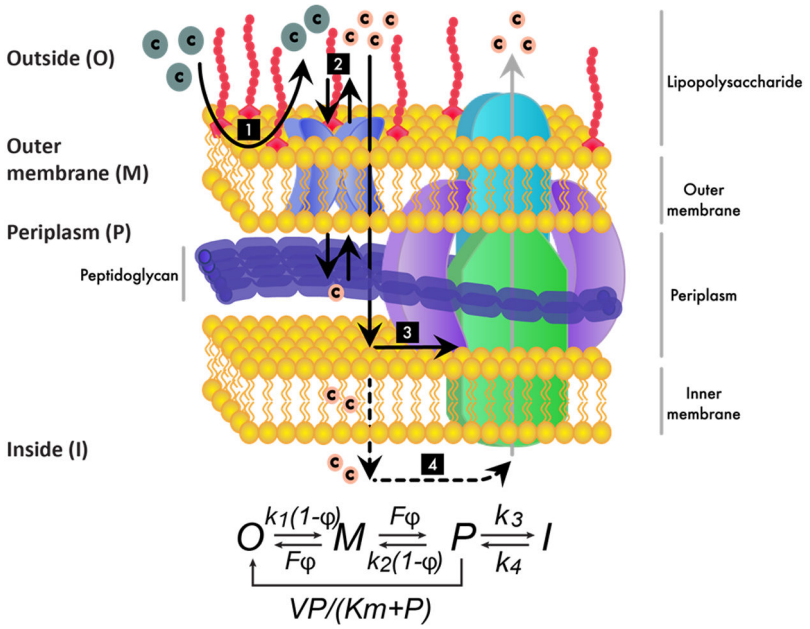

Fig. 1 |. The Gram-negative cell envelope and pathways of drug fluxes across it.

The envelope includes a network of lipopolysaccharides that limits penetration of large and hydrophobic compounds (green C) (1), porin channels that permit passage of small, hydrophilic molecules (red C) (2), and trans-envelope efflux pumps that capture their substrates from the periplasm (3) or cytosol (4) and pump them out of the cell. The kinetic scheme describes four compartments: outside the cell (O), within the outer membrane (M), in the periplasm (P), and in the cytoplasm (I). Active efflux is approximated as a Michaelis–Menten process, where V is the maximum achievable velocity, P is the concentration of drug in the periplasm, and K_M is the Michaelis constant. The binding to the membrane is postulated to be saturable, with the maximal flux F. The degree of saturation is denoted as ϕ, while k_1 through k_4 are microscopic rate constants[14]. Additional transporters can be readily integrated into this model[13].

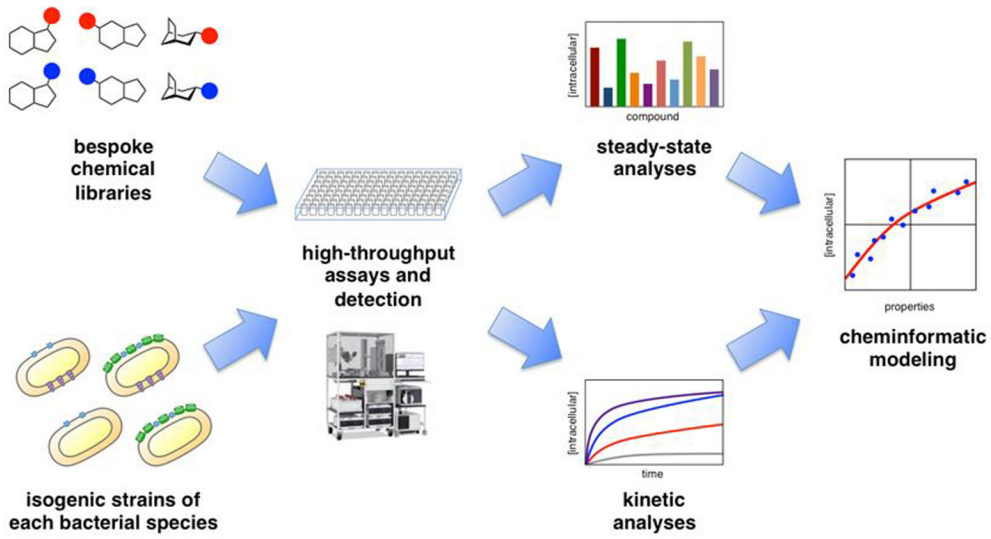

Fig. 2 |. Comprehensive approach to developing cheminformatic tools to predict Gram-negative bacterial compound accumulation.

Chemical libraries are designed to probe regions of chemical space and to assess impacts of scaffolds, regiochemistry, and substituents. For each bacterial species, four isogenic strains are used – wild-type, hyperporinated, efflux-deficient, and doubly-compromised – to dissect the contributions of the OM, IM, and efflux to net accumulation (blue = native porins, green = engineered large-bore porins, purple = efflux pumps). Compounds are screened in high-throughput assays and analyzed by high-throughput detection methods, such as solid-phase extraction–mass spectrometry. Steady-state analyses are performed for large collections of compounds while detailed kinetic analyses are carried out for smaller subsets of compounds to enable calculation of kinetic parameters. Regression and classification approaches are used to develop cheminformatic models that correlate physiochemical properties with accumulation, which are then tested further in additional analogues.

Table 1 |

Selected studies evaluating correlations between physicochemical properties and compound accumulation in bacteria.

Compounds tested (year)	Bacteria	Detection method	Correlations identified
8 β-lactams (1977)[49]	*E. coli*	β-lactamase/ iodometric	hydrophobicity [$P_{7.4}$[a]] (−)
8 cephalosporins (3 matched pairs) (1982)[50]	*E. coli M. morganii*	β-lactamase/iodometric	hydrophobicity [R_m[b]] (−)
11 cephalosporins (1983)[51]	*E. coli* (OmpF, OmpC, PhoE)	β-lactamase/iodometric	hydrophobicity [log P[c]] (−) zwitterionic (+; OmpF, OmpC) dianionic (−; OmpF, OmpC)
36 β-lactams (1985)[52]	proteoliposomes (OmpF, OmpC)	liposome swelling (OD_{400})	hydrophobicity [log P] (−) zwitterionic (+) dianionic (−) steric bulk (−)
5 tetracyclines (1992)[53]	*E. coli*	reporter gene	hydrophobicity [log K_D[d]] (−)
11 fluoroquinolones (1992)[56]	*E. coli P. aeruginosa*	intrinsic fluorescence	hydrophobicity [log $D_{7.2}$[e]] (−) inhibition x accum. ≈ activity
	S. aureus		hydrophobicity [log $D_{7.2}$[e]] (+)
8 fluoroquinolones (1992)[57]	*E. coli P. aeruginosa*	intrinsic fluorescence	hydrophobicity [$P_{app\text{-}7.2}$[e]] (−) efflux inhibition [CCCP[f]] (+)
	S. aureus		hydrophobicity [$P_{app\text{-}7.2}$[e]] (+)
15 quinolones (1993)[58]	*E. coli P. aeruginosa*	intrinsic fluorescence	not hydrophobicity [$P_{7.0}$[g]] not molecular weight
	S. aureus		not hydrophobicity [$P_{7.0}$[g]] not molecular weight
10 fluoroquinolones (2001)[59]	*E. coli*	intrinsic fluorescence	not hydrophobicity [log P[g]] not molecular weight
	S. aureus		not hydrophobicity [log P[g]] molecular weight (+)
10 sulfamoyladenosines (2014)[46]	*E. coli*	LC-MS/MS	hydrophobicity [log $D_{7.4}$[h], log P[i]] (+) polarity [log S[j], relPSA[k]] (−)
	B. subtilis		hydrophobicity (log P[j]) (+) polarity (log S[j], relPSA[k]) (−) surface area[l] (+) rotatable bonds (+)
	M. smegmatis		ring count (+) sp^3 fraction[m] (−) ring complexity[n] (−)
100 + 54 + 49 natural product-derived compounds (2017)[31]	*E. coli*	LC-MS/MS	primary amine (+) globularity (−) rotatable bonds (−) amphiphilic moment [vsurf_A[o]] (+)

Compounds tested (year)	Bacteria	Detection method	Correlations identified
20 pyrimidines + 80 pyridopyrimidinones (2018)[43]	*E. coli*	LC-MS/MS	not molecular weight hydrophobicity (log $D_{7.4}$[p]) (+) positive charge (+)

[a] Distribution coefficient in isobutanol–0.02 M phopshate buffer (pH 7.4).

[b] Reverse-phase thin-layer chromatography retention parameter in 0.05 M Na_2HPO_4–KH_2PO_4 buffer (pH 7.0) at 0% acetone (fit of data with 0–6% acetone).

[c] Partition coefficient in 1-octanol–water.

[d] Partition coefficient in chloroform–buffer (pH 7.0).

[e] Distribution coefficient in 1-octanol–0.1 M phosphate buffer (pH 7.2).

[f] Carbonyl cyanide *m*-chlorophenyl hydrazone.

[g] Partition coefficient in 1-octanol–0.1 M sodium phosphate buffer (pH 7.0).

[h] Distribution coefficient in 1-octanol–buffer (pH 7.4), calculated (Instant JChem).

[i] Partition coefficient in 1-octanol–water, calculated (Instant JChem and Virtual Computational Chemistry Lab).

[j] Solubility constant in water, calculated (Virtual Computational Chemistry Lab).

[k] Relative polar surface area, calculated (Instant JChem).

[l] van der Waals surface area, calculated (Instant JChem).

[m] sp^3 carbons ÷ carbons.

[n] Rings per ring system.

[o] Length of vector pointing from center of hydrophobic domain to center of hydrophilic domain, calculated (Molecular Operating Environment).

[p] Distribution coefficient in 1-octanol–buffer (pH 7.4), calculated (program not specified).

Table 2 |

Methods to measure intracellular compound accumulation in bacteria.

Method	Advantages	Limitations
radioactivity[54]	sensitive, quantitative	radioactive material, requires radiolabel that may alter activity and permeation, low-throughput, limited structural diversity
fluorimetry[69,70] (including fluorescence microscopy[71,72], spectrofluorimetry[73], flow cytometry[74,75])	ease of use, high-throughput, kinetics down to ms, resolution down to single cell level	requires intrinsic fluorescence or fluorescent label that may alter activity and permeation, limited structural diversity
LC-MS/MS[a,62]	label-free, quantitative to nM concentrations, in-line sample separation	low-throughput (5–20 min/sample)
SPE-MS[b,44]	label-free, high-throughput (<10 s/sample), in-line sample clean up	extensive optimization required, complex matrix can suppress signal
MALDI-MS[c,76,77]	label-free, high temporal and spatial (25 μm) resolution of drug distribution	requires appropriate matrix, moderate limit of detection
microfluidic ESI-MS[d,78,79]	label-free, high-throughput (45 s/sample), small sample size	no sample separation, frequent clogging, reproduciblity of microchip fabrication
DESI-MS[e,80,81]	label-free, ambient ionization, non-destructive, ultrahigh-throughput (<1 s/sample)	semi-quantitative, moderate limit of detection
ADE-MS[f,82]	label-free, ultrahigh-throughput (<1 s/sample)	no sample clean up, complex matrix will suppress signal
Raman spectroscopy[71,83]	label-free, *in cellulo* analysis, high-throughput	cellular background interference, requires chemical reaction of analyte
electrophysiology[84,85]	provides insights into roles of membrane potential dynamics	experimental complexity, low-throughput
biomimetic membrane models[85,86]	realistic model systems, sensitive, kinetics down to ms	concentration gradients due to reduced mass transport close to membrane may impact accumulation

[a] LC-MS/MS = liquid chromatography–tandem mass spectrometry (triple quadrupole).

[b] SPE-MS = solid-phase extraction–mass spectrometry.

[c] MALDI-MS = matrix-assisted laser desorption/ionisation mass spectrometry.

[d] ESI = electrospray ionization.

[e] DESI = desorption electrospray ionization.

[f] ADE = acoustic droplet ejection.