Top Med Chem
DOI: 10.1007/7355_2017_24
© Springer International Publishing AG 2017

The Antibiotic Future

Lynn L. Silver

Abstract Will the future of antibacterial therapy rely on an ongoing pipeline of new small molecule, direct-acting antibacterial agents that inhibit or kill bacterial pathogens, referred to here as antibiotics? What role will these small-molecule antibiotics have in the control of the bacterial infections of the future? Although there is today increased activity in the field of new antibiotic discovery, the history of this field over the past 30 years is a history of low output. This low output of new antibiotics does not encourage confidence that they can be central to the future control of bacterial infection. This low productivity is often blamed upon financial disincentives in the pharmaceutical industry, and on regulatory difficulties. But I believe that a critical underlying reason for the dearth of novel products is the fundamental difficulty of the science, coupled with a failure to directly grapple with the key scientific challenges that prevent forward motion. The future fate of antibiotic discovery will depend upon the degree to which the rate limiting steps of discovery are fully recognized, and the discovery technology turns to overcoming these blockades.

Keywords Antibiotic chemical space, Antivirulence, Combination therapy, Druggability, Entry barriers, Frequency-of-resistance, Hollow-fiber infection model, Hypersensitive screening, Monotherapy, Multi-targeting, Natural products, Synergy

Contents

1 Introduction
2 Excursion: Alternatives to Antibiotics
3 The Resistance Problem with Single Targets
  3.1 Measurement of Resistance

L.L. Silver (✉)
LL Silver Consulting, Springfield, NJ 07081, USA
e-mail: silverly@comcast.net

3.2 Overcoming Single-Target Resistance
3.3 Leveraging Single Targets by Truly Assessing Combinations
4 Old Targets new Chemical Matter
5 The Entry Problem
5.1 Gram Positives: Cytoplasmic Membrane Transit
5.2 Gram Negative Entry Barriers
6 Natural Products
7 Conclusion
References

1 Introduction

The rising tide of antibiotic resistance has compromised the usefulness of existing antibiotics against many human pathogens. While there usually are appropriate drugs still available for most infections, there are a growing number of problematic pathogens that are resistant to most antibiotics. In response to the need for new drugs and new paradigms to address the problem of antibiotic resistance, policy makers and funders have proposed that directing monetary incentives, rewards and support to small companies in the area will increase "innovation" [1–3]. The realization of innovation in this case is the introduction of new antibiotics (or other therapies) to combat rising resistance. That is, innovation is directly connected to the end product. But innovation is also a process involving the application of new technology and creative ideas at the level of individual innovative scientists. Can there be innovation without ultimate success? Will money drive innovation of either sort? Clearly financial incentives can bring more players to the table, but ultimate success will require efforts to forcefully confront the reasons for failure.

Much of antibiotic discovery over the past 20 years has focused on the discovery and exploitation of novel targets for new drugs, with little success. The challenges of this approach have been much discussed [4–10] and, in my opinion, are mainly due to the focus on inhibiting targets that are subject to rapid resistance selection and to the poverty of chemical libraries, which lack compounds with properties correlated with antibacterial activity and entry. Without an effort to seriously address these limitations, it is hard to see a rosy future for direct-acting small-molecule antibiotics.

Even though many large pharmaceutical companies have cut back their antibiotic discovery programs over the past 10 years or so, there has been a great deal of ongoing industrial and academic work in the field of antibiotic discovery. These efforts indeed have involved a great deal of innovation – creative individuals using new technologies to solve problems – of the sort mentioned above. Often, this work has been directed toward improving old classes of antibiotics in order to render them less susceptible to resistance mechanisms. A major success of current antibacterial discovery is the ongoing discovery of new combinations of often novel β-lactams and β-lactamase inhibitors. This effort has only recently produced

registered compounds which will allow the treatment of some very hard-to-treat pathogens including MDR *P. aeruginosa* (ZERBAXA®) and many carbapenem-resistant Enterobacteriaceae (CRE) (AVYCAZ®). A new aminoglycoside, plazomicin, in late clinical development is also targeted against CRE. But sometimes the efforts toward finding novel agents have been misguided: jumping head first into "genomics as panacea" without first considering the rate-limiting steps in discovery was a mistake. Can new technologies open up the field, or will they lead us astray? We must take hype with a grain of salt and continue to probe the assumptions underlying the application of new technology for its own sake. That said, it is important to bring in new viewpoints and different scientific backgrounds to attack these problems. But those new players must familiarize themselves with the field, its aims, its history, what has been tried and, importantly, what the roadblocks are.

Avycaz®
CAS 1393723-27-7
Ceftazidime·Avibactam

Zerbaxa®
CAS 1613740-46-7
Ceftolozane·Tazobactam

Plazomicin
CAS 1154757-24-0
ACHN 490

Will antibiotics, small molecules that directly inhibit the growth of or kill bacterial pathogens, be the mainstay of antibacterial therapy in the future? Is there a path to replenishing the failing pipeline? Will other modalities take the place of direct acting antibiotics? If there are to be innovative breakthroughs, then they will be, perforce, difficult to predict. Thus, it seems more productive to focus on the problems whose innovative solutions will lead to those breaks in the logjam. This focus necessitates a more historical discussion than perhaps envisioned by the editors of this book. Nonetheless, I maintain only by understanding the history of this field will we will make progress. After a small excursion this chapter will focus on the obstacles to antibacterial discovery that must be overcome in order to discover and develop small-molecule, direct-acting antibacterial agents: the antibiotics of our future.

2 Excursion: Alternatives to Antibiotics

It may be that novel, direct-acting antibiotics will continue to elude us and that the way forward for the treatment or prevention of bacterial infection lies in alternatives to antibiotics [11–16]. Indeed, interest in this field has been growing apace with strong emphasis on antivirulence approaches focused on antibodies [14] as well as small-molecule inhibitors of virulence mechanisms [15]. In any case, alternatives must be pursued.

A review [16] commissioned by the Wellcome Trust evaluated the pipeline of alternative antibacterial therapies. The authors limited their study of alternatives to "non-compound approaches that target bacteria or approaches that target the host". For example, they included antibodies but excluded small-molecules targeting virulence factors. There is clearly a great deal of effort in these "alternates-to-antibiotics" areas: antibodies and vaccines, both prophylactic and therapeutic; drugs inhibiting or modifying the activity of host factors involved in responses to bacterial infection; adjuvants that enhance the activity of antibiotics by enhancing permeability or inhibiting efflux pumps; inhibitors or blockers of antibiotic resistance functions. It is likely that some of these will take their place in the armamentarium, but most likely as adjuncts to existing antibiotics and to the small-molecule antibiotics of the future. It must be noted that many of these areas have been under study for many years with little progress. As the Wellcome review concluded, the need in these alternative approaches is not so much for discovery efforts but for "enhanced translational expertise" [16], including guidance in clinical development and regulatory paths for such alternate therapies. A recent review of antivirulence efforts, specifically including small molecules, seems quite upbeat [14]. The latter review discusses several antibodies that have been approved for prophylaxis or therapy against bacterial toxins, including those for *C. botulinum* BoNT toxins, *B. anthracis* PA toxin, and *C. difficile* toxin B (TcdB). Five antibodies (four monoclonals against toxins, one engineered bispecific antibody against two *P. aeruginosa* surface proteins) are listed as being in clinical trials and a large

variety of small molecules targeting various virulence factors and functions are under preclinical study. While antitoxin therapy has a long history [17], the development of non-antibacterial small molecules, those acting indirectly to affect infectious processes in the host, is a path less traveled.

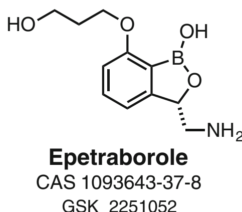
There are a number of such indirectly-acting small molecules under study for antivirulence, as described in a recent review [15]. Many of these have been uncovered in innovative screens or by in silico selection through genetic and structural study of the desired targets. In most cases, antivirulence activity is measured via surrogate in vitro assays that involve specific engineering of reporter bacterial strains. In order to develop such therapies for clinical use would require simple diagnostic tools to estimate the presence of the virulence mechanism in populations of clinical isolates (a surrogate for an MIC_{90}) as well as testing susceptibility to the compound of specific pathogens in the laboratory. Implementation of these tools may not be straightforward. It is often claimed that antivirulence approaches would discourage resistance development due to lack of selective pressure. That may be true in some cases, but resistance mutations would undoubtedly arise. Their amplification in the population would then be dependent upon the degree of selection. But resistance mutations can be useful in defining the actual molecular targets of a compound, in order to support the identification of the proposed antivirulence function as the critical target of inhibitor action. Since desirable antivirulence compounds lack antibacterial activity, it is generally difficult to select in vitro for resistance. Hence there is little evidence (for most small-molecule antivirulence compounds) that they are acting solely through the supposed target.

Other possibilities for therapeutic intervention may involve visionary synthetic biology and genome engineering approaches, including the very interesting possibility of using methods based on RNA-guided nucleases of the CAS/CRISPR (clustered, regularly-interspaced, short palindromic repeats) type [18–22]. Such proposed systems that recognize unique DNA sequences could narrowly target and kill specific pathogens or inactivate resistance mechanisms. The limitation for the CRISPR-based approach resides in design of suitable delivery methods which generally involve bacteriophage vectors. Such vectors would have to infect virtually every infecting pathogen.

3 The Resistance Problem with Single Targets

I subscribe to the view that inhibitors of single enzymes have a high likelihood of selecting for single-step resistance due to changes in the target molecule that result in large increases in MICs [9, 23–25]. Although there are exceptions (see below) I predict that most such inhibitors will fail in monotherapy, unless the resistance mutations lead to much lowered fitness. Conversely, successful monotherapeutic systemic antibiotics are those which have multiple targets, or targets that are the products of multiple genes or a pathway [23, 25]. While the potential peril of single-

targeted antibiotics was first hypothesized ~25 years ago [24], the first clear example of this was the failure due to rapid resistance development in a Phase II clinical trial of Epetraborole (GSK2251052) an inhibitor of leucyl-tRNA synthetase with excellent activity against Gram-negative pathogens [26–28]. This failure is discussed more fully below.



Sutterlin et al. discuss [29] "Antibacterial New Target Discovery" and note the potential for single-targeted compounds to yield single-step resistance in vitro. In the footnote to Table 2 of that chapter, an acceptable starting point for frequency of resistance (FoR) is stated as 10^{-8}. But what is an acceptable final FoR in vitro (and in vivo)? They note that the initial FoRs observed for a lead structure may be modulated favorably by chemical optimization, often by increasing target affinity via additional compound-target interactions. While the optimism voiced by Sutterlin et al. that in vitro results showing potentially high rates of resistance may not translate in vivo is sometimes true, there are definitely exceptions, as discussed below. However, each compound must be evaluated separately, and care taken that compounds are not mistakenly maintained in a development pipeline as a result of neglecting to test rigorously for resistance potential.

3.1 Measurement of Resistance

With advances in technology, one might think that methodologies for predicting the likelihood of clinically-important rapid resistance were well in hand. In vitro measures to ascertain the frequency of single-step resistance (FoR) generally involve plating a large inoculum of a bacterial strain on agar plates containing increasing amounts of test compound [30]. The rate of resistance (number of resistance mutations per bacterium per generation) can be measured by a Luria-Delbruck-type fluctuation test (or variations thereof) [31–35]. It is also useful to measure FoR in hypermutable (mutator) strains, where such frequencies may be increased 1,000-fold or more [36]. Such mutators occur at significant rates among clinical isolates of many pathogens and so are likely to play a role in resistance development [37–40]. There are also a variety of methods for selecting mutations by serial passage at sub-inhibitory levels of compound, which might be thought to mimic some clinical condition. In addition to revealing stable mutations that can give rise to incremental steps in resistance, serial passage can uncover unstable

adaptive changes leading to transient phenotypic resistance that requires maintenance of selective pressure. These adaptive changes may involve induction of resistance functions or target gene amplification [41]. Andersson et al. discuss the place of serial passage and resistance selection at sub-MIC levels in laying a predicate for clinical behavior [42–44].

Of course, none of these methods takes into account the horizontal genetic transfer which accounts for much clinical resistance [44]. Furthermore, in vivo growth rates, nutrient limitation, varying drug levels and the immune status of the host certainly contribute to the survival and propagation of resistant mutants. Indeed, the value of in vitro methods to predict resistance occurrence in the clinic in treatment of human infections is largely untested. While there are animal models for antibacterial efficacy that are highly predictive of clinical results, animal models for development of resistance are few and not standardized. One reason for this difference is that efficacy models are normally run with relatively low bacterial inocula, generally 10^6 or fewer infecting pathogens, and even fewer when virulent pathogens are used. This inoculum yields populations that are too small to contain pre-existing resistant mutants. The level of pre-existing mutants is likely critical to the rapid increase in resistance during therapy. Nevertheless, efforts to tie in vitro results to predictions of clinical performance should continue. An important in vitro approach, the hollow-fiber infection model (HFIM), can demonstrate the efficacy of antibiotics and the appearance of resistance under conditions of dynamic variation of drug concentration, mimicking human discontinuous dosing pharmacokinetics [45–48]. HFIM can play a useful role in relating in vitro findings to clinical outcomes, as discussed below.

What FoR determined in vitro is bound to yield rapid resistance in vivo? As noted above, while in vitro methods of FoR determination are feasible and should be routine tools in antibacterial discovery programs, there is a disconnect between in vitro results and clinical reality. This disconnect is due to the fact that there has been little clinical experience with single-targeted agents in the treatment of standard bacterial pathogens. Put another way, there is little experience with antibacterials that select rapidly for high level resistance. For the mainstays of systemic monotherapy that are multi-targeted, there may be single-step FoRs in vitro that, in the main, lead to modest increases in MICs. These single-step FoRs are often due to changes in permeability or efflux, and not to mutational changes in targets. Often there are claims in the literature that a new inhibitor has a low FoR – say a value of 10^{-8} – which is said to be in the range of that seen with standard drugs. But the amplitude of change in MIC for those standard drugs is usually not very large, while the amplitude of MIC increase for single-step resistance for a single-targeted agent can be >100-fold. In a fulminant infection, the bacterial burden is likely to be high enough to contain spontaneously-resistant mutants. Thus, while it is likely that an FoR of 10^{-6} (which might be seen if a genetic deletion can give rise to a resistant phenotype) would show overnight resistance in a clinical situation, it is not clear how low a frequency would have to be to insure that rapid resistance would not arise.

In the clinical trial, mentioned above, for complicated UTI infection of the leucyl tRNA inhibitor, GSK2251052, an in vitro resistance frequency of $\geq 10^{-8}$ yielding mutants with good fitness led to a significant rate of high-level rapid resistance in 3 of 14 patients, after 1 day of treatment [26, 27]. The mutants had MICs as high as 2,000-fold the initial MIC. This result was modeled retrospectively in an HFIM experiment [28] which showed that with a starting inoculum of 10^8 *E. coli*, resistant mutants took over the population within 24 h. This result had not been seen in preclinical animal-efficacy models, again most likely because the infecting inoculum was low.

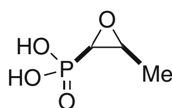
3.2 Overcoming Single-Target Resistance

There is a strong likelihood that single-step mutations leading to resistance against single targeted agents can and will arise before challenge with the agent, given that the inverse of the FoR is less than the population size. Does this result obviate the exploitation of any single target? The future of antibiotics research and development may well involve creative solutions for the exploitation of single-target inhibitors in ways that reduce resistance potential. These solutions may lie in target-inhibitor exceptions to the rapid resistance problem, including low virulence of resistant mutants; low toxicity allowing high dosing to prevent survival of resistant mutants; focus upon narrow spectrum agents which could allow design of very high affinity, high-potency inhibitors that are insusceptible to target-based resistance; and the use of combinations of single-target inhibitors to lower the probability of resistance selection. There are clearly exceptions to the "no single-targets" dictum that might be explained by special circumstances. Can we learn anything from those exceptions?

3.2.1 Low Fitness

Single-step mutations to resistance to certain antibiotics can reduce growth rates, decrease virulence, and otherwise lower the competitive fitness of the pathogen in the host milieu. These changes can counteract the effect of pre-existing resistant mutants and reduce their contribution to the infecting population. Thus, fosfomycin, an inhibitor of the MurA (UDP-*N*-acetylglucosamine-enolpyruvyl-transferase) enzyme, the first committed step of peptidoglycan synthesis, has been used traditionally as a single dose oral treatment for uncomplicated urinary tract infections (uUTI). As fosfomycin has a broad antibacterial spectrum including many MDR pathogens, is active both orally and parenterally, and has an excellent safety profile, there has been recent interest in expanding its indication. Rapid resistance through transport loss, albeit occurring at high frequency in vitro, yields lowered fitness in vivo, in the urinary tract setting. Thus, fosfomycin has been quite successful in treatment of uUTI. Very few target-based resistance mutations, which

likely arise at low frequency, have been detected in the clinic, possibly due to this lowered fitness. It is not clear whether mutational resistance, which has not been problematic for uUTI, will translate to low fosfomycin resistance in other tissue sites [49]. Furthering this caveat is a study of fosfomycin resistance development in a mouse lung model of *P. aeruginosa* infection showing that mutations did arise during treatment and that the mutants (in the *glpT* transporter) were not compromised in fitness [50]. Importantly, recent reports point to the rise of transmissible resistance due to modification of fosfomycin, including (in China) plasmids encoding both resistance to fosfomycin and the presence of the β-lactamase KPC-2 [49, 51–55].



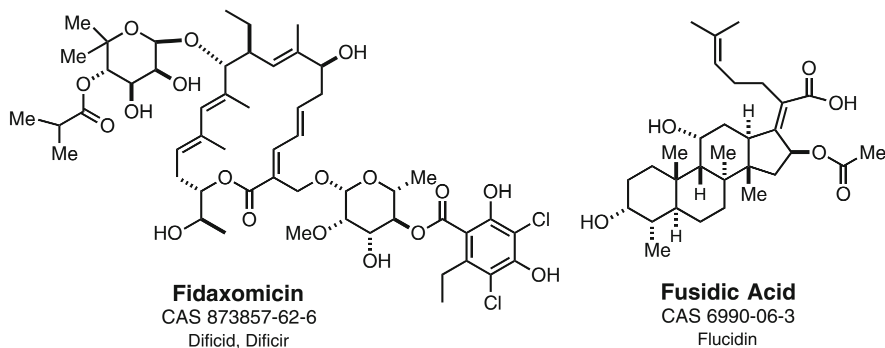
Fosfomycin
CAS 23155-02-4

3.2.2 Dosing above the Mutant Prevention Concentration (MPC)

The idea of the mutant-prevention concentration, MPC, was developed in studying resistance to the fluoroquinolones [56]. Single-step mutations with increased MICs could be selected in vitro up to a threshold concentration, above which the frequency fell dramatically (to less than 10^{-11}). This pattern is due, in the case of the fluoroquinolones, to the occurrence of mutations in the most sensitive target (DNA gyrase, GyrA, in *E. coli*) that increased the MIC to that threshold concentration, above which concentration a second mutation, in a second target (topoisomerase IV, ParC, in *E. coli*), would be required to raise the MIC further. Thus, the MPC is the concentration of drug above which a single mutation cannot afford resistance. In some cases the increase in MIC due to a single target mutation can be $>$100-fold, as with rifampicin (targeting RNA polymerase) in *S. aureus*.

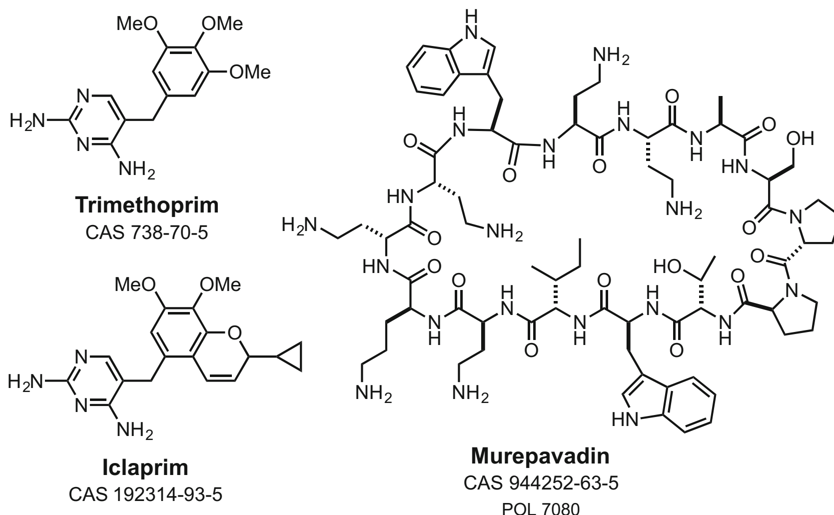
Fidaxomicin is an inhibitor of RNA polymerase with a significant FoR in vitro [57, 58], nonetheless successfully treats *C. difficile*-associated disease since very high concentrations can be maintained in the gut [59]. Essentially, this is an example of dosing above the MPC. Such dosing can be done when the drug is sufficiently safe so as to achieve very high concentrations at the infection site. Fusidic acid is an inhibitor of protein synthesis elongation factor G (Ef-G) that has been in use in Europe for many years in the oral treatment of *S. aureus* infections. Mutations in the target gene *fusA* can arise and yield resistance. Cempra Inc., in a trial for fusidic acid treatment of ABSSSi, is investigating the use of a loading dose of fusidic to kill off the pre-existing resistant mutants. Dosing above the MPC has been endorsed by many experts [60–63]. This dosing requires careful attention to the pharmacokinetic/pharmacodynamic (PK/PD) parameters for each drug, as well

as the reimagination of the concept of MIC breakpoints, perhaps evolving to an MPC breakpoint.



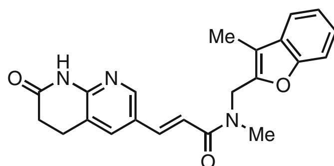
3.2.3 Avoiding Resistance by Multiple Target-Ligand Interactions

If resistance due to changes in the target-binding site is significant, it may be possible to reduce the FoR by increasing binding affinity for example by adding ligand-target interactions. This outcome was the aim in the development of iclaprim, a derivative of trimethoprim, an inhibitor of dihydrofolate reductase (DHFR), which is not yet registered. POL7080 (murepavidin) is a peptide mimetic targeting LptD of *P. aeruginosa*, a protein involved in translocation of lipopolysaccharide to the outer membrane, and is also in development. Murepavidin has an FoR of 10^{-10} [64]. Mutants contain an 18-bp duplication that apparently prevents compound binding. Presumably the lack of single-base-change resistance mutations is due to the multiple target-ligand interactions of this peptide mimetic.



3.2.4 Narrow Spectrum Agents

Other single-target agents are under development including those inhibiting FabI and LpxC. Inhibitors of LpxC (UDP-3-*O*-[acyl]-*N*-acetyl glucosamine deacetylase, the first committed step in Lipid A synthesis) are subject to various resistance mechanisms depending upon the species being inhibited [65, 66]. However, it appears that resistance in *Pseudomonas aeruginosa* is infrequent and due either to increased efflux or increased LpxC expression, generally leading to MIC increases of two- to fourfold (with exceptions) [67]. It may be possible to engineer compounds less susceptible to efflux and/or increase potency such that the compound can, if sufficiently safe, be dosed above the MPC. Thus, *P. aeruginosa*-directed LpxC inhibitors are a stated goal of Achaogen [68]. FabI, enoyl-ACP reductase, an essential enzyme in fatty acid synthesis in some bacterial species, is the target of the anti-*S. aureus*-compound, Debio1452 (AFN-1252) [69]. As this compound is being developed for a single species, the potency attained can be very high, due to the possibility of developing very tight binding inhibitors to the single isoform of the enzyme rather than to the many homologues requiring coverage for broad spectrum targets. Indeed, the MIC of this compound against *S. aureus* is ~3.9 ng/mL. Resistant mutants map solely to two sites in *fabI* and can raise MICs significantly, from 3.9 ng/mL to 250–500 ng/mL [70, 71]. These mutants appear fit. However, these MICs are easily covered by standard dosing regimens of the compound and so should not give rise to rapid resistance in the clinic.



Debio 1452
CAS 620175-39-5
API 1252

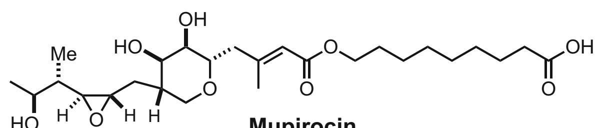
3.3 Leveraging Single Targets by Truly Assessing Combinations

In the areas of *M. tuberculosis*, HIV, HCV, and cancer, treatment with combinations of multiple single agents has become the norm. In the case of the infectious agents, while such combinations may be directed toward covering important subpopulations (such as persisters or non-growing MTB cells), these therapeutic regimens have been optimized over time, by addition of new classes of drugs, to reduce rapid selection of resistant mutants to which each of the individual drugs is subject. This process proceeded with the serial introduction of the first and following drugs for each of these pathogens, followed by appearance of resistant

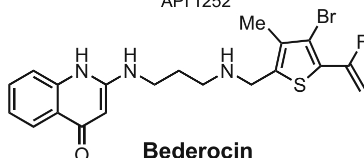
organisms. The histories of these drug regimens against HIV [72–76], HCV [77–80] and TB [81] show a progression to the use of three or four moieties as standard therapy. This progression is in contrast to the history of therapy of more "standard" (non-mycobacterial) bacterial pathogens, where the systemic agents available were not subject to rapid high-level target-based mutational resistance in the pathogen. Those standard antibiotics (including β-lactams, vancomycin, the ribosomally-targeted natural product antibiotics such as tetracycline, chloramphenicol, erythromycin, as well as the synthetic fluoroquinolones) have multiple targets or inhibit the products of multiple genes or a pathway. I believe this multitargeting is responsible for the low frequency of high-level target-based resistance [9, 23, 25]. As the discovery paradigm for standard pathogens changed in the late 1990s to wholesale pursuit of novel essential enzyme targets (as theoretically discovered through genomics), the benefits of multi-targeting were underemphasized. While not proven for every such inhibitor of single enzymes, single-targeted inhibitors (when tested) do select resistance in a single-step. Thus, in light of successful combinations of single-targeted agents for therapy of TB, HIV and HCV, it would seem reasonable to test this approach for standard pathogens, treating with combinations of single-targeted agents. If it is possible to evolve successful combinations of two or three single-targeted drugs to treat the ESKAPE pathogens, for example, then the whole of antibacterial discovery could return to the pursuit of single-targeted agents with a rational path to development and deployment – albeit one which would require regulatory buy-in and most probably, cooperation of inventors to pool the agents.

There is a great deal of literature on the clinical use of combinations of the classical (mostly multi-targeted) antibiotics to treat problematic, even MDR, infections, with varying results. Success may be based on synergistic activity, sensitization by one sub-inhibitory drug of another, or other, not well understood, mechanisms. But the idea of developing and using combinations of single-target agents against important standard bacterial pathogens in order to prevent resistance development has not been tested much. Exceptions include the use in animals and humans of combinations of various single targeted-agents, like rifampicin, fusidic acid, trimethoprim or novobiocin to treat MRSA infections [82–85], and the long standing use of combinations of trimethoprim and sulfamethoxazole, the combination of two inhibitors of the bacterial folate pathway [86].

While the hypothesis that combinations of single-targeted antibiotics should reduce resistance development in standard pathogens has been much discussed, there has been little direct testing of this in vitro with suitable controls. Recently, however, it was shown that targeting two different tRNA synthetases with a combination of inhibitors does lead to a great reduction in resistance frequency in vitro, as expected [87]. The authors showed that inhibitors of isoleucine, leucine and methionine tRNA synthetases (mupirocin [88], epetraborol (AN3365/GSK2251052) [26], and bederocin (REP8839/CRS3123) [89], respectively) which have high (10^{-7} to 10^{-8}) resistance frequencies, show $<10^{-12}$ frequencies when combined pairwise.



Mupirocin
CAS 12650-69-0
API 1252



Bederocin
CAS 757942-43-1
REP 8839

There have been a large number of HFIM studies of PK/PD and resistance development of combinations of single targeted agents in TB, for example [90–92]. Drusano et al. [63, 93] argue cogently for the use of PK/PD methods to approach ways of suppressing resistance development during monotherapy and in combination therapy. They consider both mouse models and hollow-fiber infection models (HFIM). It is explicitly proposed [93] to study combinations of antibiotics in order to suppress resistance emergence, using both standard classes of antibiotics but also preemptively studying combinations which might be used with new agents under development. I would expand this to include testing by HFIM (and, if possible, animal models) of as many of the published, validated single-target inhibitors as are available, in combinations both pairwise and in higher multiples. Table 1 lists a number of validated inhibitors subject to single mutations that significantly raise MICs. They could be used for testing in combinations against, for example, *S. aureus*, *E. coli*, *H. influenzae*, *P. aeruginosa* and permeable strains of *E. coli* and *P. aeruginosa*.

3.3.1 Problems that could Compromise Combinations

It should be noted that the feasibility of combination therapy in standard pathogens is not a given. The concept behind the use of combinations to avoid rapid resistance selection is based on initial killing (or growth inhibition) of pre-existing resistant mutants in the starting population, such that one drug will kill off the mutants resistant to the other. Theoretically, if the pre-existing rate is 10^{-8} per cell per generation, then for a combination of two drugs, a pre-existing double mutant should arise at the rate of 10^{-16}. As noted above, mutators are enriched among clinical isolates and can lead to increased resistance [37, 39, 40, 124]. This increase could require that more than two components be used in the combination. Two drugs might suffice to prevent rapid resistance during therapy, but over time if the level of either drug becomes insufficient, single mutations could arise in the surviving population (perhaps among persisters) that would then provide a

Table 1 Compounds subject to single step mutations to resistance for testing in combinations

Target	Inhibitor	Mini spectrum[a]	Resistance mechanism	Resistance frequency	In vivo?[b]	Reference for resistance
Cell wall						
MurC	Pyrazolopyrimidine	Pa effΔ, Ec tol	Target	10^{-9}	Unk[c]	[94]
MurA	Fosfomycin	Ec, Sa, Pa	Uptake	10^{-7} to 10^{-8} (Ec)	Yes	[55]
MraY	Pacidamycin	Pa	Uptake	2×10^{-6} to 10^{-8}	Yes	[95]
MurG	Murgocil	Sa	Target	10^{-7}	Unk	[96]
PBP2	Mecillinam	Ec	Target	NA[d]	Yes	[97]
Dxr	Fosmidomycin	Ec	Uptake	NA[e]	Yes	[98]
TarG	Targocil	Sa	Bypass	10^{-6}	Yes	[99]
Cell division						
FtsZ	PC190723; TXA-709	Sa	Target	10^{-8} to 3×10^{-8}	Yes	[100, 101]
MreB	CBR-4830	Pa effΔ	Target	5×10^{-9}	Unk	[102]
Lipoprotein/lipid						
LolC E	Compound 2	Ec, Ec tol, Hflu	Target	10^{-6} to 2×10^{-7} (Ec tol)	Unk	[103]
LspA	Globomycin	Ec	Bypass	10^{-7}	Yes	[104]
Fab I	Debio-1450	Sa	Target	10^{-10} to 2×10^{-9}	Yes	[105]
CoaD (PPAT)	Cycloalkyl pyrimidines	Sa	Target	10^{-7} to 10^{-9}	Yes	[106]
AccA AccD	CPD2	Sa, Ec	Target	$<10^{-9}$	Yes	[107, 108]
AccC	Pyridopyrimidines	Ec tol, Hflu	Target	10^{-8} to 10^{-9}	Yes	[109]
LpxC	Pfizer LPXC-4	Ec, Pa	Bypass, efflux, upcopy	1.5×10^{-8} to 3.4×10^{-8} (Pa)	Yes	[67]
DNA/RNA						
DHFR	Trimethoprim	Ec Sa	Target/ overexpression	10^{-9} to 2.3×10^{-10} in Sa	Yes	[110]
Tmk	Compound 39	Sa	Target	6×10^{-9}	Yes	[111]

DnaE	Nargenicin	Sa, Ec tol	Target	10^{-9}	Yes	[112]
PolC	EMAIPU	Sa	Target	NA[d]	Yes	[113]
Lig	Adenosine analogs	Sa, Hflu	Target	NA[d]	Yes	[114]
RpoB	Rifampicin	Sa, Ec lpx	Target	10^{-7} to 10^{-8}	Yes	[115]
Protein synthesis						
Tuf (Ef-Tu)	LFF571	Sa	Target	1.2×10^{-9} to $<3 \times 10^{-11}$[f]	Yes	[116]
FusA (Ef-G)	Fusidic acid	Sa, Ec tol	Target	10^{-7} to 10^{-8}	Yes	[115, 117]
RplK (L11)	Nocathiacin	Sa	Target	10^{-7} to 10^{-9}	Unk	[118]
Leu-RS	GSK2251052	Ec, Pa	Target	1×10^{-7} to 4×10^{-7}	Yes	[26]
IleRS	Mupirocin	Sa, Ec tol	Target	8×10^{-8}	Topical	[88]
ProRS	PPD-2	Ec	Target	3.3×10^{-9}	Unk	[119]
metRS	REP3123	Sa	Target	10^{-7} to 10^{-8}	Unk	[120]
PDF	GSK1322322	Sa	Bypass	5×10^{-7} to 6×10^{-8}	Yes	[121]
Other						
ClpP	ADEP4	Sa	Target	1×10^{-6}	Yes	[122]
FMN riboswitch	Ribocil	Ec lpx tol	Target	1×10^{-6}	Yes	[123]

[a]Mini spectrum: Active on *E. coli* (Ec), *P. aeruginosa* (Pa), *H. influenza* (Hflu) and permeable strains: tolC *E. coli* (Ec tol), *P. aeruginosa* efflux deletion (Pa effΔ), *E. coli* lpxC (Ec lpx), *E. coli* tolC lpx C (Ec lpx tol)
[b]Reported in vivo activity
[c]Unknown whether compound has in vivo activity
[d]Not available but target mutations obtained
[e]Not available but should be same as fosfomycin
[f]In *C. difficile*

background in which resistance to the second drug was possible. It seems important, but remains to be tested and proven, that combinations to retard resistance development would require matched pharmacokinetics of all drugs in the combination such that there is no time in which coverage is by a single drug only. A fixed dose of all components would be desired for both physician ease-of-use and patient compliance.

Treatment with multiple compounds could have further liabilities: possible increased toxicity, deleterious drug-drug interaction, and direct antagonism due to incompatible mechanisms of action. Antagonism between inhibitors may not be evident at the MIC level but could be seen in reduction in killing. For example, the killing effect of β-lactams is counteracted by drugs that inhibit protein synthesis. Projan has noted [125] that successful drug targets are not necessarily enzymes (which require very strong inhibition) but may be those functions that, if inhibited, interfere in a dominant way with cell viability – that is, they act as poison complexes. He notes that these targets include those whose inhibition leads to "stimulating autolysis, causing protein misfolding, stalling ribosomes on mRNA". I would add to this list DNA breakage, as exemplified by the fluoroquinolones acting on topoisomerases. These activities would still be subject to resistance, but their action might be subject to antagonism by inhibitors of other pathways that prevent those killing activities. Thus the effects of combinations on MBC and on killing must also be taken into account.

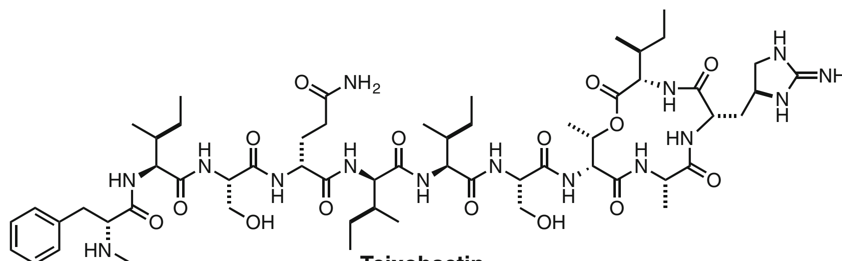
A tendency in considering the use of combinations of antibiotics is to favor combinations synergistic in terms of potency. But for this resistance-prevention use, synergy might add to selective pressure for resistance mutations, as the synergistic increase in activity would be reduced by mutations yielding resistance to one member of the combination. The idea that synergy can select for resistance mutations has been explored by Kishony's group [126]. In fact, that group demonstrated that antagonistic (as opposed to synergistic) interactions can even suppress resistance selection [127, 128]. Thus, choice of drugs in a combination must take into account intracellular drug interactions. Perhaps systems biology can uncover the right combinations of targets to be hit in order to effect sufficient antagonism to retard resistance. On the other hand, "right combinations" could be identified by empirical means.

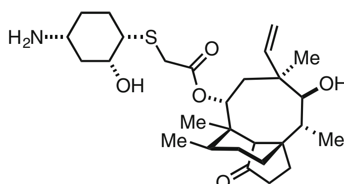
4 Old Targets new Chemical Matter

The emphasis in antibacterial discovery since 1995 has been the pursuit of inhibitors of "new targets", previously "unexploited" by marketed drugs. The motivation behind this emphasis is that inhibitors of such targets should not be subject to cross-resistance with drugs in the clinic. While resistance to the drugs in current use is in some cases target-based, it is more often based on the chemical nature of the compound. Further, since much of the history of antibacterial discovery involved empirical (kill-the-bug) screening, all possible killing targets were ostensibly

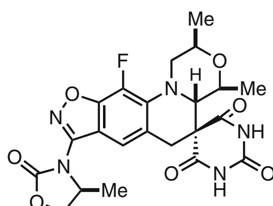
screened. What resulted was not obviously biased against or for specific targets but, in the case of natural products screening, was biased towards those targets (or receptors) selected by nature. Nevertheless, the advent of whole genome sequencing led to the search for new targets. Criteria for target suitability have traditionally included (1) essentiality to the pathogen, (2) non-homology with mammalian enzymes/targets, (3) "druggability", and (4) low likelihood of cross-resistance with existing classes. This list should be expanded to include (5) preference for an accessible location (see below, text on the entry problem) and (6) low probability of rapid resistance selection. Such rapid resistance can include resistance mutations in the target itself, or the appearance of bypasses and redundancies that might be accessed by regulatory changes or single mutations. As noted above, good antibacterial targets (as identified by successful monotherapeutic systemic drugs) are generally not the product of a single gene. It has been proposed that the already-exploited targets for antibacterials are likely to be privileged and thus are already so-called "multitargets". These targets are different from the 160 or so broad-spectrum essential gene products that have been noted as targets of interest [7]. If we accept that the 30–40 established targets [5] are privileged, it seems reasonable to continue to search for inhibitors of these targets among novel chemical matter with the purpose of finding new inhibitors that are not cross-resistant with old, and that do not themselves select for single-step resistance.

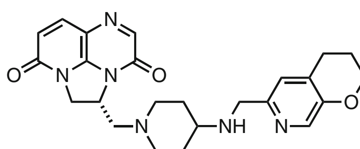
The classical targets worth attacking with new chemical matter include the cell wall pathway, rRNA of ribosomes, Lipid II, and Gyrase/Topoisomerase IV. Targets in the cell wall pathway have been reviewed [129–132]. The penicillin-binding proteins (PBPs) are demonstrated and the Mur-ligases are potential multitargets. Recent work from the Mobashery-Chang group has focused on novel oxadiazole compounds, unrelated to β-lactams, that inhibit PBPs and are active in vitro and in vivo against *S. aureus* including MRSA [133, 134]. Work toward finding developable inhibitors of multiple Mur-ligases has been in progress for many years, notably in the Tomasic group. Reported multi-Mur-ligase inhibitors – for an example, see [135] – have been discovered, but show only very limited whole-cell activity. Lipid II is an intermediate in the synthesis of peptidoglycan and is the target of the glycopeptides. It is the product of a single pathway. No single mutations in pathway members are likely to yield high-level resistance. A new Gram-positive inhibitor, teixobactin, targets both Lipid II and Lipid III, the lipid involved in teichoic acid synthesis. As expected, resistance to teixobactin has not been observed in vitro [136, 137]. Ribosomal targets have been reviewed recently [138] and chapters in this book cover the oxazolidinones and tetracyclines. Novel ribosomal inhibitors in the clinic include the systemic compound lefamulin being developed by Nabriva [139, 140]. Under preclinical study are the 50S subunit targeted ESKAPE pathogen compounds of Melinta therapeutics [141] and the Gram-negative 30S ATI-1503 (a negamycin derivative) of Appili Therapeutics [142].





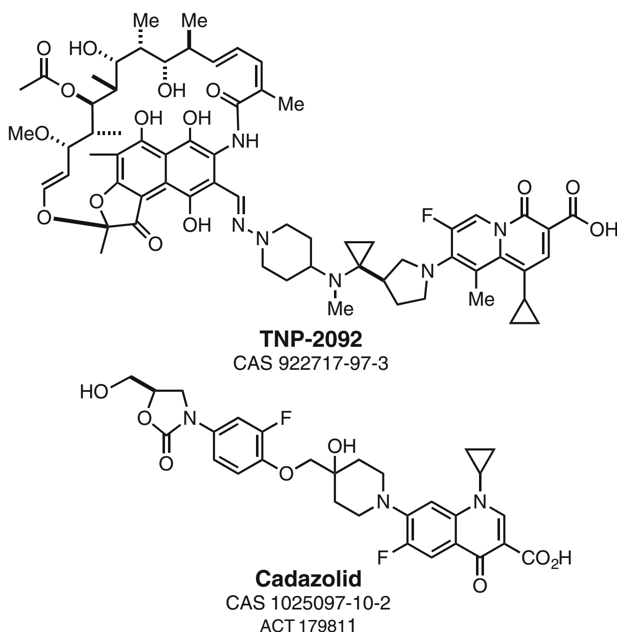
Probably the most popular set of multitargets are the bacterial type II Topoisomerases, DNA Gyrase and Topoisomerase IV. The fluoroquinolones target the A subunits of both enzymes (GyrA and ParC). Efforts toward finding new inhibitors of these enzymes that target sites separate from those targeted by the fluoroquinolones have been widespread and are addressed [143]. Non-fluoroquinolone Inhibitors of the topoisomerases that are in the clinic include zoliflodacin (ETX0914/AZD0914), a spiropyrimidinetrione being developed by Entasis for treatment of uncomplicated gonorrhea [140, 144]; and gepotadacin (GSK2140944), a triaza-acenaphthylene being developed by GSK for cUTI, uncomplicated gonorrhea and community acquired bacterial pneumonia [140, 145].





The concept of conjugating two antibiotics to make a hybrid molecule, active against the targets of each of the components and potentially less subject to resistance than either component, has been explored for many years [146]. One compound, originally discovered by Cumbre Pharmaceuticals, is a hybrid of

rifampicin (an RNA Polymerase inhibitor) and a quinolizinone (a topoisomerase inhibitor, similar to a fluoroquinolone). The compound (TNP-2092) is now under preclinical development by TenNor Pharmaceuticals for the treatment of prosthetic-joint infections [147]. A hybrid of a quinolone and an oxazolidinone, Cadazolid, is being developed by Actelion for treatment of *C. difficil*e-associated disease [58, 140].



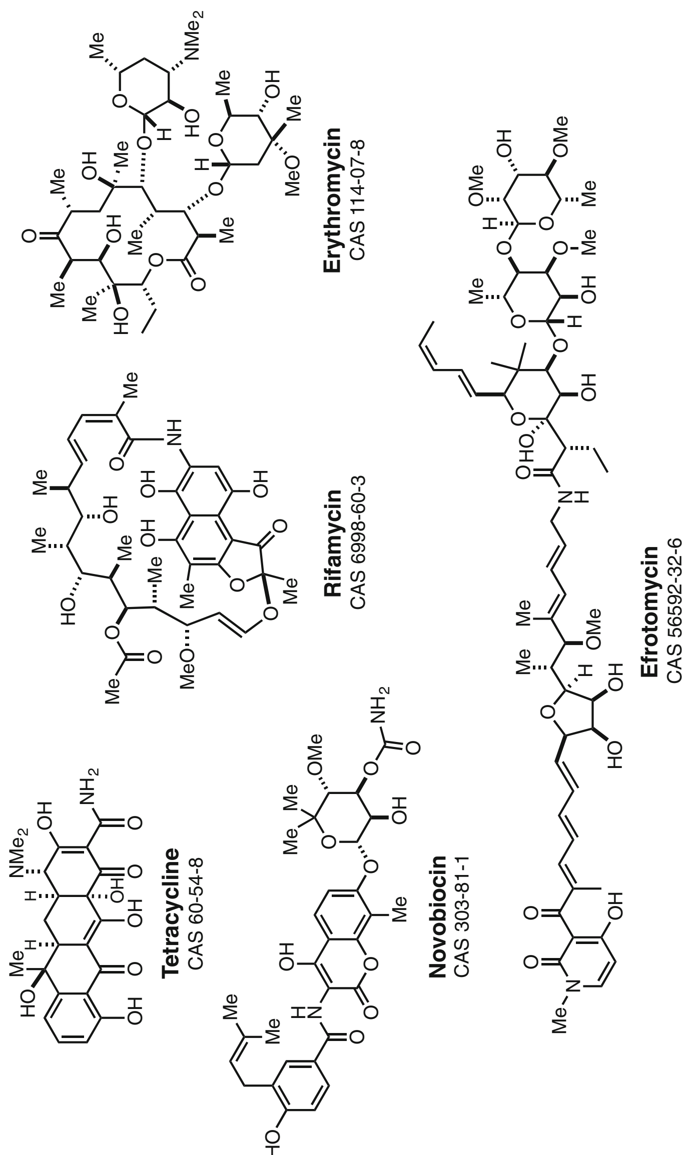
5 The Entry Problem

5.1 Gram Positives: Cytoplasmic Membrane Transit

Some classes of Gram-positive antibiotics, such as the β-lactams and glycopeptides, have extracellular targets and hence do not require membrane passage for activity. However, most of the catalogued potential genomic targets are located in the cytoplasm, as are the targets of many antibiotics in clinical or veterinary use. Obviously, to reach the cytoplasm requires properties that allow permeation of the cytoplasmic membrane. Chemical properties associated with diffusion through membranes have been uncovered, mostly based on studies with mammalian cells, synthetic membranes and liposomes. Diffusion through lipid bilayer membranes is correlated with optima of size and lipophilicity, and it is the neutral species of ionizable compounds that are preferred for entry [148].

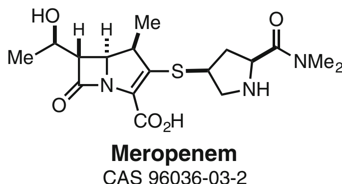
Lipinski and co-authors formulated a set of guides (the Rule of 5 or Ro5) [149] for the properties of drugs that are orally bioavailable due to permeability through intestinal membranes via diffusion. Oral absorption is more likely with compounds that have fewer than 5 H-bond donors, 10 H-bond acceptors, a molecular weight (MW) less than 500 Da, and calculated Log P (CLogP) that is less than 5 [149]. Variations of this rule also specify fewer than 10 rotatable bonds and polar surface area equal to or less than 140 $Å^2$ [150]. These "rules" have heavily influenced the composition and physicochemical characteristics of many/most industrial chemical libraries. Lipinski noted that the outliers (drugs that do not follow the Ro5) among the 2,245 orally active compounds used to generate the rule were antibiotics, antifungals, vitamins, and the cardiac glycosides. He postulated that this result was due to their ability to act as substrates for (intestinal) transporters. Later publications note that "substrates for transporters and natural products are exceptions" to the Ro5 [151, 152]. It has been argued that focus on oral bioavailability in drug discovery and expansion of chemical screening libraries to follow the Ro5 has perhaps retarded the process of drug discovery in general, as has avoidance of natural products and their semisynthetic derivatives, as they form a large percentage of successful drug classes (34% as of 2007) [152]. But the Ro5 may well be useful in identifying compounds capable of diffusion through lipid bilayer membranes in general, even though the lipid composition of membranes can vary greatly from species to species.

Clearly, there are orally-active, cytoplasmically-targeted, often large, natural product antibiotics that are among Lipinski's outliers and do not obey the Ro5, but appear to enter cells by diffusion, without the intercession of active transporters. These include erythromycin, fusidic acid, tetracycline, rifamycin, novobiocin, efrotomycin, and others. Although there may be mammalian transporters for these compounds (as proposed by Lipinski), it appears that these non-Ro5 compounds can diffuse through Gram-positive cytoplasmic membranes. Thus, some natural products have evolved to solve the membrane diffusion problem in ways outside the Ro5. For example, erythromycin and tetracycline [153, 154] although possessing many H-bond donors and acceptors (5 and 7 H-bond donors and 14 and 10 H-bond acceptors for erythromycin and tetracycline, respectively) may undergo intramolecular H-bonding [155]. Furthermore, the pK_a values of these protonatable groups are such that the molecules are ionizable to the extent that neutral species can exist at pH 7.4 in aqueous solution [153], and it is these species that can diffuse through the bilayer. Perhaps further rules could be derived for these larger natural products from QSAR studies of their permeability characteristics.



On the other hand, if molecules do adhere to Ro5, do they automatically enter the cytoplasm? Not necessarily. As an example, meropenem has no Lipinski exceptions but is unlikely to enter the cytoplasm due to its hydrophilicity [156]. It would be useful to formulate rules, or to compile exceptions, that could guide optimization of compounds for Gram-positive entry. Historically, if activity against the isolated cytoplasmic antibacterial target is measurable, then a rough estimate of relative permeation of the cytoplasmic membrane may be obtained for a series of inhibitors

by inspecting the ratio of MIC to IC_{50} or K_i of the inhibitor. However, in the absence of measurable activity against the target (or complete impermeability), obviously no ratio is obtained. Thus, there has not been much work measuring the rate or extent of uptake of a compound in the absence of activity. Recently, a number of methods have been described for this evaluation using liquid chromatography-mass spectrometry (LC-MS) [157, 158] and Raman spectroscopy [158, 159]. As noted below, the measurement of entry into Gram-negatives is complicated by the necessity of determining the compartment of accumulation.



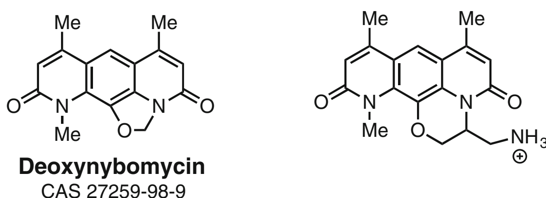
Meropenem
CAS 96036-03-2

5.2 *Gram Negative Entry Barriers*

Many reviews have discussed the barrier functions of Gram-negative bacteria [160–164]. In simplest terms, Gram negatives (like Gram-positives) are bounded by a cytoplasmic membrane (CM) but in addition a second “outer membrane” (OM), which consists of an asymmetric bilayer comprising an inner phospholipid leaflet and an outer leaflet of lipopolysaccharide. The sieving properties of these two membranes are largely orthogonal. The cytoplasmic membrane favors passage of neutral lipophilic compounds, while the outer membrane allows passage of small hydrophilic, charged compounds through water-filled protein channels called “porins”. This orthogonality makes it problematic to create compounds with physicochemical properties suitable for crossing both barriers. Additionally, powerful efflux pumps can remove compounds from the cytoplasm or periplasm. Their action is synergized by the low permeability of the outer membrane [165]. The variety of pumps and the relative promiscuity of some of them make it difficult to rationally avoid efflux, while allowing accumulation in the cytoplasm and/or periplasm. Clearly, there are compounds that have the right combination of properties to allow cytoplasmic entry by passive diffusion, but that collection of antibiotics arriving in the Gram-negative cytoplasm is quite small (in the low hundreds, representing few chemical classes). Do these compounds share chemical characteristics that enable their entry? The search for rules or guidelines for endowing small molecules with entry ability has been frustrating [164, 166].

Recently, the Innovative Medicines Initiative (IMI) TRANSLOCATION consortium and a number of academic groups have been working on the problem of Gram-negative entry, attempting to dissect the various contribution to barrier function of efflux, porins, and membrane bilayers [162, 167–169]. It may be that there is a set of physicochemical parameters that favor the diffusion of compounds

into the cytoplasm, past the various barriers [163, 170–172]. In a recent article I suggested that the set of compounds which appeared to enter the cytoplasm by diffusion were characterized as having relatively high polarity, with a cLogD between −4 and +2, and MW less than ~500 Da. They are either neutral, or have a net charge of −1 [163]. This analysis must be extended to include more physicochemical descriptors. However, the paucity of compounds known to arrive in the Gram-negative cytoplasm limits meaningful analysis. To approach the problem productively requires methodology to measure the accumulation of a large number of compounds in various compartments by activity-independent means. Published methods for analysis of compound localization include an LC/MS method for measuring accumulation of ciprofloxacin [157] and two single-cell methods, one employing tunable UV excitation combined with light microscopy [173] and another using C_{60}-secondary ion MS [174]. The exquisite sensitivity of LC-MS analysis was applied recently to an ensemble of antibacterial structures to identify the structural similarities among the compounds that accumulate within the *E. coli* bacterium [175]. Accumulation coincided generally with compounds that were rigid, had low globularity, and that paired hydrophobic structure with a sterically unencumbered amine (thus, having positive charge at physiological pH). Application of these principles to the selective Gram-positive gyrase inhibitor, deoxynybomycin (left structure), gave an analog (right structure) now having Gram-negative activity (MIC values for the racemate of 0.5–16 μg/mL, depending on strain). Moreover, the analog retained the Gram-positive activity [175]. The basis for attacking the problem of Gram-negative entry, and proposals for solutions, is reviewed in the Pew Scientific Roadmap for Antibiotic Discovery [176, 177].



5.2.1 Other Routes of Gram-Negative Entry

It seems that some antibiotics do arrive in the cytoplasm by passive diffusion. But other antibiotics use other various routes that could be emulated. Figure 1 summarizes routes of cytoplasmic entry, including the route of transit of the outer membrane by porins and the cytoplasmic membrane by diffusion (Route A in Fig. 1). Many natural product antibiotics can enter the Gram-negative cytoplasm via the active transporters that the cell deploys for the uptake of small, generally hydrophilic, molecules and to which the cytoplasmic membrane is minimally permeable. These natural products may cross the outer membrane through porins, or by use of facilitated diffusion pores (Route B). As described in an earlier section, fosfomycin

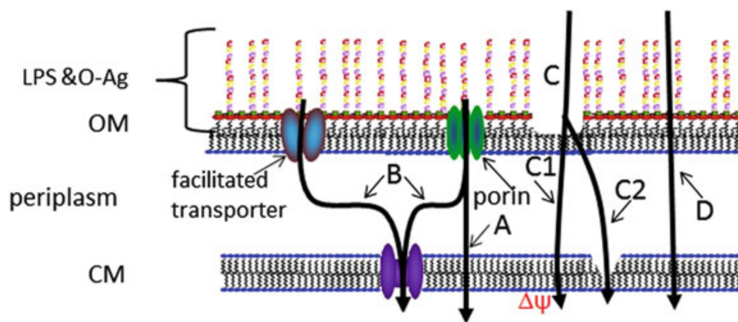
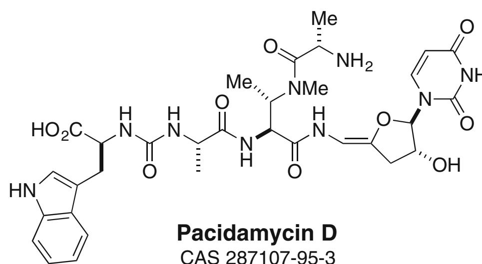


Fig. 1 Routes to the cytoplasm. *A* Entry through OM via porins and diffusion through CM. *B* Diffusion through porins, active transport through CM. *C* Self-promoted uptake of cations through OM; *C1* CM passage via Δψ or *C2* clustering of anionic lipids with passage at cluster-edges. *D* Diffusion of hydrophobic molecules through both OM and CM. *LPS* lipopolysaccharide, *O-Ag O* antigen, *OM* outer membrane, *CM* cytoplasmic membrane

is transported to the cytoplasm by either of two permeases, GlpT or Uhp. Loss of permease function leads to rapid resistance: but, in this case, the resistant mutants are not fit in UTIs. The oligopeptide permease, Opp, can transport pacidamycin, an inhibitor of the MraY enzyme, but high-level resistance to pacidamycin arises at very high frequency via mutations in *opp* [95].



Facilitated transport across the outer membrane, notably via siderophore re-uptake mechanisms has been exploited to import into the periplasm antibiotics to which iron-binding moieties such as catechol have been attached. Thus far a number of such efforts have demonstrated rapid resistance development, adaptive or mutational, due to its use of the siderophore uptake mechanism [178, 179]. However, at least two such agents using this method for periplasmic entry are still under active study [180, 181]. It is certainly worth pursuing the avenue of endowing antibiotics with the ability to use natural permeases and uptake pathways, (so-called Trojan horse approaches). Again, care should be taken to critically test for resistance emergence, not only in vitro but also in vivo. Permease loss could affect fitness of resistant mutants in the host and high FoR in vitro might discourage development of a useful compound.

A different route of cytoplasmic entry is by certain cationic compounds which can mediate their own passage through the outer membrane by means of "self-promoted uptake" [182] (Route C) and then cross the cytoplasmic membrane by less well understood mechanisms, such as that promoted by the Δψ component of the proton motive force (Route C1) or via clustering of anionic lipids in the cytoplasmic membrane leading to disruptions at the edge of such domains [183] (Route C2). Aminoglycosides are among the compounds using this route. The precise structural and physicochemical characteristics of such compounds are ripe for study. An understanding of the requirements for their entry could add another set of rules for entry.

Finally, it has been noted that early work showing very poor penetration of the outer membrane by hydrophobic molecules was likely compromised by the existence of efflux pumps, which were unknown at the time. Thus Plesiat and Nikaido have presented data showing that highly hydrophobic molecules can transit the outer membrane bilayer via the "hydrophobic route", not via porins, albeit slowly [184]. Such compounds, if they could avoid efflux, should easily enter the cytoplasm by diffusion.

5.2.2 Make new Gram-Negative Libraries

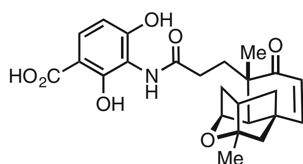
If chemical rules can be derived for any routes of entry, it should, from this microbiologist's point of view, be possible to create chemical libraries with the desired characteristics within which to screen for antibacterial activity, whether phenotypically or against in vitro targets. However, compounds in the cLogD range of −4 to +2 are poorly represented in corporate compound libraries, as this cLogD range does not match that for eukaryotic drug targeting. Initially such compounds might be gleaned from existing libraries industrial and commercial sources.

6 Natural Products

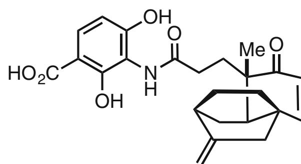
In the foregoing sections I've highlighted the target and entry problems which I feel are pre-eminent and which, if surmounted, will lead us to future antibiotics. Now let us address briefly the old source, natural products (NPs). But first a caveat: it is easy to kill bacteria with agents both synthetic and natural, but it is rare to find compounds whose toxicity is sufficiently selective to produce an acceptable therapeutic index. Microbial NPs have indeed been the source of the majority of our antibiotics, and were discovered for the most part by empirical means. While penicillins are indeed fungal products, the β-lactamase synthetic genes were apparently imported from Actinomycetes [185], which are the major source of antibacterials and in my opinion should remain the preferred producers to be exploited. Antibacterial agents are produced by many Kingdoms, but I would propose that the bulk of non-bacterial NPs are generally toxic to species unrelated to the producer,

while bacteria produce at least some antibiotics that are more selectively toxic, perhaps so the producers can co-evolve resistance mechanisms.

The value of NPs lies in the possibility of novel chemistry that has evolved through selection by forces not well understood by us to create optimized ligands for receptors we are left to define. One may regard NPs as just another chemical library, but a library incorporating chirality, fused rings, 3-dimensionality, and polar nitrogen and oxygen-containing functional groups. In short, a library that can be used to identify inhibitors of one's favorite antibacterial target. On the other hand, commercial development requires that structural novelty is found amidst the commonly found antibiotics. The process of narrowing the hits to those hits having novelty is called dereplication. It is the rate-limiting step of the process of NP antibacterial discovery. Dereplication has been addressed traditionally by biological and chemical methodologies [186–189] and also by the use of hypersensitive-phenotypic screens [190–193]. Hypersensitive screens are whole-cell screens designed to detect active compounds at concentrations below the MIC. Numerous recent reviews have addressed new methodologies for detection and discovery of novel antibiotics, especially by genome mining and by accessing previously unculturable producing organisms [194–199]. The subject is too vast for further discussion here, except to note the discovery of teixobactin, a novel inhibitor of Lipid II and III, through screening of unculturable organisms [136, 137] and of platensimycin and platencin [200, 201], by hypersensitive screening for inhibitors of FabF. They have not been developed as yet, but they represent the possibility of finding novelty among NPs.



Platensimycin
CAS 835876-32-9



Platencin
CAS 869898-86-2

7 Conclusion

Recently, the recognition of increasing antibiotic resistance has led to strong interest in the area of antibacterial discovery, yielding many policy statements from governments and NGOs promoting the need for discovery of new antibacterials to replenish the pipeline. This recognition has spurred increases in funding and a variety of efforts, mostly in academe and small companies, toward discovery of new ways to attack resistant pathogens. While many such efforts have been directed toward improvements to old antibiotic classes, there are inroads into novel areas, some new targets and new chemistry. This progress is illustrated by the recently revealed first round of funding by the CARB-X initiative supporting preclinical

development of a variety of molecules which include "3 potential new classes of antibiotics, 4 innovative non-traditional products and 7 new molecular targets" [202]. In this chapter, rather than reviewing in detail the current discovery programs (as appear in the literature, at meetings, and on websites) I have instead approached the subject of future antibiotics by noting the scientific problems that must be overcome in order to produce new successful drugs. While I have trodden this ground repeatedly in previous reviews (copiously referenced herein), it has been my experience that defining the problem is the important first step to solving the problem. I have emphasized the need to address the probability of rapid resistance development early on in the discovery process, preferably at the stage of target choice. I view the optimization of combinations of single-target inhibitors as a path to readdressing target-based discovery in the antibiotic field. Additionally, the problem of entry of compounds into the bacterial cytoplasm, especially of Gram-negatives, must be solved in order to fully address the need for new Gram-negative agents. Finally, I believe antibacterial natural products still offer a wealth of novel possibilities, which should be addressable by new methods along with hypersensitive screening. The future of antibiotics lies in addressing these obstacles.

References

1. Outterson K, Rex JH, Jinks T, Jackson P, Hallinan J, Karp S, Hung DT, Franceschi F, Merkeley T, Houchens C, Dixon DM, Kurilla MG, Aurigemma R, Larsen J (2016) Accelerating global innovation to address antibacterial resistance: introducing CARB-X. Nat Rev Drug Discov 15:589–590. doi:10.1038/nrd.2016.155
2. Hwang TJ, Carpenter D, Kesselheim AS (2014) Target small firms for antibiotic innovation. Science 344:967–969. doi:10.1126/science.1251419
3. Balasegaram M, Clift C, Røttingen JA (2015) The global innovation model for antibiotics needs reinvention. J Law Med Ethics 43(Suppl 3):22–26. doi:10.1111/jlme.12270
4. Livermore DM (2011) Discovery research: the scientific challenge of finding new antibiotics. J Antimicrob Chemother 66:1941–1944. doi:10.1093/jac/dkr262
5. Lange RP, Locher HH, Wyss PC, Then RL (2007) The targets of currently used antibacterial agents: lessons for drug discovery. Curr Pharm Des 13:3140–3154. doi:10.2174/138161207782110408
6. Overbye KM, Barrett JF (2005) Antibiotics: where did we go wrong? Drug Discov Today 10:45–52. doi:10.1016/S1359-6446(04)03285-4
7. Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL (2007) Drugs for bad bugs: confronting the challenges of antibacterial discovery. Nat Rev Drug Discov 6:29–40. doi:10.1038/nrd2201
8. Chan PF, Holmes DJ, Payne DJ (2004) Finding the gems using genomic discovery: antibacterial drug discovery strategies – the successes and the challenges. Drug Discov Today: Ther Strateg 1:519–527. doi:10.1016/j.ddstr.2004.11.003
9. Silver LL (2011) Challenges of antibacterial discovery. Clin Microbiol Rev 24:71–109. doi:10.1128/CMR.00030-10
10. Gwynn MN, Portnoy A, Rittenhouse SF, Payne DJ (2010) Challenges of antibacterial discovery revisited. Ann N Y Acad Sci 1213:5–19. doi:10.1111/j.1749-6632.2010.05828.x
11. Cegelski L, Marshall GR, Eldridge GR, Hultgren SJ (2008) The biology and future prospects of antivirulence therapies. Nat Rev Microbiol 6:17–27. doi:10.1038/nrmicro1818

12. Escaich S (2008) Antivirulence as a new antibacterial approach for chemotherapy. Curr Opin Chem Biol 12:400–408. doi:10.1016/j.cbpa.2008.06.022
13. Ruer S, Pinotsis N, Steadman D, et al. (2015) Virulence-targeted antibacterials: concept, promise, and susceptibility to resistance mechanisms. Chem Biol Drug Des 86:379–399. doi:10.1111/cbdd.12517
14. Dickey SW, Cheung GYC, Otto M (2017) Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. Nat Rev Drug Discov. doi:10.1038/nrd.2017.23
15. Johnson BK, Abramovitch RB (2017) Small molecules that sabotage bacterial virulence. Trends Pharmacol Sci 38:339–362. doi:10.1016/j.tips.2017.01.004
16. Czaplewski L, Bax R, Clokie M, et al. (2016) Alternatives to antibiotics – a pipeline portfolio review. Lancet Infect Dis 16:239–251. doi:10.1016/s1473-3099(15)00466-1
17. Lévy FM (1975) The fiftieth anniversary of diphtheria and tetanus immunization. Prev Med 4 (2):226–237. doi:10.1016/0091-7435(75)90084-5
18. Krishnamurthy M, Moore RT, Rajamani S, et al. (2016) Bacterial genome engineering and synthetic biology: combating pathogens. BMC Microbiol 16:258. doi:10.1186/s12866-016-0876-3
19. Silver LL (2014) Antibacterials for any target. Nat Biotechnol 32:1102–1104. doi:10.1038/nbt.3060
20. Bikard D, Euler C, Jiang W, et al. (2014) Development of sequence-specific antimicrobials based on programmable CRISPR-Cas nucleases. Nat Biotechnol 32:1146–1150. doi:10.1038/nbt.3043
21. Citorik R, Mimee M, Lu T (2014) Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. Nat Biotechnol 32:1141–1145. doi:10.1038/nbt.3011
22. LocusBiosciences (2016) Founded by pioneers in the CRISPR field: engineering a novel class of precision medicines. http://www.locus-bio.com/. Accessed 1 Apr 2017
23. Silver LL (2007) Multi-targeting by monotherapeutic antibacterials. Nat Rev Drug Discov 6:41–55. doi:10.1038/nrd2022
24. Silver LL, Bostian KA (1993) Discovery and development of new antibiotics: the problem of antibiotic resistance. Antimicrob Agents Chemother 37:377–383. doi:10.1128/AAC.37.3.377
25. Brotz-Oesterhelt H, Brunner NA (2008) How many modes of action should an antibiotic have? Curr Opin Pharmacol 8:564–573. doi:10.1038/nrmicro2133
26. O'Dwyer K, Spivak A, Ingraham K, et al. (2015) Bacterial resistance to leucyl-tRNA synthetase inhibitor GSK2251052 develops during treatment of complicated urinary tract infections. Antimicrob Agents Chemother 59:289–298. doi:10.1128/AAC.03774-14
27. Twynholm M, Dalessandro M, Barker K et al (2013) Termination of Phase II program due to emergence of resistance (EOR) on-therapy. Paper presented at the 53rd interscience congress on antimicrobial agents and chemotherapy, Denver
28. VanScoy BD, Bulik CC, Moseley C et al (2013) Hollow fiber infection model mimics both the time-to-resistance emergence and magnitude of *E. coli* resistance to GSK052 occurring in a Phase 2b clinical study. In: 53rd interscience congress on antimicrobial agents and chemotherapy, Denver
29. Sutterlin HA, Malinverni JC, Lee SH et al. (2017) Antibacterial new target discovery: sentinel examples, strategies, and surveying success. In: Topics in medicinal chemistry. Springer, Heidelberg, pp 1–29. doi:10.1007/7355_2016_31
30. O'Neill AJ, Chopra I (2004) Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. Expert Opin Investig Drugs 13:1045–1063. doi:10.1517/13543784.13.8.1045
31. Couce A, Blázquez J (2011) Estimating mutation rates in low-replication experiments. Mutat Res 714:26–32. doi:10.1016/j.mrfmmm.2011.06.005
32. Rosche WA, Foster PL (2000) Determining mutation rates in bacterial populations. Methods 20:4–17. doi:10.1006/meth.1999.0901

33. Young K (2006) In vitro antibacterial resistance selection and quantitation. Curr Protoc Pharmacol 34:13A.16.11–13A.16.22. doi:10.1002/0471141755.ph13a06s34
34. Hall BM, Ma C-X, Liang P, et al. (2009) Fluctuation AnaLysis CalculatOR: a web tool for the determination of mutation rate using Luria–Delbrück fluctuation analysis. Bioinformatics 25:1564–1565. doi:10.1093/bioinformatics/btp253
35. Luria SE, Delbrück M (1943) Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28:491–511. doi:10.3410/f.3620966.3337067
36. O'Neill AJ, Chopra I, Martinez JL, et al. (2001) Use of mutator strains for characterization of novel antimicrobial agents. Antimicrob Agents Chemother 45:1599–1600. doi:10.1128/aac.45.1.1599-1600.2001
37. Miller K, O'Neill AJ, Chopra I (2004) *Escherichia coli* Mutators present an enhanced risk for emergence of antibiotic resistance during urinary tract infections. Antimicrob Agents Chemother 48:23–29. doi:10.1128/aac.48.1.23-29.2004
38. Hall LMC, Henderson-Begg SK (2006) Hypermutable bacteria isolated from humans – a critical analysis. Microbiology 152:2505–2514. doi:10.1099/mic.0.29079-0
39. Oliver A (2010) Mutators in cystic fibrosis chronic lung infection: prevalence, mechanisms, and consequences for antimicrobial therapy. Int J Med Microbiol 300:563–572. doi:10.1016/j.ijmm.2010.08.009
40. Komp Lindgren P, Higgins PG, Seifert H, et al. (2016) Prevalence of hypermutators among clinical *Acinetobacter baumannii* isolates. J Antimicrob Chemother 71:661–665. doi:10.1093/jac/dkv378
41. Andersson DI, Hughes D (2009) Gene amplification and adaptive evolution in bacteria. Annu Rev Genet 43:167–195. doi:10.1146/annurev-genet-102108-134805
42. Andersson DI, Hughes D (2014) Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol 12:465–478. doi:10.1038/nrmicro3270
43. Hughes D, Andersson DI (2012) Selection of resistance at lethal and non-lethal antibiotic concentrations. Curr Opin Microbiol 15:555–560. doi:10.1016/j.mib.2012.07.005
44. Martinez J, Baquero F, Andersson D (2011) Beyond serial passages: new methods for predicting the emergence of resistance to novel antibiotics. Curr Opin Pharmacol 11:439–445. doi:10.1016/j.coph.2011.07.005
45. Tam VH, Schilling AN, Neshat S, et al. (2005) Optimization of meropenem minimum concentration/MIC ratio to suppress in vitro resistance of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 49:4920–4927. doi:10.1128/AAC.49.12.4920-4927.2005
46. Drusano GL (2003) Prevention of resistance: a goal for dose selection for antimicrobial agents. Clin Infect Dis 36(Supp 1):S42–S50. doi:10.1086/344653
47. Gumbo T, Louie A, Deziel MR, et al. (2005) Pharmacodynamic evidence that ciprofloxacin failure against tuberculosis is not due to poor microbial kill but to rapid emergence of resistance. Antimicrob Agents Chemother 49:3178–3181. doi:10.1128/aac.49.8.3178-3181.2005
48. VanScoy B, McCauley J, Bhavnani SM, et al. (2016) Relationship between fosfomycin exposure and amplification of *Escherichia coli* subpopulations with reduced susceptibility in a hollow-fiber infection model. Antimicrob Agents Chemother 60:5141–5145. doi:10.1128/AAC.00355-16
49. Silver LL (2017) Fosfomycin: mechanism and resistance. Cold Spring Harb Perspect Med 7:a025262. doi:10.1101/cshperspect.a025262
50. Rodríguez-Rojas A, Maciá MD, Couce A, et al. (2010) Assessing the emergence of resistance: the absence of biological cost in vivo may compromise fosfomycin treatments for *P. aeruginosa* infections. PLoS One 5:e10193. doi:10.1371/journal.pone.0010193
51. VanScoy BD, McCauley J, Ellis-Grosse EJ, et al. (2015) Exploration of the pharmacokinetic-pharmacodynamic relationships for fosfomycin efficacy using an in vitro infection model. Antimicrob Agents Chemother 59:7170–7177. doi:10.1128/AAC.04955-14

52. Castañeda-García A, Blázquez J, Rodríguez-Rojas A (2013) Molecular mechanisms and clinical impact of acquired and intrinsic fosfomycin resistance. Antibiotics 2:217–236. doi:10.3390/antibiotics2020217
53. Endimiani A, Patel G, Hujer KM, et al. (2010) In vitro activity of fosfomycin against *bla*KPC-containing *Klebsiella pneumoniae* isolates, including those nonsusceptible to tigecycline and/or colistin. Antimicrob Agents Chemother 54:526–529. doi:10.1128/AAC.01235-09
54. Falagas ME, Giannopoulou KP, Kokolakis GN, et al. (2008) Fosfomycin: use beyond urinary tract and gastrointestinal infections. Clin Infect Dis 46:1069–1077. doi:10.1086/527442
55. Nilsson AI, Berg OG, Aspevall O, et al. (2003) Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*. Antimicrob Agents Chemother 47(9):2850–2858. doi:10.1128/aac.47.9.2850-2858.2003
56. Dong Y, Zhao X, Domagala J, et al. (1999) Effect of fluoroquinolone concentration on selection of resistant mutants of *Mycobacterium bovis* BCG and *Staphylococcus aureus*. Antimicrob Agents Chemother 43:1756–1758
57. Leeds JA, Sachdeva M, Mullin S, et al. (2014) In vitro selection, via serial passage, of *Clostridium difficile* mutants with reduced susceptibility to fidaxomicin or vancomycin. J Antimicrob Chemother 69:41–44. doi:10.1093/jac/dkt302
58. Locher HH, Caspers P, Bruyere T, et al. (2014) Investigations of the mode of action and resistance development of cadazolid, a new antibiotic for treatment of *Clostridium difficile* infections. Antimicrob Agents Chemother 58:901–908. doi:10.1128/AAC.01831-13
59. Scott L (2013) Fidaxomicin: a review of its use in patients with *Clostridium difficile* infection. Drugs 73(15):1733–1747. doi:10.1007/s40265-013-0134-z
60. Zhao X, Drlica K (2008) A unified anti-mutant dosing strategy. J Antimicrob Chemother 62:434–436. doi:10.1093/jac/dkn229
61. Martinez MN, Papich MG, Drusano GL (2012) Dosing regimen matters: the importance of early intervention and rapid attainment of the pharmacokinetic/pharmacodynamic target. Antimicrob Agents Chemother 56:2795–2805. doi:10.1128/AAC.05360-11
62. Blondeau JM, Tillotson GS (2005) Antibiotic dosing: do we dose to cure the individual or do we treat the greater societal needs? Therapy 2:511–517. doi:10.1586/14750708.2.4.511
63. Drusano GL, Louie A, MacGowan A, et al. (2015) Suppression of emergence of resistance in pathogenic bacteria: keeping our powder dry, part 1. Antimicrob Agents Chemother 60:1183–1193. doi:10.1128/AAC.02177-15
64. Srinivas N, Jetter P, Ueberbacher BJ, et al. (2010) Peptidomimetic antibiotics target outer-membrane biogenesis in *Pseudomonas aeruginosa*. Science 327:1010–1013. doi:10.1126/science.1182749
65. Zeng D, Zhao J, Chung HS, et al. (2013) Mutants resistant to LpxC inhibitors by rebalancing cellular homeostasis. J Biol Chem 288:5475–5486. doi:10.1074/jbc.M112.447607
66. Erwin AL (2016) Antibacterial drug discovery targeting the lipopolysaccharide biosynthetic enzyme LpxC. Cold Spring Harb Perspect Med 6:a025304. doi:10.1101/cshperspect.a025304
67. Tomaras AP, McPherson CJ, Kuhn M, et al. (2014) LpxC inhibitors as new antibacterial agents and tools for studying regulation of lipid a biosynthesis in gram-negative pathogens. MBio 5:e01551–e01514. doi:10.1128/mBio.01551-14
68. Achaogen (2017) Achaogen is pursuing an advanced series of LpxC inhibitor compounds that are active against *Pseudomonas aeruginosa*. http://www.achaogen.com/lpxc-inhibitor-program/. Accessed 14 Apr 2017
69. Karlowsky JA, Kaplan N, Hafkin B, et al. (2009) AFN-1252, a FabI inhibitor, demonstrates a *Staphylococcus*-Specific Spectrum of activity. Antimicrob Agents Chemother 53:3544–3548. doi:10.1128/aac.00400-09
70. Yao J, Rock CO (2016) Resistance mechanisms and the future of bacterial enoyl-acyl carrier protein reductase (FabI)antibiotics. Cold Spring Harb Perspect Med 6:a027045. doi:10.1101/cshperspect.a027045

71. Yao J, Maxwell JB, Rock CO (2013) Resistance to AFN-1252 arises from missense mutations in *Staphylococcus aureus* enoyl-acyl carrier protein reductase (FabI). J Biol Chem 288:36261–36271. doi:10.1074/jbc.M113.512905
72. Moore RD, Chaisson RE (1999) Natural history of HIV infection in the era of combination antiretroviral therapy. AIDS 13:1933–1942
73. Pirrone V, Thakkar N, Jacobson JM, et al. (2011) Combinatorial approaches to the prevention and treatment of HIV-1 infection. Antimicrob Agents Chemother 55:1831–1842. doi:10.1128/AAC.00976-10
74. Larder B, Darby G, Richman D (1989) HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science 243:1731–1734. doi:10.1126/science.2467383
75. Larder B, Kemp S, Harrigan P (1995) Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science 269:696–699. doi:10.1126/science.7542804
76. Ho DD, Bieniasz PD (2008) HIV-1 at 25. Cell 133(4):561–565. doi:10.1016/j.cell.2008.05.00
77. Aghemo A, De Francesco R (2013) New horizons in hepatitis C antiviral therapy with direct-acting antivirals. Hepatology 58:428–438. doi:10.1002/hep.26371
78. Lange CM, Jacobson IM, Rice CM, et al. (2014) Emerging therapies for the treatment of hepatitis C. EMBO Mol Med 6:4–15. doi:10.1002/emmm.201303131
79. Kwong AD (2014) The HCV revolution did not happen overnight. ACS Med Chem Lett 5:214–220. doi:10.1021/ml500070q
80. Shahid I, Almalki WH, Hafeez MH, et al. (2016) Hepatitis C virus infection treatment: an era of game changer direct acting antivirals and novel treatment strategies. Crit Rev Microbiol 42:535–547. doi:10.3109/1040841x.2014.970123
81. Kerantzas CA, Jacobs Jr WR (2017) Origins of combination therapy for tuberculosis: lessons for future antimicrobial development and application. MBio 8:e01586–e01516. doi:10.1128/mBio.01586-16
82. Arathoon EG, Hamilton JR, Hench CE, et al. (1990) Efficacy of short courses of oral novobiocin-rifampin in eradicating carrier state of methicillin-resistant *Staphylococcus aureus* and in vitro killing studies of clinical isolates. Antimicrob Agents Chemother 34:1655–1659. doi:10.1128/AAC.34.9.1655
83. Walsh TJ, Standiford HC, Reboli AC, et al. (1993) Randomized double-blinded trial of rifampin with either novobiocin or trimethoprim-sulfamethoxazole against methicillin-resistant *Staphylococcus aureus* colonization: prevention of antimicrobial resistance and effect of host factors on outcome. Antimicrob Agents Chemother 37:1334–1342. doi:10.1128/AAC.37.6.1334
84. Howden BP, Grayson ML (2006) Dumb and dumber – the potential waste of a useful antistaphylococcal agent: emerging fusidic acid resistance in *Staphylococcus aureus*. Clin Infect Dis 42:394–400. doi:10.1086/499365
85. Mandell GL, Moorman DR (1980) Treatment of experimental staphylococcal infections: effect of rifampin alone and in combination on development of rifampin resistance. Antimicrob Agents Chemother 17:658–662. doi:10.1128/aac.17.4.658
86. Huovinen P (2001) Resistance to trimethoprim-sulfamethoxazole. Clin Infect Dis 32:1608–1614. doi:10.1086/320532
87. Randall CP, Rasina D, Jirgensons A, et al. (2016) Targeting multiple aminoacyl-tRNA synthetases overcomes the resistance liabilities associated with antibacterial inhibitors acting on a single such enzyme. Antimicrob Agents Chemother 60:6359–6361. doi:10.1128/AAC.00674-16
88. Hurdle JG, O'Neill AJ, Ingham E, et al. (2004) Analysis of mupirocin resistance and fitness in *Staphylococcus aureus* by molecular genetic and structural modeling techniques. Antimicrob Agents Chemother 48:4366–4376. doi:10.1128/AAC.48.11.4366-4376.2004
89. Ochsner UA, Jarvis TC (2013) Aminoacyl-tRNA synthetase inhibitors. In: Gualerzi C, Brandi L, Fabbretti A, Pon C (eds) Antibiotics: targets, mechanisms and resistance. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp 387–410. doi:10.1002/9783527659685.ch16

90. Drusano GL, Neely M, Van Guilder M, et al. (2014) Analysis of combination drug therapy to develop regimens with shortened duration of treatment for tuberculosis. PLoS One 9: e101311. doi:10.1371/journal.pone.0101311
91. Srivastava S, Sherman C, Meek C, et al. (2011) Pharmacokinetic mismatch does not lead to emergence of isoniazid or rifampin-resistant *Mycobacterium tuberculosis*, but better antimicrobial effect: a new paradigm for anti-tuberculosis drug scheduling. Antimicrob Agents Chemother 55:5085–5089. doi:10.1128/AAC.00269-11
92. Pasipanodya JG, Gumbo T (2011) A new evolutionary and pharmacokinetic-pharmacodynamic scenario for rapid emergence of resistance to single and multiple anti-tuberculosis drugs. Curr Opin Pharmacol 11:457–463. doi:10.1016/j.coph.2011.07.001
93. Drusano GL, Hope W, MacGowan A, et al. (2015) Suppression of emergence of resistance in pathogenic bacteria: keeping our powder dry, part 2. Antimicrob Agents Chemother 60:1194–1201. doi:10.1128/AAC.02231-15
94. Hameed PS, Manjrekar P, Chinnapattu M, et al. (2014) Pyrazolopyrimidines establish MurC as a vulnerable target in *Pseudomonas aeruginosa* and *Escherichia coli*. ACS Chem Biol 9:2274–2282. doi:10.1021/cb500360c
95. Mistry A, Warren MS, Cusick JK, et al. (2013) High-level pacidamycin resistance in *Pseudomonas aeruginosa* is mediated by an Opp oligopeptide permease encoded by the *opp-fabI* operon. Antimicrob Agents Chemother 57:5565–5571. doi:10.1128/aac.01198-13
96. Mann PA, Muller A, Xiao L, et al. (2013) Murgocil is a highly bioactive staphylococcal-specific inhibitor of the peptidoglycan glycosyltransferase enzyme MurG. ACS Chem Biol 8:2442–2451. doi:10.1021/cb400487f
97. Barbour AG, Mayer LW, Spratt BG (1981) Mecillinam resistance in *Escherichia coli*: dissociation of growth inhibition and morphologic change. J Infect Dis 143:114–121. doi:10.1093/infdis/143.1.114
98. Sakamoto Y, Furukawa S, Ogihara H, et al. (2003) Fosmidomycin resistance in adenylate cyclase deficient (cya) mutants of *Escherichia coli*. Biosci Biotechnol Biochem 67 (9):2030–2033. doi:10.1271/bbb.67.2030
99. Wang H, Gill CJ, Lee SH, et al. (2013) Discovery of wall teichoic acid inhibitors as potential anti-MRSA β-lactam combination agents. Chem Biol 20:272–284. doi:10.1016/j.chembiol.2012.11.013
100. Haydon DJ, Stokes NR, Ure R, et al. (2008) An inhibitor of FtsZ with potent and selective anti-staphylococcal activity. Science 321:1673–1675. doi:10.1126/science.1159961
101. Kaul M, Mark L, Zhang Y, et al. (2015) TXA709, an FtsZ-targeting benzamide prodrug with improved pharmacokinetics and enhanced in vivo efficacy against methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 59:4845–4855. doi:10.1128/AAC.00708-15
102. Robertson GT, Doyle TB, Du Q, et al. (2007) A novel indole compound that inhibits *Pseudomonas aeruginosa* growth by targeting MreB is a substrate for MexAB-OprM. J Bacteriol 189:6870–6881. doi:10.1128/jb.00805-07
103. McLeod SM, Fleming PR, MacCormack K, et al. (2015) Small-molecule inhibitors of gram-negative lipoprotein trafficking discovered by phenotypic screening. J Bacteriol 197:1075–1082. doi:10.1128/JB.02352-14
104. Inukai M, Takeuchi M, Shimizu K (1984) Effects of globomycin on the morphology of bacteria and the isolation of resistant mutants. Agric Biol Chem 48(2):513–518. doi:10.1271/bbb1961.48.513
105. Kaplan N, Albert M, Awrey D, et al. (2012) Mode of action, in vitro activity, and in vivo efficacy of AFN-1252, a selective antistaphylococcal FabI inhibitor. Antimicrob Agents Chemother 56:5865–5874. doi:10.1128/AAC.01411-12
106. de Jonge BL, Walkup GK, Lahiri SD, et al. (2013) Discovery of inhibitors of 4′-phosphopantetheine adenylyltransferase (PPAT) to validate PPAT as a target for antibacterial therapy. Antimicrob Agents Chemother 57:6005–6015. doi:10.1128/AAC.01661-13

107. Freiberg C, Brunner NA, Schiffer G, et al. (2004) Identification and characterization of the first class of potent bacterial acetyl-CoA carboxylase inhibitors with antibacterial activity. J Biol Chem 279:26066–26073. doi:10.1074/jbc.M402989200
108. Freiberg C, Pohlmann J, Nell PG, et al. (2006) Novel bacterial acetyl coenzyme a carboxylase inhibitors with antibiotic efficacy in vivo. Antimicrob Agents Chemother 50:2707–2712. doi:10.1128/AAC.00012-06
109. Miller JR, Dunham S, Mochalkin I, et al. (2009) A class of selective antibacterials derived from a protein kinase inhibitor pharmacophore. Proc Natl Acad Sci U S A 106:1737–1742. doi:10.1073/pnas.0811275106
110. Vickers AA, Potter NJ, Fishwick CWG, et al. (2009) Analysis of mutational resistance to trimethoprim in *Staphylococcus aureus* by genetic and structural modelling techniques. J Antimicrob Chemother 63:1112–1117. doi:10.1093/jac/dkp090
111. Kawatkar SP, Keating TA, Olivier NB, et al. (2014) Antibacterial inhibitors of gram-positive thymidylate kinase: SAR and chiral preference of a new hydrophobic binding region. J Med Chem 57:4584–4597. doi:10.1021/jm500463c
112. Painter RE, Adam GC, Arocho M, et al. (2015) Elucidation of DnaE as the antibacterial target of the natural product, nargenicin. Chem Biol 22:1362–1373. doi:10.1016/j.chembiol.2015.08.015
113. Kuhl A, Svenstrup N, Ladel C, et al. (2005) Biological characterization of novel inhibitors of the gram-positive DNA polymerase IIIC enzyme. Antimicrob Agents Chemother 49:987–995. doi:10.1128/aac.49.3.987-995.2005
114. Mills SD, Eakin AE, Buurman ET, et al. (2011) Novel bacterial NAD+-dependent DNA ligase inhibitors with broad spectrum activity and antibacterial efficacy in vivo. Antimicrob Agents Chemother 55:1088–1096. doi:10.1128/aac.01181-10
115. O'Neill AJ, Cove JH, Chopra I (2001) Mutation frequencies for resistance to fusidic acid and rifampicin in *Staphylococcus aureus*. J Antimicrob Chemother 47:647–650. doi:10.1093/jac/47.5.647
116. Leeds JA, LaMarche MJ, Brewer JT, et al. (2011) In vitro and in vivo activities of novel, semisynthetic thiopeptide inhibitors of bacterial elongation factor Tu. Antimicrob Agents Chemother 55:5277–5283. doi:10.1128/aac.00582-11
117. Sulavik MC, Houseweart C, Cramer C, et al. (2001) Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. Antimicrob Agents Chemother 45:1126–1136. doi:10.1128/aac.45.4.1126-1136.2001
118. Pucci MJ, Bronson JJ, Barrett JF, et al. (2004) Antimicrobial evaluation of nocathiacins, a thiazole peptide class of antibiotics. Antimicrob Agents Chemother 48:3697–3701. doi:10.1128/aac.48.10.3697-3701.2004
119. Montgomery JI, Smith JF, Tomaras AP, et al. (2014) Discovery and characterization of a novel class of pyrazolopyrimidinedione tRNA synthesis inhibitors. J Antibiot 68:361–367. doi:10.1038/ja.2014.163
120. Ochsner UA, Young CL, Stone KC, et al. (2005) Mode of action and biochemical characterization of REP8839, a novel inhibitor of methionyl-tRNA synthetase. Antimicrob Agents Chemother 49:4253–4262. doi:10.1128/aac.49.10.4253-4262.2005
121. Min S, Ingraham K, Huang J, et al. (2015) Frequency of spontaneous resistance to peptide deformylase inhibitor GSK1322322 in *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*. Antimicrob Agents Chemother 59:4644–4652. doi:10.1128/AAC.00484-15
122. Conlon BP, Nakayasu ES, Fleck LE, et al. (2013) Activated ClpP kills persisters and eradicates a chronic biofilm infection. Nature 503:365–370. doi:10.1038/nature12790. http://www.nature.com/nature/journal/vaop/ncurrent/abs/nature12790.html#supplementary-information
123. Howe JA, Wang H, Fischmann TO, et al. (2015) Selective small-molecule inhibition of an RNA structural element. Nature 526:672–677

124. Matsushima A, Takakura S, Fujihara N, et al. (2010) High prevalence of mutators among *Enterobacter cloacae* nosocomial isolates and their association with antimicrobial resistance and repetitive detection. Clin Microbiol Infect 16:1488–1493. doi:10.1111/j.1469-0691.2010.03145.x
125. Projan SJ (2008) Whither antibacterial drug discovery? Drug Discov Today 13(7–8):279–280. doi:10.1016/j.drudis.2008.03.010
126. Chait R, Vetsigian K, Kishony R (2012) What counters antibiotic resistance in nature? Nat Chem Biol 8:2–5. doi:10.1038/nchembio.745
127. Baym M, Stone LK, Kishony R (2016) Multidrug evolutionary strategies to reverse antibiotic resistance. Science 351:aad3292. doi:10.1126/science.aad3292
128. Yeh PJ, Hegreness MJ, Aiden AP, et al. (2009) Drug interactions and the evolution of antibiotic resistance. Nat Rev Microbiol 7:460–466. doi:10.1038/nrmicro2133
129. Silver LL (2013) Viable screening targets related to the bacterial cell wall. Ann N Y Acad Sci 1277:29–53. doi:10.1111/nyas.12006
130. Bugg TDH, Braddick D, Dowson CG, et al. (2011) Bacterial cell wall assembly: still an attractive antibacterial target. Trends Biotechnol 29:167–173. doi:10.1016/j.tibtech.2010.12.006
131. Katz AH, Caufield CE (2003) Structure-based design approaches to cell wall biosynthesis inhibitors. Curr Pharm Des 9:857–866. doi:10.2174/1381612033455305
132. Schneider T, Sahl H-G (2010) An oldie but a goodie – cell wall biosynthesis as antibiotic target pathway. Int J Med Microbiol 300:161–169. doi:10.1016/j.ijmm.2009.10.005
133. Janardhanan J, Chang M, Mobashery S (2016) The oxadiazole antibacterials. Curr Opin Microbiol 33:13–17. doi:10.1016/j.mib.2016.05.009
134. Spink E, Ding D, Peng Z, et al. (2015) Structure-activity relationship for the oxadiazole class of antibiotics. J Med Chem 58:1380–1389. doi:10.1021/jm501661f
135. Tomasic T, Zidar N, Kovac A, et al. (2010) 5-Benzylidenethiazolidin-4-ones as multitarget inhibitors of bacterial Mur ligases. ChemMedChem 5:286–295. doi:10.1002/cmdc.200900449
136. Ling LL, Schneider T, Peoples AJ, et al. (2015) A new antibiotic kills pathogens without detectable resistance. Nature 517:455–459. doi:10.1038/nature14098
137. Homma T, Nuxoll A, Brown Gandt A, et al. (2016) Dual targeting of cell wall precursors by teixobactin leads to cell lysis. Antimicrob Agents Chemother 60:6510–6517. doi:10.1128/AAC.01050-16
138. Arenz S, Wilson DN (2016) Bacterial protein synthesis as a target for antibiotic inhibition. Cold Spring Harb Perspect Med 6:a025361. doi:10.1101/cshperspect.a025361
139. Eyal Z, Matzov D, Krupkin M, et al. (2016) A novel pleuromutilin antibacterial compound, its binding mode and selectivity mechanism. Sci Rep 6:39004. doi:10.1038/srep39004
140. The-Pew-Charitable-Trusts (2014) Antibiotics currently in clinical development. http://www.pewtrusts.org/en/multimedia/data-visualizations/2014/antibiotics-currently-in-clinical-development. Accessed 14 Apr 2017
141. Melinta (2017) ESKAPE pathogen program. http://melinta.com/pipeline/eskape-pathogen-program/. Accessed 14 Apr 2017
142. Appili (2017) Pipeline. http://www.appilitherapeutics.com/pipeline/. Accessed 14 Apr 2017
143. Tse-Dinh Y-C (2016) Targeting bacterial topoisomerases: how to counter mechanisms of resistance. Future Med Chem 8:1085–1100. doi:10.4155/fmc-2016-0042
144. Kern G, Palmer T, Ehmann DE, et al. (2015) Inhibition of *Neisseria gonorrhoeae* type II topoisomerases by the novel spiropyrimidinetrione AZD0914. J Biol Chem 290(34):20984–20994. doi:10.1074/jbc.M115.663534
145. Biedenbach DJ, Bouchillon SK, Hackel M, et al. (2016) In vitro activity of gepotidacin, a novel triazaacenaphthylene bacterial topoisomerase inhibitor, against a broad spectrum of bacterial pathogens. Antimicrob Agents Chemother 60:1918–1923. doi:10.1128/aac.02820-15
146. Barbachyn MR (2008) Recent advances in the discovery of hybrid antibacterial agents. Annu Rep Med Chem 43:281–290. doi:10.1016/S0065-7743(08)00017-1

147. Ma Z, Lynch AS (2016) Development of a dual-acting antibacterial agent (TNP-2092) for the treatment of persistent bacterial infections. J Med Chem 59:6645–6657. doi:10.1021/acs.jmedchem.6b00485
148. Missner A, Pohl P (2009) 110 years of the Meyer–Overton rule: predicting membrane permeability of gases and other small compounds. ChemPhysChem 10:1405–1414. doi:10.1002/cphc.200900270
149. Lipinski C, Lombardo F, Dominy B, et al. (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 23:3–25. doi:10.1016/S0169-409X(96)00423-1
150. Veber DF, Johnson SR, Cheng H-Y, et al. (2002) Molecular properties that influence the oral bioavailability of drug candidates. J Med Chem 45:2615–2623. doi:10.1021/jm020017n
151. Lipinski CA (2003) Physicochemical properties and the discovery of orally active drugs: technical and people issues. In: Hicks M, Kettner C (eds) Molecular informatics: confronting complexity. Beilstein-Institut, Bozen
152. Zhang MQ, Wilkinson B (2007) Drug discovery beyond the 'rule-of-five'. Curr Opin Biotechnol 18:478–488. doi:10.1016/j.copbio.2007.10.005
153. McFarland JW, Berger CM, Froshauer SA, et al. (1997) Quantitative SAR among macrolide antibacterial agents: in vitro and in vivo potency against *Pasteurella multocida*. J Med Chem 40:1340–1346. doi:10.1021/jm960436i
154. Nikaido H, Thanassi DG (1993) Penetration of lipophilic agents with multiple protonation sites into bacterial cells: tetracyclines and fluoroquinolones as examples. Antimicrob Agents Chemother 37:1393–1399
155. Rezai T, Yu B, Millhauser GL, et al. (2006) Testing the conformational hypothesis of passive membrane permeability using synthetic cyclic peptide diastereomers. J Am Chem Soc 128:2510–2511. doi:10.1021/ja0563455
156. Li XZ, Ma D, Livermore DM, et al. (1994) Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to β-lactam resistance. Antimicrob Agents Chemother 38:1742–1752. doi:10.1128/aac.38.8.1742
157. Cai H, Rose K, Liang L-H, et al. (2009) Development of a LC/MS-based drug accumulation assay in *Pseudomonas aeruginosa*. Anal Biochem 385:321–325. doi:10.1016/j.ab.2008.10.041
158. Davis TD, Gerry CJ, Tan DS (2014) General platform for systematic quantitative evaluation of small-molecule permeability in bacteria. ACS Chem Biol 9:2535–2544. doi:10.1021/cb5003015
159. Carey PR, Heidari-Torkabadi H (2015) New techniques in antibiotic discovery and resistance: Raman spectroscopy. Ann N Y Acad Sci 1354:67–81. doi:10.1111/nyas.12847
160. Nikaido H, Vaara M (1985) Molecular basis of bacterial outer membrane permeability. Microbiol Rev 49:1–32
161. 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
162. Zgurskaya HI, López CA, Gnanakaran S (2015) Permeability barrier of gram-negative cell envelopes and approaches to bypass it. ACS Infect Dis 1:512–522. doi:10.1021/acsinfecdis.5b00097
163. Silver LL (2016) A gestalt approach to gram-negative entry. Bioorg Med Chem 24:6379–6389. doi:10.1016/j.bmc.2016.06.044
164. Manchester JI, Buurman ET, Bisacchi GS, et al. (2012) Molecular determinants of AcrB-mediated bacterial efflux implications for drug discovery. J Med Chem 55:2532–2537. doi:10.1021/jm201275d
165. Nikaido H, Pagès J-M (2012) Broad specificity efflux pumps and their role in multidrug resistance of gram negative bacteria. FEMS Microbiol Rev 36:340–363. doi:10.1111/j.1574-6976.2011.00290.x
166. Tommasi R, Brown DG, Walkup GK, et al. (2015) ESKAPEing the labyrinth of antibacterial discovery. Nat Rev Drug Discov 14:529–542. doi:10.1038/nrd4572

167. Krishnamoorthy G, Wolloscheck D, Weeks JW, et al. (2016) Breaking the permeability barrier of *Escherichia coli* by controlled hyperporination of the outer membrane. Antimicrob Agents Chemother 60:7372–7381. doi:10.1128/AAC.01882-16
168. Scorciapino M, Acosta-Gutierrez S, Benkerrou D, et al. (2017) Rationalizing the permeation of polar antibiotics into gram-negative bacteria. J Phys Condens Matter 29:113001. doi:10.1088/1361-648X/aa543b
169. Graef F, Vukosavljevic B, Michel JP, et al. (2016) The bacterial cell envelope as delimiter of anti-infective bioavailability – an in vitro permeation model of the gram-negative bacterial inner membrane. J Control Release 243:214–224. doi:10.1016/j.jconrel.2016.10.018
170. Lewis K (2010) Challenges and opportunities in antibiotic discovery. In: Choffnes E, Relman DA, Mack A (eds) Antibiotic resistance: implications for global health and novel intervention strategies: workshop summary. National Academies Press, Washington, pp 233–256
171. Silver LL (2008) Are natural products still the best source for antibacterial discovery? The bacterial entry factor. Expert Opin Drug Discov 3:487–500. doi:10.1517/17460441.3.5.487
172. Lewis K (2013) Platforms for antibiotic discovery. Nat Rev Drug Discov 12(5):371–387. doi:10.1038/nrd3975
173. Cinquin B, Maigre L, Pinet E, et al. (2015) Microspectrometric insights on the uptake of antibiotics at the single bacterial cell level. Sci Rep 5:17968. doi:10.1038/srep17968
174. Tian H, Six DA, Krucker T, et al. (2017) Subcellular chemical imaging of antibiotics in single bacteria using C60-secondary ion mass spectrometry. Anal Chem 89:5050–5057. doi:10.1021/acs.analchem.7b00466
175. Richter MF, Drown BS, Riley AP, et al. (2017) Predictive compound accumulation rules yield a broad-spectrum antibiotic. Nature 545:299–304. doi:10.1038/nature22308
176. Pewtrusts (2016) A scientific roadmap for antibiotic discovery. http://www.pewtrusts.org/en/research-and-analysis/reports/2016/05/a-scientific-roadmap-for-antibiotic-discovery. Accessed 14 Apr 2017
177. Shore CK, Coukell A (2016) Roadmap for antibiotic discovery. Nat Microbiol 1:16083. doi:10.1038/nmicrobiol.2016.83
178. Kim A, Kutschke A, Ehmann DE, et al. (2015) Pharmacodynamic profiling of a siderophore-conjugated monocarbam in *Pseudomonas aeruginosa*: assessing the risk for resistance and attenuated efficacy. Antimicrob Agents Chemother 59:7743–7752. doi:10.1128/AAC.00831-15
179. Tomaras AP, Crandon JL, McPherson CJ, et al. (2013) Adaptation-based resistance to siderophore-conjugated antibacterial agents by *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 57:4197–4207. doi:10.1128/AAC.00629-13
180. Ghosh M, Miller PA, Mollmann U, et al. (2017) Targeted antibiotic delivery: selective siderophore conjugation with daptomycin confers potent activity against multi-drug resistant Acinetobacter baumannii both in vitro and in vivo. J Med Chem 60:4577–4583. doi:10.1021/acs.jmedchem.7b00102
181. Ito A, Nishikawa T, Matsumoto S, et al. (2016) Siderophore cephalosporin cefiderocol utilizes ferric iron transporter systems for antibacterial activity against *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 60:7396–7401. doi:10.1128/AAC.01405-16
182. Hancock REW (1984) Alterations in outer membrane permeability. Annu Rev Microbiol 38:237–264. doi:10.1146/annurev.mi.38.100184.001321
183. Epand RM, Epand RF (2009) Lipid domains in bacterial membranes and the action of antimicrobial agents. Biochim Biophys Acta 1788:289–294. doi:10.1016/j.bbamem.2008.08.023
184. Plesiat P, Nikaido H (1992) Outer membranes of gram-negative bacteria are permeable to steroid probes. Mol Microbiol 6:1323–1333. doi:10.1111/j.1365-2958.1992.tb00853.x
185. Miller JR, Ingolia TD (1989) Cloning and characterization of β-lactam biosynthetic genes. Mol Microbiol 3:689–695. doi:10.1111/j.1365-2958.1989.tb00217.x
186. Nakashima T, Takahashi Y, Omura S (2016) Search for new compounds from Kitasato microbial library by physicochemical screening. Biochem Pharmacol 134:42–55. doi:10.1016/j.bcp.2016.09.026

187. Genilloud O (2014) The re-emerging role of microbial natural products in antibiotic discovery. Antonie Van Leeuwenhoek 106:173–188. doi:10.1007/s10482-014-0204-6
188. Harvey AL, Edrada-Ebel R, Quinn RJ (2015) The re-emergence of natural products for drug discovery in the genomics era. Nat Rev Drug Discov 14:111–129. doi:10.1038/nrd4510
189. Gaudencio SP, Pereira F (2015) Dereplication: racing to speed up the natural products discovery process. Nat Prod Rep 32:779–810. doi:10.1039/c4np00134f
190. Silver LL (2015) Natural products as a source of drug leads to overcome drug resistance. Future Microbiol 10:1711–1718. doi:10.2217/fmb.15.67
191. Silver LL (2012) Rational approaches to antibacterial discovery: pre-genomic directed and phenotypic screening. In: Dougherty TJ, Pucci MJ (eds) Antibiotic discovery and development. Springer, New York, pp 33–75. doi:10.1007/978-1-4614-1400-1_2
192. Monciardini P, Iorio M, Maffioli S, et al. (2014) Discovering new bioactive molecules from microbial sources. J Microbial Biotechnol 7:209–220. doi:10.1111/1751-7915.12123
193. Abrahams Garth L, Kumar A, Savvi S, et al. (2012) Pathway-selective sensitization of *Mycobacterium tuberculosis* for target-based whole-cell screening. Chem Biol 19:844–854. doi:10.1016/j.chembiol.2012.05.020
194. Adamek M, Spohn M, Stegmann E, et al. (2017) Mining bacterial genomes for secondary metabolite gene clusters. Methods Mol Biol 1520:23–47. doi:10.1007/978-1-4939-6634-9_2
195. Lewis K (2016) New approaches to antimicrobial discovery. Biochem Pharmacol 134:87–98. doi:10.1016/j.bcp.2016.11.002
196. Olano C, Méndez C, Salas J (2014) Strategies for the design and discovery of novel antibiotics using genetic engineering and genome mining. In: Villa TG, Veiga-Crespo P (eds) Antimicrobial compounds. Springer, Berlin Heidelberg, pp 1–25. doi:10.1007/978-3-642-40444-3_1
197. Bachmann B, Lanen S, Baltz R (2014) Microbial genome mining for accelerated natural products discovery: is a renaissance in the making? J Ind Microbiol Biotechnol 41:175–184. doi:10.1007/s10295-013-1389-9
198. Müller R, Wink J (2013) Future potential for anti-infectives – how to exploit biodiversity and genomic potential. Int J Med Microbiol 304:3–13. doi:10.1016/j.ijmm.2013.09.004
199. Wohlleben W, Mast Y, Stegmann E, et al. (2016) Antibiotic drug discovery. Microb Biotechnol 9:541–548. doi:10.1111/1751-7915.12388
200. Wang J, Soisson SM, Young K, et al. (2006) Platensimycin is a selective FabF inhibitor with potent antibiotic properties. Nature 44:358–361. doi:10.1038/nature04784
201. Wang J, Kodali S, Lee SH, et al. (2007) Discovery of platencin, a dual FabF and FabH inhibitor with in vivo antibiotic properties. Proc Natl Acad Sci U S A 104(18):7612–7616. doi:10.1073/pnas.0700746104
202. CARB-X. CARB-X injects up to $48 million to accelerate first powered by CARB-X portfolio of drug discovery and development projects to tackle antibiotic resistance. http://www.carb-x.org/press. Accessed 14 Apr 2017