Appropriate Targets for Antibacterial Drugs

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Successful small-molecule antibacterial agents must meet a variety of criteria. Foremost is the need for selectivity and safety: It is easy to kill bacteria with chemicals, but difficult to do it without harming the patient. Other requirements are possession of a useful antibacterial spectrum, no cross-resistance with existing therapeutics, low propensity for rapid resistance selection, and pharmacological properties that allow effective systemic dosing. Choosing molecular targets for new antibiotics does seem a good basis for achieving these criteria, but this could be misleading. Although the presence of the target is necessary to insure the desired spectrum, it is not sufficient, as the permeability and efflux properties of various species, especially Gram-negatives, are critical determinants of antibacterial activity. Further, although essentiality (at least in vitro), lack of close human homologs, lack of target-based cross-resistance, and presence in important pathogens can be predicted based on the target, the choice of a single enzyme as a target may increase the likelihood of rapid resistance selection. In fact, it is likely that the low output of antibacterial target-based discovery is because of difficulty of endowing lead enzyme inhibitors with whole-cell activity and to the propensity for such inhibitors (if they can gain entry) to select rapidly for resistance. These potential problems must be reckoned with for success of novel target-based discovery.

The specter of increasing antibiotic resistance has raised recognition that the world is reliant on antibiotics for the maintenance and improvement of world health. Whether multi-drug-resistant (MDR) pathogens become prevalent and lead to a postantibiotic era before we are able to put the brakes on the resistance phenomenon is uncertain. But, it is clear that there are movements underway to address the looming problem of antibiotic resistance by various means, including increased surveillance to map the course of resistance spread, development of rapid diagnostics to insure early selection of suitable therapeutics, improved regulatory pathways, incentives for discovery of new agents, and studies of alternate therapeutic routes.

However, discovery of new agents is not a given, even if incentivized. The rate of discovery of developable novel antibacterial agents has been decreasing. Although it is true that large pharmaceutical companies have been leaving the area, their exit was not only for financial and regulatory reasons, but it followed many years of focused pursuit of new antibiotics with little success. The highly productive years of screening natural products for discovery of new antibiotics via mostly empirical (“kill-the-
bug”) screens had ended in the early 1980s and the output of novel useful antibacterial classes fell dramatically. More rational methods of drug discovery, using whole-cell-directed phenotypic screens were instituted (Singh et al. 2011; Mills and Dougherty 2012; Silver 2012), but this too was generally unproductive, turning up leads but few drugs. Fosfomycin, thienamycin, and the early β-lactamase inhibitors, found through such screens, were exceptions (Gadebusch et al. 1992; Silver 2012). Thus, when antibiotic resistance started its dramatic increase in the 1980s and 1990s, starting with methicillin-resistant Staphylococcus aureus (MRSA), the industry looked for new ways to attack the problem. One direction that proved productive was reevaluation and development of previously discovered antibiotics targeting Gram-positives, for example, daptomycin, and modification of existing classes to cover the resistant species, as with the β-lactams active against MRSA (Livermore 2006). By the mid-1990s, however, genomics and bioinformatics, along with high-throughput technologies for chemical synthesis and compound screening, transformed the discovery process to the search for inhibitors of novel targets.

THE ADVENT OF GENOMICS

It is often remarked that the molecular targets of existing antibacterial agents are limited. And this is true; there are estimated to be about 40 targets of marketed agents (Lange et al. 2007). In the mid-1990s, under threat of the increase in antibiotic resistance and aided (or goaded) by the advent of genomics, this apparently narrow set of exploited targets led many in industry and academia to search for new targets, with the idea that identification of hitherto unexploited targets would lead to discovery of new agents that were not cross-resistant with existing classes of drugs. This proposed lack of cross-resistance was based on the assumption that cross-resistance is a function of the molecular target (which is not always true). Thousands of scholarly reviews (e.g., Chan et al. 2002; Isaacson 2002; Lerner and Beutel 2002; McDevitt et al. 2002; Mills 2006; Monaghan and Barrett 2006), described the new paradigm of antibiotic discovery based on identification and prioritization of new targets and the proliferation of high-throughput techniques to find and optimize inhibitors of these targets.

In fact, there are many but not an unlimited number of essential gene products in bacteria, 160 to 170 shared by a broad spectrum of bacteria (Forsyth et al. 2002; Payne et al. 2007) and 400 or so in Salmonella typhimurium and Escherichia coli (Schmid et al. 1989; Black and Hare 2000). Interestingly, few, if any, of these were discovered through genomics. Rather, they were known from microbial genetics studies starting in the 1960s, based on conditional lethal mutants that defined most of the essential functions of bacteria. Because the earliest methods for antibiotic discovery used empirical screens, all of the essential functions of bacteria should have been subject to discovery. It is not necessary to know the target of a new antibacterial a priori and very few of the antibacterial drugs in use were discovered by target-directed screening. Still, target-based discovery was a rational approach and it gained widespread adherence.

Genomics does provide a great deal of useful information on the distribution of essential genes among species and forms the basis for greatly improved understanding of mechanisms of antibiotic action and resistance, bacterial physiology, and metabolic networks. But the genomic approach of providing a fertile field of unexploited targets for discovery of inhibitors that could be turned into drugs has been unproductive. The reasons for this have been the subject of general reviews (Overbye and Barrett 2005; Gwynn et al. 2010; Livermore 2011; Silver 2011; Chopra 2012) and has been documented in reports of the extensive novel target-directed screening programs of GlaxoSmithKline (Philadelphia, PA) (Payne et al. 2007) and AstraZeneca (Wilmington, DE) (Tommasi et al. 2015). The main barriers have been (1) the high probability of rapid resistance arising to inhibitors of the single enzymes chosen via genomics studies (Silver 2007; Brotz-Oesterhelt and Brunner 2008), and (2) the inappropriateness of current chemical libraries as sources of antibac-
materials, especially because of the difficulty of compound entry into bacterial cells (Silver 2011; Brown et al. 2014; Tommasi et al. 2015).

Once it was recognized that the narrow focus on novel “unexploited” targets, although heavily pursued, was not productive, the area slowly turned to appreciation that the tried-and-true targets are indeed worthy, somehow privileged, and should be pursued with novel chemical matter (Projan 2002; Lange et al. 2007).

THE NATURE OF “GOOD” ANTIBACTERIAL TARGETS

The molecular targets of the successful antibacterials are relatively few but they are almost uniformly involved in pathways of macromolecular synthesis; indeed, these are the essential functions of bacteria that cannot be satisfied by feeding of intermediates. Notably, only a few targets of the main classes of antibacterials used in systemic monotherapy are essential enzymes. As listed in Table 1, most of these systemic monotherapeutic agents have nonprotein targets. Most target ribosomal RNA, intermediates in cell-wall synthesis, or membranes. Only the β-lactams and fluoroquinolones target enzymes and these, notably, each target at least two enzymes. On the other hand, there are many registered antibacterials, listed in Table 2, that do target single essential enzymes. In column 3 of Tables 1 and 2, the relative frequency and function of single-step endogenous chromosomal mutations to high-level resistance (more than sixteenfold minimum inhibitory concentration [MIC]) seen in vitro are shown, whereas in column 4 of both tables, the major forms of clinically important resistance are noted. It can be seen that, for the monotherapeutic agents of Table 1, the occurrence of single-step mutations giving significant resistance in vitro is rare to low, whereas for the single-targeted agents in Table 2, single-step in vitro resistance is stan-

### Table 1. Antibacterial classes used in systemic monotherapy

<table>
<thead>
<tr>
<th>Drug class</th>
<th>Target</th>
<th>High-level(^a) single-step resistance in vitro</th>
<th>Major clinical resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Lactams</td>
<td>Multiple PBPs</td>
<td>Endogenous β-lactamase, porin loss, efflux</td>
<td>HGT β-lactamases, endogenous β-lactamases, porin loss, efflux</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Lipid II</td>
<td>Rare</td>
<td>HGT vancomycin-resistance cassettes; stepwise cell-wall changes</td>
</tr>
<tr>
<td>Macrolides</td>
<td>50S RNA</td>
<td>Rare</td>
<td>HGT MLS(_{B}) ribosome methylation, efflux</td>
</tr>
<tr>
<td>Oxazolidinones</td>
<td>50S RNA</td>
<td>Rare</td>
<td>HGT cfr ribosome methylation</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>50S RNA</td>
<td>Rare</td>
<td>HGT modification</td>
</tr>
<tr>
<td>Pleuromutilins</td>
<td>50S RNA</td>
<td>Rare</td>
<td>HGT cfr target methylation</td>
</tr>
<tr>
<td>Streptogramins</td>
<td>50S RNA</td>
<td>Rare</td>
<td>HGT MLS(_{B}) ribosome methylation, efflux, modification</td>
</tr>
<tr>
<td>Lincomycins</td>
<td>50S RNA</td>
<td>Rare</td>
<td>HGT MLS(_{B})</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>30S RNA</td>
<td>Low (some efflux)</td>
<td>HGT efflux, ribosome protection</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>30S RNA</td>
<td>Low (ribosomal proteins)</td>
<td>HGT modification</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Gyrase, Topo IV</td>
<td>Rare</td>
<td>Stepwise two or more than two target mutations, efflux</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>Membranes</td>
<td>Rare</td>
<td>Stepwise membrane changes</td>
</tr>
<tr>
<td>Polymyxins</td>
<td>Membranes</td>
<td>Low (mutations modifying LPS)</td>
<td>Stepwise LPS changes, HGT modification</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>DNA (?)</td>
<td>Low (loss of reductase, entry)</td>
<td>HGT nim (alternate reductase) genes</td>
</tr>
</tbody>
</table>

\(^a\)PBPs, Penicillin binding proteins; HGT, horizontal gene transfer; LPS, lipopolysaccharide.

\(^{\text{a}}\)Minimum inhibitory concentrations (MICs) raised more than sixteenfold.

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Clinical resistance to the agents in Table 1 is generally caused by horizontal gene transfer (HGT) with contribution of the in vitro types of resistance seen in only a few cases. These observations and other supporting evidence led to the multitarget hypothesis (Silver and Bostian 1993; Silver 2007; Brotz-Oesterhelt and Brunner 2008) that successful systemic monotherapeutic agents are those that have low levels of endogenous single-step resistance, and this is due to their targeting of the products of multiple genes or of pathways.

For the single-targeted agents, the types of resistance seen in vitro are generally the same as those seen in the clinic, with few exceptions. In fact, few of the agents in Table 2 are used in systemic monotherapy. Rifampicin is almost always used in combination, as are trimethoprim and sulfamethoxazole, presumably limiting the selection of resistance. Fidaxomicin is used in nonsystemic treatment of Clostridium difficile; although resistance due to target mutations can occur, dosing is extremely high and presumably explains why resistance is rarely seen. Fusidic acid has been used for treatment of S. aureus skin infections, generally in combination with rifampicin to retard resistance development. However, it is now being developed for monotherapeutic use with a high loading dose to decrease resistance development (Fernandes 2016).

Of the single-enzyme inhibitors, only fosfomycin is currently used as monotherapy and that mainly for urinary tract infections (UTIs). Interestingly, the prevalent in vitro mutations to fosfomycin resistance, caused by loss of permeases necessary for active transport across the cytoplasmic membrane, are only rarely seen in vivo (in UTI). It seems that these transport mutants have reduced growth rates in media and urine in the presence of fosfomycin and are not maintained in the bladder (Nilsson et al. 2003). Recently, it has been proposed to use fosfomycin for more systemic indications against MDR pathogens, and it remains to be seen whether the low resistance frequencies, caused by endogenous mutations, extend to other sites of infection (Karageorgopoulos et al. 2012).

Inhibitors of FabI and LpxC are in various stages of development, and progress in these two areas is reviewed in the literature by Yao and Rock (2016) and Erwin (2016), respectively. In each case, the propensity of inhibitors to select for resistance is carefully reviewed. According to Yao and Rock, for FabI inhibitors, which have a narrow spectrum (Staphylococci only),...
it may be possible to design extremely potent enzyme inhibitors that are able to be dosed at levels that overcome target-based MIC increases. For single-target inhibitors with broader spectra, the need to inhibit homologs across many species may limit the potency attainable. The potential for clinically important resistance will have to be critically monitored.

Not listed in Table 2 is the inhibitor of leucyl-tRNA synthetase, GSK2251052, which failed in a phase II trial for complicated UTIs due to high-level resistance seen after 1 day of treatment (O’Dwyer et al. 2014). The mutants were highly fit and were shown to arise at a high rate in vitro. This has proved a cautionary tale for the antibacterial discovery community and has fortunately increased attention to the possibility of rapid resistance development.

TARGET LOCATION

As noted above, one of the reasons for failure of target-based antibacterial discovery, especially that done by screening for inhibition of enzyme activity, is the inability to endow such enzyme inhibitors with the ability to enter and be retained in bacterial cells. Entry into Gram-positives is generally attainable, as their permeability barrier is the cytoplasmic membrane; thus, neutral, nonpolar compounds are preferred. It is Gram-negative entry that is highly problematic as there are no simple rules for the physicochemical properties that can guide the entry of a molecule through the outer membrane, avoiding efflux and penetrating the cytoplasmic membrane. Thus, cytoplasmic targets are generally a poor bet for Gram-negative agents. However, it is often possible to afford entry into the periplasm. Thus, extracellular or periplasmic targets are most attractive for Gram-negatives. Still, the resistance—nodulation—division (RND) efflux pumps exert their power in the periplasm and must be avoided. It may be that the β-lactam antibiotics owe much of their success, aside from their multiple targets, to periplasmic location and covalent interaction with those targets that would allow escape from the sweep of efflux pumps.

NONESSENTIAL TARGETS

It is often posited that virulence functions of pathogens would make good targets, even though they are nonessential to the survival of the bacteria, because inhibitors of those functions would exert little selective pressure for their loss. There is little proof of this and some evidence to the contrary. A recent review (Ruer et al. 2015) discusses in detail the probability of resistance development against antivirulence agents, noting that resistance has been shown to occur in some cases of quorum-sensing inhibitors (Maeda et al. 2012) but that there may be some virulence functions nonessential for survival that might have low resistance potential. Mutations will occur regardless of the presence of inhibitors and it is their selection that is responsible for maintenance in the population. If inhibitors of virulence genes have no effect on growth or adherence, then it is conceivable that selective pressure could be negligible. But more studies are required (Garcia-Contreras et al. 2015). As with all target/inhibitor pairs, rapid resistance development, its maintenance, and spread must be tested and monitored.

CONCLUSIONS

The standard list of target criteria should emphasize low-resistance potential and accessibility to inhibitors. The classically useful antibiotics (in Table 1) define targets that have shown their value and validity. For use as monotherapeutic systemic agents, it would seem that, with possible exceptions, a significant frequency of single-step spontaneous resistance is not acceptable because those mutations would be present in a sufficiently large infectious load and, if fit, could compete with susceptible siblings. The agents in Table 1 have very low single-step resistance rates, presumably caused by their so-called multitargeting. Notably, few of these classical targets are proteinaceous enzymes. New agents should be sought, using novel chemical matter that attack these validated targets. Inhibitors of single enzymes, which otherwise meet target criteria, may theoretically avoid resistance selection by use in combinations, by being safe
enough for dosing at high enough levels to overcome resistance (above the mutant-prevention concentration), or by selecting mutants that have a high-fitness cost that prevents retention of resistance after removal of the selective pressure.

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