The role of genomics in antimicrobial discovery

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Introduction

The majority of today’s most effective classes of antimicrobials originated many decades ago as natural products isolated from soil-colonizing bacteria and fungi. Several of these antibiotics were amenable to semi-synthetic chemistry, and as a result the antimicrobial industry has excelled at fine-tuning these existing classes of antibiotics to improve their spectrum, efficacy and safety. Conversely, there are very few examples of novel synthetic antimicrobials. Linezolid, an oxazolidinone antimicrobial,1 represents the first significant synthetic compound class introduced to the market in >25 years, since compounds of the quinolone class were optimized into today’s very successful fluoroquinolones.2

In 1969, US Surgeon General William Stewart testified before US Congress that it was time to “…close the book on infectious diseases…” . In addition, during the 1980s, antimicrobial research and development had limited appeal to the pharmaceutical industry due to the large number of effective products on the market and increasing generic substitution.3 In contrast to this earlier situation, there is presently a driving need to discover new antimicrobials to combat the escalating threats of drug resistance, as well as new and re-emerging infectious diseases.4 This shift in the unmet medical needs associated with infectious disease has resulted in renewed interest in antimicrobial discovery.3 In order to confront resistance effectively, it is necessary to develop novel classes of compounds aimed at existing targets or new targets not yet exploited by current antimicrobial therapies. In addition, it would be prudent for governing authorities to mandate and enforce policies for more appropriate antimicrobial use in medicine and agriculture so that these new classes remain effective for as long as possible.5 The present article will focus on genomics-related technologies as they are currently applied to the discovery of small molecule antibacterial therapies. These concepts are, in many cases, also applicable to antifungal and antiviral research.

Microbial genomics

New technologies are driving significant change in antimicrobial discovery and development. One such technology is microbial genomics, which in recent years has had a considerable impact on the overall paradigm of antimicrobial discovery. At the forefront of this technology was the high throughput sequencing and assembly of the Haemophilus influenzae genomic DNA sequence in 1995.6 This landmark accomplishment was the first free-living whole-organism genomic sequence ever published. A second, much smaller genome of Mycoplasma genitalium was sequenced later in the same year.7 The availability of this second completed genomic sequence facilitated the first detailed comparison of two different whole-genome sequences and ushered in the concept of the ‘minimum gene set for cellular life’, which predicted the minimum number of genes required for a bacterium to sustain independent life.8 This in turn has prompted continuous development of sophisticated bioinformatic tools to carry out detailed comparative analyses on an ever-increasing directory of whole sequenced genomes. These tools have evolved at a rapid pace to keep up with the large volumes of sequence being deposited into databases. Informatics and data management continue to be critical issues associated with genomics research. Currently, there are 79 completely sequenced bacterial genomes publicly available, including >40 human pathogens.9,10 Additionally, there are >100 genome-sequencing projects worldwide, at various stages of completion.10 In contrast, there are few whole-genome sequences of fungi publicly available, partially due to their size and complexity. The yeast Saccharomyces cerevisiae genome
was sequenced in 1996 and remains the workhorse model in the fungal world.11

Genomics and target-based antimicrobial discovery

As genome sequences have been completed, functional genomic studies were conducted to test the minimum gene set hypothesis. These studies included using genome-scale transposon mutagenesis to identify all non-essential genes in the first two sequenced genomes (H. influenzae and M. genitalium), and thus inferring the essential genes, expression of anti-sense RNA to probe suspected essential genes in Staphylococcus aureus, and knocking out conserved genes of unknown function in Escherichia coli and Bacillus subtilis to identify novel ‘broad-spectrum’ essential genes. Among other important findings, these studies have generated a valuable inventory of essential genes and cellular processes from which to further select and validate antibacterial targets. This inventory of genes forms the basis for the genome-driven target-based approach now commonly applied at the earliest stages of the drug discovery process. This is different from the classic whole-cell screening approaches, which first identified an antimicrobial compound and later sought to establish the cellular target of that compound.

First, a target gene is selected as having a specific conservation profile based on comparative genomic analysis (e.g. narrow spectrum if conserved in one or a few medically relevant bacterial species, or broad spectrum if generally conserved among all bacteria). Next, the target gene must be proven to be essential for in vitro growth, usually by means of genetic manipulation (e.g. gene knockout) in the relevant bacteria. Once validated, the target gene is cloned and sequenced, and its corresponding protein product expressed in an optimized expression system (e.g. Pichia pastoris, Baculovirus or E. coli). The target protein is then purified and a robust biochemical assay is developed that is suitable for screening a large and diverse collection of low molecular weight compounds in order to identify target inhibitors or ‘hits’. The hits are subsequently characterized with respect to potency, mechanism of inhibition and enzyme spectrum and selectivity. When focusing on potential broad-spectrum targets, it is beneficial to express and characterize isoforms from several genetically diverse bacterial species. This has proven to be a useful predictor of bacterial spectrum potential for a given compound. Suitable potent hits with acceptable isozyme spectra are further screened against a panel of microbes for cellular activity. Two of the primary difficulties in converting an enzyme inhibitor into an antimicrobial are firstly, getting past the membrane barrier, and secondly, once inside the cell, to prevent efflux of the compound by one of a number of multidrug resistance efflux pumps (MDRs). In order to determine whether an enzyme inhibitor can get into a cell and inhibit growth, without introducing the complication of efflux, antimicrobial activities can be evaluated using recombinant bacterial strains lacking one or more efflux pumps. Once whole-cell activity is achieved, it is important to ascertain whether the antimicrobial effect is linked to the intended target or via an unintended mechanism. This can be accomplished using defined mechanism of action (MOA) assays, which become decisive in guiding medicinal chemistry efforts with respect to optimizing potency, spectrum and selectivity. As lead compound series are identified and optimized, DMPK (drug metabolism and pharmacokinetic) studies are undertaken and animal models of infection are applied. This is most certainly not a linear process as described above, but an iterative one with frequent decision points along the way, optimizing desired compound properties.

Genomics and genetics play a critical role throughout this process, but it is clear that once a robust assay is subjected to screening, the quality and diversity of the compound collection, combined with the accuracy of follow-up assays, are ultimately what decides the fate of a given target. The genomics-driven, target-based approach using high throughput screening (HTS) as a means to identify chemical starting points is a relatively recent strategy employed in drug discovery. Given this, it will understandably take a period of time for the industry to fine-tune the approach and deliver late-stage lead compounds. However, proof-of-principle success stories are beginning to appear, of which some examples are provided below.

Peptide deformylase

Peptide deformylase (PDF) is a good example of a protein target with potential to facilitate the discovery of a broad-spectrum antibacterial drug. PDF is responsible for removing the N-formyl group from the N-terminal methionine following translation. PDF is encoded by the def gene, which is present in all pathogenic bacteria, including Mycoplasma and Chlamydia species, and which does not share a functionally equivalent gene in mammalian cells. def is an essential gene for bacterial growth and survival, and has been validated as such in E. coli. PDF contains three highly conserved catalytic domains and belongs to the matrix metallo-protease (MMP) family of enzymes. Owing to this, a focused library of metallo-enzyme inhibitors was screened using a robust PDF assay. From this screen, a 7 nM selective inhibitor (BB-3497) of bacterial PDF was identified and characterized as having activity against relevant Gram-positive bacteria and some Gram-negative bacteria. The availability of the solved crystal structure of PDF lends additional value to this target in that a highly rational approach to drug design can be employed during optimization. This is a good proof-of-principle illustration of the genomics-driven, target-based approach: starting with a conserved gene and leading to an antimicrobial compound.
Methionyl tRNA synthetase

Amino acyl tRNA synthetases charge tRNA molecules with their corresponding amino acid, an essential step in protein synthesis. This is a validated target class in that mupirocin inhibits isoleucyl tRNA synthetase and is marketed as a topical antibiotic Bactroban. There are 20 tRNA synthetases, most of which correspond to attractive broad-spectrum antibacterial targets. In a recent paper by Jarvest et al., a potent nanomolar inhibitor (IC50 = 350 nM) was described that came out of high throughput screening of S. aureus methionyl tRNA synthetase for enzyme inhibition. The inhibitor was competitive with methionine (substrate) and had a Ki of 100 nM, but did not have antibacterial activity. Medicinal chemists improved the enzyme potency to 1.0 nM and subsequently achieved antibacterial activity against S. aureus and Enterococcus faecalis (MIC <0.06–0.125 mg/L). They confirmed that this activity was linked to the desired MOA and showed in vivo efficacy in a rat abscess model of infection. This is a good example of a target-based project where an enzyme inhibitor identified by HTS can be successfully optimized into an antibacterial.

Target-based whole-cell screening assays

In an effort to capitalize on genomics and yet minimize the risk of not achieving whole-cell activity with hits from HTS, there is an alternative approach combining genomics with the classic whole-cell screening approach. Targets are identified and validated in the same manner as described earlier in this paper, however, instead of screening an isolated enzyme, screening would be carried out using a genetically altered strain of bacteria such that it would respond in a measurable way when a target of interest is inhibited. The response could be measured as growth inhibition (absorbance) or induction of a linked reporter gene (e.g. luminescence or fluorescence). A secondary enzyme assay would provide confirmation of target inhibition. If successful, target-directed inhibitors with a microbiological profile could be identified directly from HTS. This would allow medicinal chemists to focus on other important properties such as potency and DMPK without the diversion of permeability or efflux issues. An example of this is provided below.

LpxC

Lipid A is an essential component of the outer membrane of Gram-negative bacteria. LpxC is a metallo-enzyme that carries out the second step in lipid A biosynthesis and is an essential protein required for growth of Gram-negative bacteria. Clements et al. reported on screening of an lpxC mutant of E. coli, predicted to be hypersusceptible to LpxC inhibitors, with a collection of low molecular weight compounds containing metal chelating groups. The idea was that the mutants would help to identify compounds that possessed antibacterial activity and were specific for LpxC. Several compounds with MICs < 1 mg/L were identified by this screen, including two sulphonamide derivatives of α-(R)-amino hydroxamic acid. Screening against a broader panel of pathogens revealed that the compounds were specifically active against Gram-negative pathogens with MICs versus wild-type E. coli of 1–2 mg/L, and had corresponding IC50s versus E. coli LpxC of 160–400 nM. This report validates the use of genomics, genetics and whole-cell screening to identify target-specific inhibitors with antibacterial activity for further development.

DNA microarrays

High-density DNA microarrays allow researchers to monitor the relative expression levels of thousands of genes (an entire microbial genome) on no more than a square inch of solid surface. DNA microarray experiments generate an almost inconceivable amount of functional information regarding the coordinated gene expression that occurs under various microbial growth conditions. Such growth conditions include strains that are treated with subinhibitory concentrations of antimicrobials. Ng et al. published a recent example, where they determined transcription profiles for Streptococcus pneumoniae subjected to sublethal concentrations of antibiotics representing four different classes of translation inhibitors. In their paper, they identified many genes whose expression was either increased or decreased in response to the treatments. This information could provide the basis for developing a specific MOA assay to confirm target inhibition for whole-cell-active compounds, or it could help in identifying the targets of antibacterial compounds with unknown MOAs. In many cases, the DNA microarray technology is still undergoing validation and its potential is yet to be fully realized.

Conclusions

One of the most commonly asked questions in the antimicrobial discovery arena is: ‘Has genomics delivered?’ Genomics and genetics have definitely identified and delivered novel essential targets for target-based discovery efforts. Genomics has delivered information regarding the overall conservation, and hence therapeutic potential of antimicrobial targets (broad or narrow spectrum). It can tell us whether there is a similar gene in the human genome, alerting us to potential mechanism-based toxicity. Genomics has delivered information about active site conservation and has played a fundamental role in structural biology as it is applied to drug discovery and it has facilitated cloning and expression of any gene of interest within days instead of weeks or months. However, genomics
has not, and will not, hand us drugs. This requires appropriate chemical starting points to be present in the compound collections used for screening. Moreover, the industry needs to gain more experience in efficiently converting enzyme inhibitors into antimicrobial compounds with in vivo efficacy, which includes achieving a better understanding of what it takes for a compound to enter a bacterial cell and not be subject to various efflux mechanisms. Genomics has facilitated the identification of potential efflux pumps, but now physiological studies are required to understand their regulation and substrate specificity. In fact, efflux pumps have been considered by some researchers as targets in their own right, which, if inhibited, may potentiate antimicrobials that otherwise would be inactive due to being pumped.\textsuperscript{16} A case in point was illustrated by Lomovskaya et al.,\textsuperscript{22} where efflux pump inhibitors were identified that potentiated the antimicrobial effect of levofloxacin by eight-fold in Pseudomonas aeruginosa.

Natural products have provided us with the vast majority of successful antibiotics to date. However, their route of synthesis has taken thousands of years for natural evolution to perfect. The ultimate goal of the genomics-driven, target-based approach is to discover novel antimicrobials that operate via a novel mechanism of action.\textsuperscript{23,24} This is not an easy task, but indisputably important. We should not make false promises. Genomics is not the Holy Grail. It is an exciting and promising approach in trying to solve a serious problem in health care by delivering novel targets that will help us find novel antimicrobials in our efforts to cure patients from infections.

References