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 regula-

tory 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-

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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 re-evaluation 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 academe 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 GlaxoSmith-Kline (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-

terials, 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 sys-

temic 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

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

PBPs, Penicillin binding proteins; HGT, horizontal gene transfer; LPS, lipopolysaccharide.

^aMinimum inhibitory concentrations (MICs) raised more than sixteenfold.

Table 2. Antibacterial classes targeting single enzymes

Drug class	Target	High-level ^a single-step resistance in vitro	Major clinical resistance
Rifampicin	RNA polymerase	Changes in <i>rpoB</i> at many sites	Changes in <i>rpoB</i> at fewer sites
Trimethoprim	Dihydrofolate reductase	Altered DHFR (<i>folA</i>)	Altered DHFR, HGT altered DHFR
Sulfonamides	Dihydropterate synthase	Altered DHPS (<i>folP</i>)	Altered <i>folP</i> , HGT altered DHPS
Fidaxomicin	RNA polymerase	Changes in <i>rpoC</i> , <i>rpoB</i>	Low, <i>rpoC</i> or <i>rpoB</i>
Fosfomycin	MurA	Loss of permeases	HGT-modifying enzymes
Fusidic acid	Elongation factor G	Mutations in <i>fusA</i> (EfG)	Mutations in <i>fusA</i> ; HGT target protection FusB,C,D
Fab I inhibitors	Enoyl (acyl carrier protein) reductase	Mutations in <i>fabI</i>	Mutations in <i>fabI</i>
LpxC inhibitors	Lipid A deacetylase	Mutations in <i>fabZ</i> , efflux, <i>lpxC</i>	Mutations in <i>fabZ</i> , efflux

DHFR, Dihydrofolate reductase; DHPS, dihydropteroate synthase; HGT, horizontal gene transfer.

^aMinimum inhibitory concentrations (MICs) raised more than sixteenfold.

dardly seen. 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 mono-

therapeutic 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

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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.

REFERENCES

*Reference is also in this collection.

- Black T, Hare R. 2000. Will genomics revolutionize antimicrobial drug discovery? *Curr Opin Microbiol* **3**: 522–527.
- Brotz-Oesterhelt H, Brunner NA. 2008. How many modes of action should an antibiotic have? *Curr Opin Pharmacol* **8**: 564–573.
- Brown DG, May-Dracka TL, Gagnon MM, Tommasi R. 2014. Trends and exceptions of physical properties on antibacterial activity for Gram-positive and Gram-negative pathogens. *J Med Chem* **57**: 10144–10161.
- Chan PF, Macarron R, Payne DJ, Zalacain M, Holmes DJ. 2002. Novel antibacterials: A genomics approach to drug discovery. *Curr Drug Targets Infect Disord* **2**: 291–308.
- Chopra I. 2012. The 2012 Garrod Lecture: Discovery of antibacterial drugs in the 21st century. *J Antimicrob Chemother* **68**: 496–505.
- * Erwin AL. 2016. Antibacterial drug discovery targeting the lipopolysaccharide biosynthetic enzyme LpxC. *Cold Spring Harb Perspect Med* doi: 10.1101/cshperspect.a025304.
- * Fernandes P. 2016. Fusidic acid: A bacterial elongation factor inhibitor for the oral treatment of acute and chronic staphylococcal infections. *Cold Spring Harb Perspect Med* **6**: a025437.
- Forsyth RA, Haselbeck RJ, Ohlsen KL, Yamamoto RT, Xu H, Trawick JD, Wall D, Wang L, Brown-Driver V, Froelich JM, et al. 2002. A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*. *Mol Microbiol* **43**: 1387–1400.
- Gadebusch HH, Stapley EO, Zimmerman SB. 1992. The discovery of cell wall active antibacterial antibiotics. *Crit Rev Biotechnol* **12**: 225–243.
- Garcia-Contreras R, Maeda T, Wood TK. 2015. Can resistance against quorum-sensing interference be selected? *ISME J* **10**: 4–10.
- Gwynn MN, Portnoy A, Rittenhouse SE, Payne DJ. 2010. Challenges of antibacterial discovery revisited. *Ann NY Acad Sci* **1213**: 5–19.
- Isaacson RE. 2002. Genomics and the prospects for the discovery of new targets for antibacterial and antifungal agents. *Curr Pharm Des* **8**: 1091–1098.
- Karageorgopoulos DE, Wang R, Yu XH, Falagas ME. 2012. Fosfomycin: Evaluation of the published evidence on the emergence of antimicrobial resistance in Gram-negative pathogens. *J Antimicrob Chemother* **67**: 255–268.
- Lange RB, Locher HH, Wyss PC, Then RL. 2007. The targets of currently used antibacterial agents: Lessons for drug discovery. *Curr Pharm Des* **13**: 3140–3154.
- Lerner CG, Beutel BA. 2002. Antibacterial drug discovery in the post-genomics era. *Curr Drug Targets Infect Disord* **2**: 109–119.
- Livermore DM. 2006. Can β -lactams be re-engineered to beat MRSA? *Clin Microbiol Infect* **12**: 11–16.
- Livermore DM. 2011. Discovery research: The scientific challenge of finding new antibiotics. *J Antimicrob Chemother* **66**: 1941–1944.
- Maeda T, Garcia-Contreras R, Pu M, Sheng L, Garcia LR, Tomas M, Wood TK. 2012. Quorum quenching quinary: Resistance to antivirulence compounds. *ISME J* **6**: 493–501.
- McDevitt D, Payne DJ, Holmes DJ, Rosenberg M. 2002. Novel targets for the future development of antibacterial agents. *J Appl Microbiol* **92**: 28S–34S.
- Mills SD. 2006. When will the genomics investment pay off for antibacterial discovery? *Biochem Pharmacol* **71**: 1096–1102.
- Mills SD, Dougherty TJ. 2012. Cell-based screening in antibacterial discovery. In *Antibiotic discovery and development* (ed. Dougherty TJ, Pucci JM), pp. 901–929. Springer, Boston.
- Monaghan RL, Barrett JE. 2006. Antibacterial drug discovery—Then, now and the genomics future. *Biochem Pharmacol* **71**: 901–909.
- Nilsson AI, Berg OG, Aspevall O, Kahlmeter G, Andersson DI. 2003. Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*. *Antimicrob Agents Chemother* **47**: 2850–2858.
- O'Dwyer K, Spivak A, Ingraham K, Min S, Holmes DJ, Jakielaszek C, Rittenhouse S, Kwan A, Livi GP, Sathe G, et al. 2014. Bacterial resistance to leucyl-tRNA synthetase inhibitor GSK2251052 develops during treatment of complicated urinary tract infections. *Antimicrob Agents Chemother* **59**: 289–298.
- Overbye KM, Barrett JE. 2005. Antibiotics: Where did we go wrong? *Drug Discov Today* **10**: 45–52.
- 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.
- Projan SJ. 2002. New (and not so new) antibacterial targets—From where and when will the novel drugs come? *Curr Opin Pharmacol* **2**: 513–522.
- Ruer S, Pinotsis N, Steadman D, Waksman G, Remaut H. 2015. Virulence-targeted antibacterials: Concept, promise, and susceptibility to resistance mechanisms. *Chem Biol Drug Des* **86**: 379–399.
- Schmid MB, Kapur N, Isaacson DR, Lindroos P, Sharpe C. 1989. Genetic analysis of temperature-sensitive lethal mutants of *Salmonella typhimurium*. *Genetics* **123**: 625–633.
- Silver LL. 2007. Multi-targeting by monotherapeutic antibacterials. *Nat Rev Drug Discov* **6**: 41–55.
- Silver LL. 2011. Challenges of antibacterial discovery. *Clin Microbiol Rev* **24**: 71–109.
- Silver LL. 2012. Rational approaches to antibacterial discovery: Pre-genomic directed and phenotypic screening. In *Antibiotic discovery and development* (ed. Dougherty TJ, Pucci MJ), pp. 33–75. Springer, Boston.

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- Silver LL, Bostian KA. 1993. Discovery and development of new antibiotics: The problem of antibiotic resistance. *Antimicrob Agents Chemother* **37**: 377–383.
- Singh SB, Young K, Miesel L. 2011. Screening strategies for discovery of antibacterial natural products. *Expert Rev Anti Infect Ther* **9**: 589–613.
- Tommasi R, Brown DG, Walkup GK, Manchester JI, Miller AA. 2015. ESKAPEing the labyrinth of antibacterial discovery. *Nat Rev Drug Discov* **14**: 529–542
- * 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.