Postgenomic strategies in antibacterial drug discovery

Heike Brötz-Oesterhelt^{1†} & Peter Sass²

¹AiCuris, Wuppertal, Germany

²Institute of Medical Microbiology, Immunology & Parasitology, Pharmaceutical Microbiology Section, University of Bonn, Germany

[†]Author for correspondence: Institute for Pharmaceutical Biology, University of Duesseldorf, Universitätsstrasse 1, Building 26.23.U1, Germany ■ Tel.: +49 211 811 4180 ■ Fax: +49 211 811 1923 ■ heike.broetz-oesterhelt@uni-duesseldorf.de

Review
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During the last decade the field of antibacterial drug discovery has changed in many aspects including bacterial organisms of primary interest, discovery strategies applied and pharmaceutical companies involved. Target-based high-throughput screening had been disappointingly unsuccessful for antibiotic research. Understanding of this lack of success has increased substantially and the lessons learned refer to characteristics of targets, screening libraries and screening strategies. The 'genomics' approach was replaced by a diverse array of discovery strategies, for example, searching for new natural product leads among previously abandoned compounds or new microbial sources, screening for synthetic inhibitors by targeted approaches including structure-based design and analyses of focused libraries and designing resistance-breaking properties into antibiotics of established classes. Furthermore, alternative treatment options are being pursued including anti-virulence strategies and immunotherapeutic approaches. This article summarizes the lessons learned from the genomics era and describes discovery strategies resulting from that knowledge.

This article is dedicated to Professor Dr Harald Labischinski in homage to his constant effort in the discovery and development of novel antibiotics.

Antibiotic therapy under resistance pressure

Since the 1940s we have experienced the impressive potential of antibiotics in reducing morbidity and mortality thereby increasing the quality of life. However, over the last two decades this achievement is increasingly threatened by bacterial resistance development. Nowadays, infections caused by multidrug-resistant bacteria present daily challenges to infectious disease physicians in hospitals throughout the world and these pathogens are spreading into the community [1,2]. In developed countries, the most problematic bacterial species in terms of high incidence of infection, attributable morbidity and resistance factors undermining standard antibiotic therapy include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus*, extended-spectrum β -lactamase producing *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [1–3]. Several treatment options currently exist for the Gram-positive pathogens from this list, which reflects the focused effort of antibiotic companies in this sector over the last decade. Drugs launched since 2000 (particularly linezolid, daptomycin, tigecycline and telavancin) plus the prospect of several additional agents in late-stage development (e.g., anti-MRSA cephalosporins, the glycopeptide-cephalosporin hybrid

TD-1792, the tetracycline derivative amadacycline, and a new streptogramin combination plus several quinolones and oxazolidinones [4]) have, for the time being, averted the immediate threat of MRSA. However, it is important that, with the exception of the lipopeptide daptomycin and the oxazolidinone linezolid, this list lacks further new antibiotic classes, for which there is still a high demand, and that there is also continued need for orally available drugs and bactericidal agents with good tissue penetration. In addition, it is noteworthy that several agents in development for the therapy of infections caused by multiresistant isolates have recently encountered difficulties in the regulatory approval process (e.g., ceftobiprole, iclaprim, oritavancin, dalbavancin, cethromycin), which will postpone or even prevent their market entries either worldwide or in particular countries.

Regarding Gram-negative pathogens, the current treatment situation is far more worrisome because new anti-Gram-negative agents are scarce in pharmaceutical pipelines and predominantly in early stages of development [4]. This asymmetry can, to a certain extent, be attributed to the fact that this bacterial spectrum had received less attention from the pharmaceutical industry from the mid-1990s onwards, but it is also important to bear in mind that it is much easier to discover novel agents with antibacterial activity against

Keywords

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- drug discovery ■ genomics
- natural product ■ rational design ■ resistance
- screening ■ structure ■ target

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Gram-positive than against Gram-negative species and that nonfermenters are especially difficult to target. The outer membrane of Gram-negative bacteria plays an important role in this respect, because its penetration requirements are directly opposed to those of the cytoplasmic membrane. A broad collection of highly efficient efflux pumps further aggravates antibiotic attack. Based on our personal experience we estimate that, when screening random and unbiased libraries of diverse low molecular weight chemical entities, the hit rate for compounds with initial activity against *Escherichia coli* is 1–2 orders of magnitude lower than that for *S. aureus*, and for nonfermenters such as *P. aeruginosa* the yield is especially low. As a result, we have to cope with an increasing number of reports on infections caused by multidrug-resistant Gram-negative microorganisms for which antibacterial treatment options are very limited [5]. European surveillance data from 2008 indicate that 3.1% of invasive *E. coli* isolates and 14% of *Klebsiella pneumoniae* blood isolates showed combined resistance to fluoroquinolones, aminoglycosides and third-generation cephalosporins. Similarly, 17% of European *P. aeruginosa* isolates were shown to be resistant to three or more compounds or compound classes, respectively (including piperacillin/tazobactam, ceftazidime, carbapenems, fluoroquinolones and aminoglycosides) [201]. The recovery of *P. aeruginosa* isolates susceptible only to polymyxins from critically ill patients has left colistin as the only available therapy in these cases. It is characteristic of our current situation that we have no choice but treat these patients with a drug that had been shelved for decades due to strong nephrotoxicity and neurotoxicity and that would most probably never obtain regulatory approval by current standards. Even worse, outbreaks of colistin-resistant pandrug-resistant *P. aeruginosa* isolates have already been described [6,7]. Despite the rarity of isolation of pandrug-resistant Gram-negative bacteria so far, there is great concern, as clinicians are practically left with no treatment options for patients who carry these bacteria. Industry is aware of the urgent need for novel resistance breaking compounds against Gram-negative bacteria and among the companies that remained active in antibiotic research many shifted their present attention to the discovery of new anti-Gram-negative agents, which resulted in several distinct approaches in discovery and early development [4,8].

We have to face the fact that bacterial resistance development and spread is an inevitable consequence of any antibiotic treatment. The

very short generation times characteristic for bacteria facilitate development of resistance traits by a rapid mutation and selection process and the ability to disseminate genetic material across species borders promotes their spread [9]. In addition, versatile efflux pumps [10] and efficient penetration barriers [11], as well as the ability to react to antibiotic exposure by elaborate physiological responses in order to reduce the detrimental effects on cell structure and metabolism [12] constitute an impressive intrinsic defense strategy of bacteria against antibiotic attack. As a result, we cannot completely prevent the erosion of efficacy of our current antibiotic arsenal over time by bacterial resistance development [202], even by the most prudent application regimens or hygienic measures [9,13]. However, it should be emphasized that proper hygiene, skilful antibiotic treatment and restricted use play an important role, as obvious from the large geographic differences in resistance prevalence and that we need globally improved measures in order to prolong the lifespan of our current antibiotics [202]. However, even against our newest antibiotic classes resistance has already been reported, emphasizing the need for novel antibiotics (FIGURE 1) [14,15]. It is also crucial to bear in mind that bacterial resistance is not the only factor that defines medical need. Severe nosocomial infections in critically ill or immunocompromised patients can cause life-threatening illnesses and death, even if the causative pathogens fall below the susceptibility breakpoints of the applied antibiotics. In a situation where we face an aging, less immunocompetent patient population and where medical progress allows for treatments such as cancer chemotherapy and organ transplantation, we have additional need for highly effective, bactericidal and highly penetrative antibiotics that complement our current armamentarium. Therefore, the continuous discovery and development of novel antibacterial agents with unprecedented mechanisms of action devoid of cross-resistance to currently used antibiotics as well as the exploration of alternative treatment strategies are necessary to ensure efficient antibacterial therapy in the future.

The classical approach: screening for whole-cell antibacterial activity

Without exception, all currently used antibiotic classes were discovered through an empirical serendipitous approach by screening natural product extracts or synthetic compounds from various sources for promising antibacterial activity against bacteria of the desired spectrum. The vast majority

of them were discovered in a highly productive period from the 1940s to the 1960s (FIGURE 1). Then a gap of almost four decades followed until the first representative of a new class entered the market in 2000. What happened in the meantime to prevent prolongation of this streak of success? One reason that is often forgotten is that the pharmaceutical industry was busy and productive in optimizing the originally discovered predecessor molecules and developed a multitude of considerably improved follow-up compounds that were superior with respect to efficacy, bacterial spectrum, tolerability and dosing interval. It cannot be denied that the perception of having basically solved the problem of bacterial infections together with commercial considerations as discussed below had triggered a decline in antibacterial research and development efforts. However, the hundreds of antibacterial natural products described in the scientific literature [16] demonstrate that considerable effort had continuously been invested into

the discovery of new antibacterial lead structures. Such screening programs seem to have led either to the rediscovery of already known antibiotic classes or to compounds that were inferior in their overall profile or too complex for chemical modification at that time. One feasible explanation is that the low hanging fruit, that is to say, the natural product antibiotics that are readily produced under laboratory conditions by easily culturable organisms, had already been picked. Furthermore, regulatory demands on the efficacy and safety of new antibiotics have substantially increased over time, in parallel with the improved therapeutic standard and technical progress in measuring these parameters. As a result, the requirements that a new antibiotic chemical entity has to fulfill in order to obtain regulatory approval are much higher today than in the early days of antibiotic discovery and many agents that had been approved at that time would not be able to achieve this goal by current standards.

Figure 1. Overview of systemically applied broad-spectrum antibiotic classes. The approximate year of introduction into human therapy is indicated along with concomitant occurrence of bacterial resistance. ESBL: Extended spectrum β -lactamase; VISA: Vancomycin intermediately resistant *Staphylococcus aureus*; VRE: Vancomycin-resistant *Enterococcus*; VRSA: Vancomycin highly resistant *Staphylococcus aureus*.

The genomic approach: the unfulfilled promise

The lack of success from screening of microbial extracts together with the discovery of the enormous potential of the synthetic quinolone class had led most pharmaceutical companies to redirect their screening efforts from natural product libraries to synthetic low molecular weight compounds. The enormous amount of bacterial genomic information, which has become available from 1995 onwards, triggered further major alterations in antibacterial screening strategies and led to the advent of the 'genomics era'. Today more than 1000 fully sequenced eubacterial genomes are available for comparative analyses in antibiotic drug discovery [203]. Rather than phenotypic screening for antibacterial activity, inhibition of rationally preselected targets was at that time considered to be the key to the identification of novel antibacterial lead structures. Central to this paradigm shift was the belief that bacteria would contain a rich number of hitherto unknown and therefore unexploited targets for antibiotic therapy, which were for the first time accessible by genome analysis. Bioinformatics was employed to compare the genomes of important bacterial pathogens and to contrast them with the available eukaryotic genomes. With the intention in mind to select targets that are broadly conserved among the desired bacterial spectrum but absent or evolutionary distant in eukaryotes, similar and often overlapping lists of potential target candidates were assembled by pharmaceutical companies, which represented in the range of 150–350 targets, depending on the broadness of the desired bacterial spectrum [17,18]. For target validation (FIGURE 2), the respective genes were then subjected to knockout analyses, mutation studies and inducible gene expression experiments to identify the targets that are essential for bacterial survival under *in vitro* growth conditions. Such experiments were often conducted for one Gram-positive and one Gram-negative model organism only, because the workload for mutating every target species of interest would have been enormous. Target proteins were then expressed, purified and screened, wherever possible under miniaturized high-throughput assay conditions, against libraries in the range of 1 million synthetic compounds, many of which had been obtained by combinatorial and parallel synthesis methods. At the time, considerable effort had been invested into the very first step of antibacterial drug discovery, that is to say, evaluating the quality of novel targets, but it turned out that this

investment led to very limited success in identifying suitable leads for further optimization into antibacterial development candidates (FIGURE 3).

Illustrative examples of large screening campaigns include those of GlaxoSmithKline (GSK) and Pfizer [17,19]. In the years 1995–2001, researchers from GSK had selected more than 350 genes of interest by comparative genome analyses of *S. aureus*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. Of these, 127 were identified as being essential in at least one of these organisms and 67 were screened as purified proteins in high-throughput screening (HTS) against the corporate compound collection, which ranged from 260,000 to 530,000 compounds. Of these 67 assays, only 16 yielded hits, five of them resulted in leads and only one lead series was progressed to development; the fatty acid biosynthesis inhibitor series, which relies on FabI (enoyl-acyl carrier protein [ACP] reductase) as the target [17]. However, as the enoyl-ACP reductase function is performed in several important pathogens by enzymes unrelated to FabI (e.g., FabK in *S. pneumoniae* [20], or FabV in *P. aeruginosa* [21]), this target is only suitable for a narrow spectrum approach and the resulting inhibitor was outlicensed by GSK to the biotech company Affinium. Their candidate AFN-1252 is currently being developed for oral treatment of *S. aureus* infections and Phase I studies were initiated in March 2008. Similarly, Pfizer reported that only four leads resulted from 65 HTS campaigns and that none of them was developed to the point of reaching clinical trials [19]. In a somewhat different approach, Cubist had concentrated on one specific target class, which they explored extensively [22]. They predominantly focused on aminoacyl-tRNA synthetases and of the 20 representatives of this target family, which are all indispensable for bacterial survival, they screened 17 enzymes against a rather small library of 50,000 compounds, also without success. Other companies including Wyeth, Bristol Meyers Squibb or Bayer HealthCare had comparable experiences with their target-related HTS approaches, which led to the industry-wide conclusion that it made little scientific or economic sense to simply keep screening more targets in the same manner. Why was the success rate so low in relation to the enormous effort invested into this screening strategy?

Lessons learned from the genomics era

Over the years considerable knowledge has accumulated that explains why the target-based HTS approach was not as successful as anticipated.

Figure 2. The antibacterial drug discovery process. In the search for novel antibiotic classes, two prominent strategies have been followed over the last decade: the genomics approach starting from an unprecedented rationally selected target and an alternative approach starting from a compound with interesting antibacterial activity. For the latter approach, the term reverse genomics had been coined to indicate that the genomic target is identified only in a second step. Due to the aid of powerful functional genomics techniques, the speed of target identification has considerably improved and in most cases the target is known at the start of a lead structure optimization program. From in-depth hit evaluation onwards both approaches are processed similarly. HTS: High-throughput screen; MIC: Minimal inhibitory concentration.

The reasons are related to the targets themselves and their selection procedure, the screening process and the compound pools employed as source of inhibitors.

Lessons from targets

The first explanation lies in the targets themselves. The previous perception that there would be a wealth of novel and unexplored broad-spectrum targets, which could be easily exploited for antibiotic therapy, turned out to be wrong. Apart from the characteristics used for target selection (i.e., target conservation, evolutionary distance to human homologs and reduction of *in vitro* growth rate upon target depletion), a target has to possess several further equally important prerequisites (Box 1). It is absolutely essential that the target is not only mandatory for bacterial survival *in vitro* but also in the nutrient-rich environment of the human body and that the bacteria have no mechanism for circumventing the target reaction

by acquiring its reaction product from the host. An illustrative example is fatty acid biosynthesis, which had been heavily investigated during the genomics era and resulted in leads such as AFN-1252 (GSK/Affinium) [23], platensimycin (Merck) [24], platencin (Merck) [25] and the pyrrolidine diones (Bayer) [26]. Interestingly, later studies revealed that major low-GC Gram-positive pathogens are resistant to fatty acid biosynthesis inhibitors *in vitro*, when their culture broth is supplemented with unsaturated exogenous fatty acids in the concentration that is present in human serum or when human serum is added [27]. Along the same line, infectivity of *Streptococcus agalactiae* mutants in mice was not diminished by the deletion of essential fatty acid biosynthesis genes, thereby questioning the need for *de novo* fatty acid biosynthesis under *in vivo* infection conditions in compartments where exogenous fatty acids are present [27]. It is also crucial that antibiotics have a low propensity to select for resistant mutants

Figure 3. High attrition rates in target-based high-throughput screening and reasons for failures at the different stages of the profiling cascade.

HTS: High-throughput screening; MIC: Minimal inhibitory concentration; SAR: Structure–activity relationship.

within a bacterial population, which means that point mutations in the target gene must not allow for a noteworthy increase in MIC. Indeed, all antibiotics that have been successfully applied in systemic monotherapy for many years do not rely on single targets or on targets that are encoded by a single gene [28,29]. An additional aspect is that for full coverage of the desired bacterial spectrum the target has to be validated for each and every bacterial species *in vitro* as well as in the infection model. Targets can encode an essential function in one organism but can be dispensable in another by means of a biochemical bypass reaction or redundant enzyme equipment. The enoyl-ACP reductase mentioned above is one example, as it is encoded by distinct isoforms (FabI, FabK, FabL, FabV) in different bacterial species with varying sensitivity for known fatty acid biosynthesis inhibitors [20,21,30]. To complicate the situation further, genetic diversity might even occur for different strains within the same species. When researchers from GSK investigated quinolinone derivatives as exploratory inhibitors of methionyl-tRNA synthetase in a panel of 101 clinical *S. pneumoniae*

isolates, they observed that 20% were intrinsically resistant to their antibiotic due to the presence of a second unrelated methionyl-tRNA synthetase [31]. Knowing these potential pitfalls is a first important step, but this does not solve the problem of identifying novel broad-spectrum targets of high quality. Validation of the essential nature of a previously uninvestigated target for all species of the intended bacterial spectrum *in vitro* as well as in the host during the infection process is mandatory and methodologically highly challenging, especially if at the beginning of a screening campaign a model target inhibitor is not available. Techniques for *in vivo* target validation have been described but only sporadically applied [32,33]. This makes targets and metabolic pathways that have been validated by therapeutically applied antibiotics especially valuable. Along the same line, an analysis of genes expressed by *Salmonella enterica* in a mouse model of typhoid fever revealed that almost all of the *in vivo* expressed and essential genes are either absent in other pathogens, thus limiting their usefulness as broad-spectrum targets, or belong to pathways already addressed by current antibiotics or development compounds [34]. A further lesson that can be learned from therapeutically proven antibiotics is that complex mechanisms (e.g., by interfering with more than a single target or inhibiting a target in a different way than simply preventing its catalytic function) seem beneficial for exerting bactericidal activity [28].

Lessons from screening strategies & screening libraries

The targets are only one part of the problem. The assay set-up and screening strategies applied play another important role. When isolated targets are screened in cell-free biochemical assay formats, penetration through the bacterial cell envelope is not a selection criterion for hits. As a result, a large percentage of the hits obtained will not be able to enter the cell and, consequently, will not exert antibacterial activity [17]. A way to overcome this is the design of whole-cell assays that allow for measurement of the function of the desired target in the intact bacterial cell. This can be achieved, for instance, on the basis of the differential sensitivity of target-depleted strains. The target might either be silenced by antisense RNA [35,36] or be downregulated by expression behind a regulated promoter [37]. In both cases an inhibitor of the desired target will display differential antibacterial activity against mutant versus wild-type strain. The charm of these methodologies is that they can, in principle, be

applied to the analysis of all gene-encoded targets. The drawback is that two detection points (i.e., mutant plus wild-type) are required for each time point and inhibitor concentration, doubling the screening effort. A further technical limitation during HTS is that the inhibitor concentration needs to fall in the range where it prevents growth of the mutant but not yet that of the wild-type and that hitting this often small detection window cannot be guaranteed for all compounds from a large library. Another interesting strategy for whole-cell screening is to go beyond the single target to the analysis of metabolic pathways and target areas. Our knowledge on bacterial regulons responding to certain types of metabolic disturbances including antibiotic attack is constantly growing [12] and examples have been described where this information was exploited for the construction of bacterial reporter strains that reacted to drug-induced inhibition of certain target areas by emission of a luminescent signal [38–40].

Although initial knowledge gaps have probably led to early mistakes in certain cases, such as insufficiently validating unprecedented targets or choosing suboptimal screening strategies, this does not explain the industry-wide lack of success of almost all antibacterial high-throughput target-based campaigns. When natural product libraries were screened with the same assays, proof-of-principle was obtained that known natural product inhibitors of given targets or target areas could successfully be detected [39]. So, why were novel chemical entities suitable as starting points for antibacterial drug discovery so rarely discovered in the large ‘big-pharma’ screening libraries? The problem resides within the compound collections themselves. It is now widely acknowledged that the characteristics of antibiotics differ in important aspects from those of drugs in other therapeutic sectors, such as cancer, cardiovascular, metabolic or central nervous system diseases and in particular that parenteral antibiotics do not follow the Lipinski’s ‘rule of five’ [41–43]. In general, the chemical structures of antibiotics are more complex than those of drugs marketed in other fields and contain more stereocenters, rotatable bonds, proton donors and acceptors and, often, multiple pharmacophores. As far higher serum and tissue concentrations are needed for efficient treatment of bacterial infections than for obtaining pharmacological effects in other disease areas, it is also important that antibiotics possess sufficient solubility and free fractions (i.e., the compound amount that is not tightly bound to serum components such as albumine or α -glycoprotein)

to achieve these drug levels. This necessity plus the penetration requirements of the bacterial cell envelope lead to a physicochemical profile that significantly differs from that of other drugs (FIGURE 4) [42]. In general, antibiotics are less lipophilic than other drugs and higher molecular weights are tolerated for compounds that target extracytoplasmic structures of Gram-positive bacteria. The dilemma is that the distribution of physicochemical parameters of the big-pharma compound libraries follows the average needs of all therapeutic sectors, with the result that only a rather small subset of screening compounds has a profile suitable for antibacterials. HTS will only be capable of identifying compounds that already exist in corporate collections. Increasing understanding of this problem has, in recent years, led to the conclusion that antibacterial screening approaches should rather be based on focused libraries, with more natural product-like physicochemical properties [17,44].

A probable additional counterproductive aspect was the managerial attitude that dominated the high-throughput era. High attrition rates of early hits and leads were expected from this approach, as it relied strongly on quantity and the philosophy was to terminate presumably unsuccessful projects very rapidly. As a result, the periods companies allowed for compound optimization were often too short to meet the substantial optimization requirements of antibacterial leads.

Box 1. Lessons learned from the high-throughput screening era.

The targets

Targets need to be validated in every species (pay attention to subpopulations).
Targets need to be validated *in vivo*.
Single gene targets are prone to rapid resistance development.
Antibacterial polypharmacology (multiple targets, multiple binding sites) is beneficial.
Complex mechanisms, rather than simple inhibition of enzyme function, seem more efficient in achieving bactericidal activity.
Therapeutically validated targets are especially valuable.

The assay formats

Screens against isolated targets often yield hits without cell penetration.
MIC assays often yield nonspecific compounds.
Whole-cell assays increase the chance of specific membrane permeable hits (drawback: limited detection window).

The compounds

Large ‘big-pharma’ compound libraries contain few antibiotic-like structures.
Physicochemistry of antibacterials is different.

Postgenomic approaches

When companies realized that the HTS campaigns as described above make little sense in antibacterial research, substantial shifts in the field occurred. Several big players left the whole area of antibiotic research for economic reasons, as discussed briefly below, mid-sized spin-offs were formed from big-pharma research units that developed products of their parent companies further and a variety of novel biotechnology companies were founded to seize the opportunity of reduced competition. Today, there is no longer a predominant research strategy followed by most, rather, diverse and complementary approaches are being pursued, some of them new, others well-known but technically improved. Several such strategies are illustrated by selected examples in TABLE 1 and corresponding compound structures are depicted in FIGURE 5. Although this collection gives a good impression of the current strategies that are applied in the search for novel antibiotics, it should be noted that this

compilation is only exemplary and that there is no claim of completeness, neither in terms of research programs that have been or still are being pursued, nor with regard to compound classes or inventing companies. For the leads, development candidates or launched products provided as examples, the focus here is on the strategy that led to their discovery. For their status within the discovery or development process, their intended indication as well as strengths and potential weaknesses, the interested reader is referred to other excellent reviews [4,41,45,46].

Natural product research

When the small molecule synthetics failed to deliver suitable leads for antibacterial drug discovery, interest in natural products flared up again [46–49]. This is not astonishing, since approximately 3/4 of all currently used antibiotic classes originate from natural products (FIGURE 1). Although the reasons why natural products are

Figure 4. Physicochemistry of antibiotics compared with drugs in other therapeutic sectors.

Comparing a set of 147 antibacterial compounds to 4623 compounds from the commercially available CMC database reveals major differences between antibiotics and ‘other’ drugs but also between antibacterial agents that target Gram-positive compared with Gram-negative bacteria. Antibiotics with exclusive activity against Gram-positive bacteria have much less restriction in molecular weight than other drugs, while antibiotics directed against Gram-negative species demonstrate a remarkable polarity. It seems that in order to penetrate the Gram-negative cell envelope and to efficiently evade efflux either highly polar compounds or zwitterionic compounds (as in the case of sulfa-drugs and quinolones), which are capable of changing their charge states in the process of crossing the two membranes, are required. It is reasonable to suggest that passage through a polar porin of the outer membrane is more efficient for a polar compound, while penetration of the cytoplasmic membrane can be achieved by passive diffusion in a nonpolar state, active uptake or forced entry of highly cationic compounds (e.g., the proton motive force-dependent self-driven uptake of highly charged aminoglycosides).

CMC: Comprehensive Medicinal Chemistry; Mw: Molecular weight.

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advantageous compared to synthetics in antibacterial drug discovery are not fully understood, it is obvious that natural products strongly surpass synthetics in terms of structural diversity, functional complexity and unique molecular architectures [41]. Their antibacterial activity surpasses that of synthetic screening hits in most cases, which is no wonder considering that antibiotic-producing strains have co-evolved over millions of years with other microorganisms in competition for the same ecological niche. In addition, complex mechanisms of action have often developed, which are multilayered, directed against more than a single target and would probably not have been invented by rational design. Plausible calculations in expert reports led to the conclusion that we have so far discovered only a small percentage of all antibacterial agents that are produced by microbial communities in diverse habitats world wide [47,50]. The majority of the antibacterials known to date are produced by rather easily culturable *Streptomyces* species and even among the streptomycetes a large pool of additional antibiotics can still be expected [47]. Therefore, one reasonable strategy towards future antibiotic drugs is the search for novel natural product leads by improved modern technologies. New habitats are currently being explored and innovative culture conditions are being developed to promote the growth of previously unculturable organisms [51,52]. An interesting pilot study demonstrated that the growth of previously unculturable organisms could be supported by culturing them together with other species from their natural habitat [53]. Modern molecular biology now allows the heterologous expression of foreign biosynthetic gene clusters in hosts that can be cultured more easily and even DNA pools of whole environmental communities are investigated by metagenomic techniques [47]. It can be expected that in the long term such methodical advances will uncover unexplored microbial sources and provide interesting new antibacterial lead structures, although the effort that has to be invested will remain higher than in the early days of antibacterial drug discovery, when there was still a choice of easily accessible antibacterial classes and when regulatory standards were lower.

To fill the medium term gap in their antibiotic pipelines, in the late 1990s companies decided to revisit their already available libraries of natural product extracts or to mine the scientific literature for so far under-evaluated natural products. TABLE 1 provides several examples of structural classes that emerged from that effort. Wyeth, for instance, initiated a program, in which they

re-investigated shelved fractions of their own natural product collection, which had been previously discarded due to narrow spectrum activity. From a fraction of *Streptomyces hygroscopicus* LL-AC98 they obtained the glycopeptide class of mannopeptimycins. The antibiotic complex of this strain had been known at Wyeth since the 1950s and was described in 1970 [301], but structure elucidation was only performed at the beginning the 2000s [54]. Components of the natural product complex showed interesting antibacterial activity against MRSA and penicillin-resistant streptococci and moderate activity against VRE [55]. Subsequently semisynthetic analogues were developed with improved enterococcal activity and safety profiles [56]. Mannopeptimycins inhibit peptidoglycan synthesis at the level of transglycosylation by targeting the membrane-bound precursor lipid II, but their binding site differs from that of vancomycin, which explains their activity against VRE [57].

Another novel natural product that addresses the same target is the peptide antibiotic plectasin that was isolated by Novozymes from the saprophytic fungus *Pseudoplectanina nigrella* [58]. The structure of the oligopeptide plectasin (40 amino acids) closely resembles that of defensins from invertebrates [58]. However, in contrast to many other defensins, which according to current knowledge are believed to act rather nonspecifically at the level of the cytoplasmic membrane by dissipating the membrane potential or by interfering with the structure and function of diverse membrane-associated components [59], plectasin has a defined prokaryotic target, namely lipid II [59,60]. Like the mannopeptimycins, plectasin binds to lipid II in a region other than the D-alanyl-D-alanine terminus, thereby retaining activity against vancomycin-resistant isolates. While the natural product plectasin showed rather low activity against staphylococci, a recently reported derivative, NZ2114, displayed improved activity against staphylococci, while retaining its good activity against streptococci [61,62]. Apart from the proof that new interesting natural products can still be found, the two examples of the mannopeptimycins and plectasins teach a lesson with respect to antibacterial targets. Both classes confirm that it is possible to obtain resistance-breaking qualities by addressing a novel binding site at a therapeutically validated target and they highlight the value of natural products as tool compounds in target validation and identification. The lipodepsipeptide friulimicin, which was discovered by Hoechst Marion Roussel in 2000 [63], also has

Figure 5. Selected antibiotics discussed in this article.

an interesting Gram-positive resistance-breaking spectrum and was recently shown to have an unprecedented target within the therapeutically validated target area of peptidoglycan synthesis. Friulimicin binds to undecaprenyl-pyrophosphate,

thereby sequestering the bacterial lipid carrier and preventing its function in the synthesis of peptidoglycan and wall teichoic acids [64].

In a similar attempt to make use of their existing natural product library, Merck re-screened its

Figure 5. Selected antibiotics discussed in this article (cont.).

culture extract collection for inhibitors of selected targets using RNA silencing in intact bacterial cells. In an assay in which antisense RNA of a key enzyme in fatty acid biosynthesis (the keto-acyl-ACP synthase II [FabF]), was expressed in

S. aureus in order to make FabF rate-limiting for cellular survival, they discovered platensimycin [24,65]. The compound inhibited FabF isolated from *S. aureus* with an IC_{50} of 48nM by binding to the acyl-enzyme intermediate formed in the

Table 1. Selected prominent examples of postgenomic strategies for the discovery of novel antibiotics.

Discovery approach	Compound class (examples)	Compound origin	Target	Companies
Antibiotic strategies				
Screening for novel natural products	Mannopeptimycins	Natural product derivative	Lipid II	Wyeth
	Plectasins (NZ2114)	Natural product derivative	Lipid II	Novozymes/Sanofi-Aventis
	Friulimicin	Unmodified natural product	Undecaprenyl-diphosphate	Hoechst/CombiNature/MerLion
	CB-182804 (undisclosed lipopeptide)	Unknown	Unknown	Cubist
	Platensimycin, platencin	Unmodified natural product	FabF	Merck
Re-evaluation of underexploited natural products	Tetrahydropyrimidinones (Tan 1057, NP)	Natural product derivatives	Ribosomal peptidyltransferase center	Bayer
	Biphenomycins (biphenomycin B, NP)	Natural product and derivatives	Novel target in translation, undisclosed	Bayer/AiCuris
	Pyrrolidine diones (moiramide B, NP)	Natural product derivatives	Acetyl-CoA carboxylase	Bayer/InterMed Discovery
	ADEPs (A54556 A, NP)	Natural product derivatives	Clp protease	Bayer/AiCuris
	Daptomycin	Unmodified natural product	Bacterial cytoplasmic membrane/ envelope	Cubist
	Ramoplanin	Unmodified natural product	Lipid II	Biosearch Italia/ Genome Therapeutics/ IntraBiotics/Oscient/ Nanotherapeutics
	Pleuromutilin (BC-3205)	Natural product derivative	Peptidyl transferase center	Nabriva
Structure-based drug design (new classes)	m-terphenyls (RX B72)	Synthetic, rational design	A-site, 50S ribosomal subunit	Rib-X
	PC 190723 series	Synthetic, rational design	FtsZ	Prolysis
	Pyrrolamides	Synthetic, rational design	GyrB	AstraZeneca
Optimization of established classes	Anti-MRSA cephalosporins (ceftobiprole, ceftaroline)	Established natural product-derived class	Penicillin-binding proteins including PBP2a	Basilea/Johnson & Johnson, Forest
	Tetracyclines (tigecycline, amadacycline)	Established natural product-derived class	16S rRNA at A-site with additional contacts	Wyeth, Paratek/Bayer/ Novartis
	Ketolides (telithromycin)	Established natural product-derived class	23S rRNA domain V plus II	Hoechst/Sanofi-Aventis
	Glycopeptides (telavancin, oritavancin)	Established natural product-derived class	Lipid II, D-alanine-D-alanine terminus, plus additional interactions (interpeptide bridge, cytoplasmic membrane or penicillin-binding proteins)	Theravance/Astellas, Lilly/InterMune/ Targanta/The Medicines Company
	Diamino-pyrimidines (iclaprim)	Synthetic, rational design	Dihydrofolate reductase	Roche/Arpida
	Oxazolidinone (torezolid)	Synthetic	23S rRNA at ribosomal A-site	Dong-A/Trius
	Quinolones (delafloxacin, WCK-771, fleroxacin)	Synthetic	Topoisomerases II and IV	Wakunaga/Abbott/ Rib-X, Wockhardt, Combinature/MerLion
	Neoglycosides (ACHN-490)	Established natural product-derived class	16S rRNA close to A-site	Achaogen
	Monobactams (BAL30072)	Synthetic, natural product origin	Penicillin-binding protein 3 plus 2	Basilea

KPC: *Klebsiella pneumoniae carbapenemase*; MRSA: *Methicillin-resistant Staphylococcus aureus*; NP: *Natural product*.

Table 1. Selected prominent examples of postgenomic strategies for the discovery of novel antibiotics (cont.).

Discovery approach	Compound class (examples)	Compound origin	Target	Companies
Nonantibiotic strategies				
Inhibition of resistance factors	β -lactamase inhibitor (NXL-104)	Synthetic	β -lactamases class A (including KPC), class C and partly class D	Novoxel
	Efflux pump inhibitors	Synthetic	Efflux pumps (e.g., AcrAB-TolC, Mex-pumps)	Mpex
Virulence inhibition	Mar regulon inhibitors (P005784)	Synthetic	Resistance factor and virulence factor expression	Paratek
	Inhibitors of teichoic acid and lipopolysaccharide synthesis	Synthetic	Virulence factor expression	Mutabilis
Biologicals as antibacterial agents	Therapeutic antibody fragment (KB001, KBPA 101, pagibaximab)	Natural product derivatives	Virulence factor antagonization	e.g., KaloBios, Kenta Biotech, Biosynexus, Medarex
	Phage-derived endolysins	Natural product	Peptidoglycan	Profos, academic groups
Immunostimulation	Mimics/derivatives of host defense peptides	Natural product derivatives	Innate immunity, immune-regulatory cascades	e.g., Migenix, Genaera, Ardea, AM-Pharma, Inimex

KPC: *Klebsiella pneumoniae* carbapenemase; MRSA: Methicillin-resistant *Staphylococcus aureus*; NP: Natural product.

course of the condensation reaction. MICs are in the low $\mu\text{g/ml}$ range for staphylococci, streptococci and enterococci. *In vivo* activity was reported in the treatment of a disseminating *S. aureus* infection in mice, although a continuous infusion regimen had to be applied in order to demonstrate this effect [24,66]. All of these examples are against Gram-positive bacteria only. However, first discovery approaches are now also being reported for Gram-negative bacteria. Cubist, for instance, has reported the development of a membrane-active lipopeptide (CB-182804) with bactericidal activity against *Pseudomonas*, *Acinetobacter*, *Escherichia* and *Klebsiella*. The compound is currently in Phase I trials for parenteral therapy of multidrug-resistant Gram-negative bacteria, but to our best knowledge, details about structure and profile have not been disclosed.

Other companies such as Bayer put an effort into the in-depth investigation of previously described but underexploited natural products [41]. After literature mining, several natural product complexes were selected, for which interesting antibacterial activities had been previously reported, but which had been rather poorly characterized otherwise (TABLE 1). Bayer employed functional genomics technologies such as proteomics and transcriptional profiling [67,68] in combination with a versatile methodical platform for mode of action studies to elucidate their mechanisms. Two novel classes of translation inhibitors were identified. The dihydropyrimidinones derived

from the natural product Tan-1057 bind to the large ribosomal subunit and inhibit the peptidyltransferase reaction [67,69] and the biphenomycins interfere with bacterial protein synthesis by another mechanism without cross-resistance to known translation inhibitors [41]. Furthermore, the pseudopeptide pyrrolidine diones, based on the natural product moiramide B, address a new target in fatty acid biosynthesis, the acetyl coenzyme A carboxylase [26]. In addition, a group of acyldepsipeptides designated ADEPs work by dysregulating the Clp protease and initiate bacterial death by degradation of essential bacterial proteins [70,71]. Despite the complexity of the molecules, routes for total syntheses were elaborated in all cases [41], thus, allowing broad compound derivatization using current medicinal chemistry technology. After detailed biological evaluation of the natural products, their weaknesses were addressed in optimization programs. For the pyrrolidine diones and the ADEPs, antibacterial activities were remarkably increased reaching MIC values of 0.01 and 0.05 $\mu\text{g/ml}$, respectively, for *S. aureus* and 0.25 and 0.02 $\mu\text{g/ml}$, respectively, for *S. pneumoniae* [72,73]. For both classes, physicochemical properties were improved in parallel and, in the case of the ADEPs also chemical and metabolic stability [72,73]. For the biphenomycins it was possible to improve antibacterial *in vitro* activity [41] and in the dihydropyrimidinone class, target-related toxicity could be reduced. While the natural product Tan-1057

bound with similar affinities to prokaryotic and eukaryotic ribosomes [69], new derivatives demonstrated improved selectivity indices during *in vitro* transcription/translation in parallel with lower cytotoxicity against eukaryotic cells and improved tolerability in mice [41,74]. In the course of the optimization programs all four classes had *in vitro* and *in vivo* antibacterial activities against important Gram-positive pathogens well in the range of marketed antibiotics, without being affected by pre-existing cross-resistances. These examples demonstrate that previously described compound classes also bear considerable potential that can be exploited when investigating and improving these leads with current technologies.

The marketed drug daptomycin (Cubist) is another interesting example of a natural product that had long been neglected, but which has now found a highly successful application. Eli Lilly and Co., discovered daptomycin in the late 1980s [75] and initiated clinical trials for the treatment of Gram-positive infections, applying 3 mg/kg every 12 h. When transient skeletal muscle myopathy was observed during Phase I trials, Lilly discontinued all development efforts. The drug remained unused for several years until Cubist licensed the compound and discovered that it was surprisingly well tolerated after once daily dosing, which allows the muscles sufficient time for recovery between two applications [76]. When the drug was launched in 2003 for the treatment of complicated skin and soft tissue infections by Gram-positive bacteria at 4 mg/kg/day to be applied in a single 30 min infusion, it addressed a substantial medical need and became a commercial success. Along the same line the glycopeptide ramoplanin A2 is another 'old' natural product that was discovered in 1984 by Biosearch Italia [77]. The compound has substantial activity against the most important Gram-positive pathogens including staphylococci, streptococci, enterococci and clostridia and a convincing target, again, lipid II. However, due to the lack of a suitable formulation for intravenous application and no oral bioavailability, the drug was shelved until the beginning of this decade and then passed through several biotech companies. At present, the unmodified natural product is being developed by Nanotherapeutics and is expected to enter Phase III trials for the oral treatment of *Clostridium difficile*-associated diarrhea, an indication where lack of oral bioavailability is an advantage. In addition, injectable formulations are under investigation [302] and a derivatization program on the basis of a successful total synthesis explores the chance of improved derivatives

[78]. A third prominent case of a long-neglected natural product class is that of the pleuromutilins, a family of protein synthesis inhibitors that bind to the peptidyl transferase center on the 50S ribosomal subunit in a region partly overlapping with both A-site and P-site, thereby blocking peptide bond formation [79]. The natural product pleuromutilin was discovered in 1950 and a derivative, tiamulin, was approved for veterinary use in 1979. However, for human therapy the first representative of this class, retapamulin (GSK) was only introduced in 2007. Due to low oral bioavailability and insufficient metabolic stability, retapamulin is restricted to topical use but a new congener suitable for oral application (BC-3205) is currently being investigated in Phase I clinical trials by Nabriva [80].

Although the various examples presented here prove the potential of natural products for current antibacterial drug discovery, the risks of this approach are also important to consider. Apart from the positive characteristics of natural product antibacterials as described above, there are additional important factors, which are no optimization parameters during natural evolution, but which are indispensable for a successful drug (e.g., safety/tolerability, chemical and metabolic stability, human pharmacokinetics, producibility and cost of goods). Consequently, many natural products require extensive optimization prior to development, which is an often arduous and sometimes impossible task. Obtaining sufficient material for in-depth lead profiling can be a hurdle at the beginning of a new discovery program, when fermentation yields are still low. A laborious route for total synthesis may need to be developed prior to a meaningful optimization program. Complex synthetic access may limit the number of derivatives that can be explored and high production costs may exclude the use of the drug in certain indications. As a result, several of the examples discussed above have limitations that seem to have prevented their progression beyond their current state. Platensimycin has insufficient metabolic stability, friulimycin demonstrated unfavorable human pharmacokinetics in Phase I studies, the ADEPs have a rather high potential for resistance induction, the pyrrolidine dione class demonstrates only moderate *in vivo* activities and for the dihydropyrimidinones no progress was reported for years. Although natural product research is one prominent pillar of post-genomic antibacterial research, it is important that complementary strategies are followed in parallel, as described in the following section.

Structure-based design & focused synthetic approaches

Methodical advances in x-ray crystallography, nuclear magnetic resonance spectroscopy and computational tools made a significant contribution to the technical progress in antibacterial drug discovery. Over the last decade, not only the genes but also the structures of a large number of antibacterial targets became available and often structures of target protein homologues were solved for several bacterial species, offering comparison and broader applicability during modeling studies. Structures with high impact for antibacterial drug discovery include those of several topoisomerases, RNA polymerase, a large array of aminoacyl-tRNA synthetases, peptide deformylase, diverse ribosomal regions, various cytoplasmic and membrane-bound enzymes of peptidoglycan biosynthesis, diverse metabolic enzymes (e.g., those involved in fatty acid and isoprenoid biosynthesis) and cell division machinery (for a constantly updated overview see, for example, the protein database [204]). Importantly, not only the structures of the target apo-proteins were solved, but often also co-structures with substrates, reaction intermediates and inhibitors (including known antibiotics, hits, leads and exploratory tool compounds), thereby generating valuable information on active site topology, drugable regions and pharmacophore structure. This information is increasingly used in antibacterial drug discovery and the recent literature contains a variety of examples where structure-based design was employed by industry and academia for the identification of hits and lead structures from new structural classes as well as for the optimization of established antibiotics [81,82]. The structures are employed in different discovery strategies, including virtual screening of commercial or virtual compounds, fragment-based screening, *de novo* compound design on the basis of target substrates, products or reaction intermediates as well as for guiding chemistry campaigns by HTS. In addition, for interesting compounds discovered by empirical medicinal chemistry programs the co-structure with the target is often determined in the course of the project in order to understand the binding mode, to rationalize the structure–activity relationship (SAR) and to pave the road for further compound optimization. It is beyond the scope of this article to discuss the technical details, strengths and limitations of these approaches and for further information on these aspects the reader is directed to [44,83–86]. Instead, selected important cases will be described here, which exemplify the application of these techniques in the search for novel antibacterial agents.

Rational design was, for example, instrumental in the discovery of iclaprim, the successor of trimethoprim within the therapeutically established class of diaminopyrimidine antibiotics. Iclaprim had been investigated by Arpida in Phase III clinical trials for the treatment of staphylococcal skin and skin-structure infections [87] but failed to meet the noninferiority standards in the US FDA regulatory process. Trimethoprim is a competitive inhibitor of dihydrofolate reductase (DHFR), thereby preventing the biosynthesis of tetrahydrofolate, an essential cofactor in several one-carbon transfer reactions. A single amino acid exchange in the active site of *S. aureus* DHFR (F98Y) results in the loss of a crucial hydrogen bond between trimethoprim and its target and forms the main cause of trimethoprim resistance in this species [88]. Structural information from a trimethoprim–*S. aureus* DHFR co-crystal [88] and thorough understanding of the resistance mechanism was used by Roche in modeling studies with the aim to design diaminopyrimidines with improved activity against the DHFR of Gram-positive bacteria [89]. In iclaprim, which resulted from this effort, the trimethoxyphenyl side chain of trimethoprim is replaced by a dimethoxychromene substituent. This enlarged moiety allows for additional hydrophobic interactions with the target and a general gain of binding energy, resulting in a 20-fold higher affinity of iclaprim compared with trimethoprim at wild-type level (K_i 0.3 and 6 nM, respectively) [90]. When the F98Y mutation occurs, target affinity of both inhibitors decreases by the same factor (20-fold, K_i = 6 nM and 100 nM, respectively). However, according to the principle that potency may beat resistance, iclaprim still maintains low inhibition constants and corresponding MICs in the low $\mu\text{g/ml}$ range, while the MICs for trimethoprim are far beyond therapeutic concentrations [90].

In another program focused on rational design, Rib-X used high-resolution crystal structures of known protein biosynthesis inhibitors bound to the bacterial ribosome as a molecular framework for the design of new antibacterial scaffolds, which competed with the established antibiotics for target interaction. They discovered a series of m-terphenyls including RX-B72, which bind to the ribosomal A-site in a region that largely overlaps with the oxazolidinone binding site. The best compounds from that series demonstrated single-digit MIC values against important Gram-negative pathogens [91].

In efforts to develop novel FtsZ inhibitors undertaken by Polysis, the template for rational design was not provided by an established

antibiotic but by a small exploratory compound, almost devoid of antibacterial activity. FtsZ can be regarded as the pacemaker protein for cell division in bacteria. It is structurally and functionally homologous to mammalian tubulin and undergoes GTP-dependent polymerization at mid-cell at the time of cell division to form a ring structure that recruits further cell division proteins [92]. If the activity of FtsZ is disturbed, including its ability to interact with its partners, cell division is prevented leading to cell mass increase and eventually cell lysis. The simple structure of 3-methoxybenzamide (3-MBA) was selected as a starting point for the program. It had previously been reported to inhibit FtsZ and to induce filamentation in *Bacillus subtilis* as a proof of target inhibition in the intact bacterial cell [93]. Although 3-MBA possesses only marginal antibacterial activity (MIC against *B. subtilis* = 4000 µg/ml), its principle ability to enter Gram-positive cells, its small size, which leaves room for compound expansion, and its corresponding high ligand efficacy (i.e., the free energy of binding per heavy atom) made it an attractive starting point for inhibitor design [94,95]. Classical exploration of the SAR around 3-MBA was supported by docking studies on the basis of the crystal structure of the FtsZ apoprotein from *B. subtilis* and by mutational analyses. The ligand was predicted to bind to a cleft adjacent to the GTP binding site of FtsZ and all mutations, obtained by selecting *S. aureus* resistant against an improved congener from the program, mapped to this region. On the basis of the suggested docking model, the small 3-MBA nucleus was grown in size and yielded PC190723 as the most progressed congener reported from this program so far [94,95]. In PC190723, the 3-methoxy group is replaced by a thiazolopyridine ring attached to the benzamide via an ether bridge. The activity of the compound is almost exclusively focused on staphylococci, with MICs of 0.5–1 µg/ml and moderate efficacy in a murine model of systemic staphylococcal infection. Further optimization is necessary, because *in vivo* efficacy is still 10-fold inferior to vancomycin and a resistance rate of 2×10^{-8} impedes use of the compound in monotherapy.

In cases where a suitable chemical structure that could serve as starting point for a structure-driven approach is not available, fragment-based screening is an option [44,96]. This technique is starting to show its potential in the first development compounds now entering clinical trials, however, these are in areas other than antibacterial research. Abbott is a pioneer in this technology, other companies have integrated it as a part of their discovery platform and AstraZeneca has

reported using it in diverse disease areas including antibacterial lead discovery [44]. The central idea of the fragment-based approach is to screen a comparably small library (in the range of 1000 compounds) of low molecular weight synthetic structures (typically 150–250 Da) for inhibitors that bind to the target of interest. The use of structurally diverse generic synthetic libraries has been reported here with the option to extract a suitable subset of structures for a particular screening program, as well as the design of tailored libraries that exploit already available structural information of a particular target family (e.g., kinases). In the library design for their antibacterial fragment-based screening program, AstraZeneca took care to ensure that not only the molecular weight was lower than in their corporate collection used for HTS but also the clogP (the decadic logarithm of the calculated distribution coefficient between octanol and water), which is used as a measure for lipophilicity, and the nonpolar surface area, reflecting the potential for hydrophobic interactions with the target. Subsequently, this design allowed the addition of hydrophobic substituents to the fragments, which is a common strategy in medicinal chemistry for increasing binding affinity, without exceeding the upper limit of preferred logP. One technical challenge is that due to their limited size the initial fragments have comparably low binding affinities (in the 0.1–10 mM range) and that the screening method has to be able to detect such weak binders reliably. Biophysical methods are often used here, such as nuclear magnetic resonance, surface plasmon resonance or thermal shift assays, which rely on the change in temperature that occurs when a target slightly unfolds upon fragment binding. Biochemical assays can also be used, if they are robust enough to allow extension of the inhibitor concentration to the mM range and if control assays are run in parallel to correct for potential artifacts (e.g., compound autofluorescence and quenching effects). Once binders with suitable affinities are identified, their co-structures with the target guide further optimization programs. Here, the method of expanding the nucleus of a single fragment by growing it within the binding site has so far been more successful than attempts to join two neighboring fragments, because it proved rather difficult to find suitable linkers that retained the optimal orientation and position of the two fragments [44,96]. As a result of this effort, AstraZeneca reported a pyrrolamide series as novel inhibitors of the DNA-gyrase β -subunit. In recent derivatives, a bis-chlorinated pyrrolamide is linked to a carboxy-thiazolyl moiety

via a fluoro-piperidine linker. Most potent representatives displayed bactericidal activity with sub- $\mu\text{g/ml}$ MIC₉₀ values against *S. pneumoniae*, *H. influenzae* and *Moraxella catarrhalis*. However, *in vivo* efficacy requires further improvement [97,98]. The described examples demonstrate that there are alternatives to classical HTS for the discovery of novel inhibitors of bacterial targets and, due to the smaller number of compounds that need to be processed, more attention can be paid to the compilation of screening libraries as well as to the assay setup, which has to comply with medium-throughput requirements only. Structure-based design has proven instrumental in improving potency and selectivity at the target level, but as the conditions that mediate compound uptake through the bacterial cell envelope and that prevent efflux are still incompletely understood; rational design with respect to the latter features is not possible at present. Furthermore, it is obvious that a given structure has to succeed with regard to a variety of further parameters including pharmacokinetics, tolerability and bioavailability. Experience has taught that, using our current methodology, attempts to combine these disparate features into novel exploratory classes are not always successful. Therefore, it is also of high importance that we keep searching for novel resistance-breaking structures within known antibiotic classes that have already proven their therapeutic potential.

Optimizing established antibiotic classes

Several companies have explored the option of introducing novel structural elements into established antibiotics with the aim of improving activities against wild-type strains and resistant isolates. The detailed knowledge of class- and compound-specific characteristics, a profound understanding of the SAR and in many cases molecular information of target–inhibitor interactions are advantages during the optimization of established classes. In fact, most antibiotics that are currently in the later stages of pharmaceutical development pipelines originate from these efforts, which proves the importance of this approach [4]. Here, resistance was circumvented by, for example:

- Increasing affinity for an as yet unaffected protein of a target family (e.g., ceftaroline and ceftobiprole) [99,100];
- Increasing affinity to the original target by establishing additional interactions with the target site or neighboring regions (e.g., iclaprim, torezolid, tigecycline, amadacycline) [89,101–103];

- Including a further binding domain at the same target (e.g., telithromycin, oritavancin) [103–105];
- Introducing an additional mode of action into the antibiotic (e.g., telavancin) [106];
- Evading bacterial efflux pumps (e.g., tigecycline, amadacycline) [107,108].

Interesting examples in earlier stages of the antibacterial drug discovery chain include two compounds with activity against multi-resistant Gram-negative isolates. The neoglycoside ACHN-490 (Phase I) was modified to resist attack by almost all aminoglycoside-degrading enzymes and the monobactam BAL-30072 (preclinical) benefits from facilitated uptake into bacterial cells via a siderophore mechanism and from increased stability against certain β -lactamases [109–112]. For most of these molecules potency was already improved at the susceptible wild-type level and although target-related resistance mutations do often also affect these new derivatives of known classes, their activity seems high enough to maintain therapeutic efficacy against a large proportion of currently resistant isolates. Aside from the examples listed above, the same principle of ‘potency beats resistance’ applies to the new quinolones in development (e.g., delafloxacin and WCK 771), which demonstrate good activity against quinolone-resistant Gram-positive bacteria, especially staphylococci, although the mechanistic basis for this potency increase is less understood due to the lack of target co-structures [113,114]. This general increase of wild-type activity is beneficial in severely ill patients, where highly effective antibiotics are necessary. Another quinolone in development, finafloxacin, relies entirely on the concept of improved activity under infection conditions [115]. While the compound is slightly inferior to established quinolones in standard MIC determinations that are performed at neutral pH, its activity surpasses that of all other marketed quinolones in acidic environments, for example the gastrointestinal or urogenital tract, or during infection conditions that are accompanied by acidification of the environment.

Alternative treatment strategies & narrow spectrum therapy

Apart from the antibiotic approach that relies on an active small molecule that inhibits bacterial growth, a variety of further treatment principles are currently being explored by industry and academia. Extension beyond the scope of classical antibiotics can be envisaged in several ways, either by leaving the requirement of

direct bacterial growth inhibition, by leaving the realm of small molecules, or even by shifting the focus of therapeutic intervention completely away from the invading bacterial pathogen to stimulation of the immune system of the host.

β -lactamase inhibitors are the prototype of agents without own growth-inhibitory activity in the treatment of bacterial infections. In 1980, the first β -lactamase inhibitor, clavulanic acid, was introduced into therapy in combination with amoxicillin, thereby efficiently protecting the potent semisynthetic penicillin-derivative from inactivating hydrolysis by the resistance factor. This combination principle proved highly successful and has preserved the value of potent β -lactams for decades [116]. However, the inhibitory activity of clavulanic acid, as well as of all other β -lactamase inhibitors in clinical use, is restricted to class A β -lactamases and to date more than 500 different β -lactamase types have been described, many of which fall into the structural classes B, C and D. Therefore, it is of interest that several novel β -lactamase inhibitors with broader inhibition spectra are currently in development [117]. A notable example is NXL104 from Novexel, a diazabicyclo(3.2.1.)octanone, which is distinguished by its novel structure, lacking a β -lactam ring, and a remarkably long deacylation rate. The compound shows broad inhibition of β -lactamases from class A, including the recently emerging carbapenemase KPC, from class C and partly from class D, but does not affect class B enzymes [117,118]. NXL104 is currently in Phase II studies in combination with ceftazidime for the treatment of complicated urinary tract and intra-abdominal infections and in Phase I in combination with ceftaroline for the treatment of MRSA infections. It is also interesting that the core structure of this non- β -lactam seems to offer potential beyond the use as a β -lactamase inhibitor. For compounds that carry the diazabicyclo[3.2.1.]octanone core with additional substituents Novexel reported antibacterial activity against drug-resistant *P. aeruginosa* in a patent application [303]. The company also reported the development of a new anti-*Pseudomonas* compound with undisclosed structure (NXL105), which acts by inhibiting bacterial transpeptidases involved in cell wall biosynthesis. It is tempting to presume that the diazabicyclo[3.2.1.]octanone containing structure is NXL105 and that this core also has transpeptidase inhibitory activity.

Although the principle of protecting an antibiotic by an inhibitor of its corresponding resistance factor proved highly effective in the case of β -lactams, this concept has found no clinical

application beyond this class so far. Efflux pumps have been investigated at Microcide and later Mpex and proof-of principle has been obtained in *in vitro* studies that their inhibition can substantially lower the MIC for quinolones and other antibiotics, particularly in efflux pump overexpressing strains. Several classes of exploratory inhibitors have been described [10], but clinical investigations have not been initiated so far, probably reflecting the significant obstacles associated with this approach. Generally, pathogens are equipped with a redundant set of efflux pump systems and a single antibiotic is often transported by more than one pump. Also, broad-spectrum efflux pumps possess distinct binding sites for different antibiotic substrates and it is difficult to prevent efflux of a broad range of substrates by a single inhibitor simultaneously. Additional problems of previous inhibitors include unfavorable pharmacokinetics and toxicity [119].

A second approach that relies on agents without growth-inhibitory potential *in vitro* is the inhibition of bacterial virulence factors. Growing knowledge about the physiology of pathogens and hosts over the last decade has boosted our molecular understanding of the factors that promote bacterial pathogenicity in the course of the infection process. This includes the structures that promote adhesion, colonization and dissemination, the components responsible for cell communication and regulatory functions, the factors that facilitate evasion of the immune system and the machineries for toxin production and excretion. As a result, the recent scientific literature is full of examples suggesting the evaluation of specific virulence factors for future antibacterial therapy and inhibitors have been described that display effects in animal models of infection [120]. However, so far only a few companies have initiated discovery programs in this area, which can probably be attributed to the difficulties attached to this approach. First, the often very narrow activity spectrum of the anticipated drugs due to the often high species-specificity of the pathogenicity factor; second a therapeutic rationale that does not aim at pathogen eradication but rather is synergistic with antibiotic intervention; and finally, obstacles in setting up simple *in vitro* assays for the measurement of inhibitory activity. Paratek, for instance, is searching for inhibitors of MAR proteins (for multiple adaptation response), a family of transcriptional regulators that control a large stress response regulon in *Enterobacteriaceae*,

which promotes antibiotic resistance [121]. Inhibiting induction of this regulon increases the sensitivity of bacteria to antibiotic treatment. Furthermore, P005784 (an inhibitor of ExsA, which is a MAR protein that governs the exotoxin-producing type III secretion system), reduced infectivity of *P. aeruginosa* in mice [122]. Mutabilis is investigating inhibitors of heptosyl transferases involved in the biosynthesis of lipopolysaccharides of Gram-negative bacilli [304] as well as compounds interfering with D-alanylation of lipoteichoic acids in streptococci [120,123]. Their DltA inhibitor MUTB-370, part of a series of imidazoloquinoline derivatives, reduced the D-alanine content in lipoteichoic acids and possessed an antibacterial effect in a mouse model of systemic *S. agalactiae* infection at 10 mg/kg [124]. The lower D-alanine level in the bacterial cell wall increases the net negative surface charge and sensitizes the bacteria against the activity of defensins of the innate immune system and against cationic antibiotics.

A rather progressed example of a virulence factor inhibitor is the humanized antibody Fab fragment KB001. KaloBios is currently evaluating the agent in Phase II for the treatment of *P. aeruginosa* infections. KB001 binds to PcrV, a protein located at the tip of the Type III secretion system and blocks the injection needle, thereby preventing the delivery of exotoxins to host cells [125]. Several further therapeutic antibodies are currently in development, including KBPA101 (Kenta Biotech) targeting the O-polysaccharide moiety of *P. aeruginosa* serotype O11, an agent that is being co-developed with a diagnostic test for rapid serotyping; Pagibaximab (Biosynexus) directed against lipoteichoic acids of *S. aureus* and a combination of two antibodies targeting *C. difficile* exotoxins A and B (Medarex, now acquired by Bristol-Meyers Squibb) [126–128]. The use of therapeutic monoclonal antibodies or fragments thereof has been very successfully established in other therapeutic areas, especially in cancer and immunosuppressive therapy. They may also represent a useful complement to antibiotic therapy, leading to a more rapid resolution of infections in combination with classical antibiotics.

Other biologicals currently under exploratory investigation for the potential treatment of bacterial infections are endolysins derived from bacteriophages. Endolysins are cell-wall hydrolytic enzymes, which are produced by phages at the terminal stage of the phage lytic cycle. These enzymes cut the peptidoglycan at various positions, and possess glucosaminidase,

muramidase, amidase and endopeptidase functions; some enzymes even possess two cleavage functionalities. An interesting feature of endolysins is their very rapid lytic action, which is in principle appealing, because it is also directed against resting cells and bacteria living in biofilm communities [129,130]. First, therapeutic effects were demonstrated in bacterial infection models in mice (e.g., during the treatment of *S. pneumoniae* or *Bacillus cereus* septicemia) [131], however, several hurdles remain that may prevent systemic application in humans. For example, several endolysins were shown to be immunogenic in rabbits and their half-life is rather short [131].

In principle, it may also be possible to interfere with bacterial infections by stimulating the human immune system such that it more efficiently eliminates the invading pathogen. In particular viral disease immune modulators form the current standard therapy (e.g., recombinant α -interferons and nucleoside analogues for hepatitis B as well as pegylated interferons and ribavirin against hepatitis C). A variety of further immune modulatory agents are currently in development for HCV- and HIV-therapy, including interferons, Toll-like receptor agonists and immunoglobulins [132]. For bacterial infections, this approach is still in its infancy. Several biotechnology companies are performing clinical trials for derivatives of host defense peptides involved in innate immunity. Most of these agents are being evaluated for topical use, the most progressed including omiganan by Migenix/Cutanea, pexiganan by Genaera/Access Pharmaceuticals and iseganan by Ardea Biosciences [133]. An advantage of topical application is the largely reduced risk of systemic toxicity, an aspect that is particularly important in immunotherapy because of potentially severe inflammatory responses. However, hFL1–11 (AM-Pharma), a peptide derived from lactoferrin, demonstrated safety during systemic application in hematopoietic stem-cell transplant recipients. Furthermore, IMX-942 (Inimex), a synthetic pentapeptide modeled after innate defence regulator-1, has been applied in Phase I trials by the parenteral route.

It is obvious that most approaches discussed in this section are still a long way from the hospital bed, at least in the antibacterial field and that further investigations are required to assess their efficacy or risk:benefit ratio in this therapeutic sector. It is also important to note that, apart from the broad-spectrum β -lactamase inhibitors, all agents discussed in

this paragraph have a narrow activity spectrum that is in many cases restricted to a single bacterial species. The same applies to several of the novel antibiotic classes presented above and further compounds compiled in another recent review [134]. In strong contrast, current medical practice still requires empirical broad-spectrum therapy in order to assure rapid adequate therapy, thereby precluding the use of such agents. The reason is that diagnostic tools still have not developed to the point where rapid, robust and economic assays are available for most of the relevant bacterial species. However, much technical progress has also been achieved in diagnostics, especially in methodologies relying on DNA and RNA amplification and on pathogen identification by MS [135,136]. In the medium term we can therefore expect improvements in *in vitro* diagnostics to impact on the collection of antibacterial agents considered suitable for certain indications. As the time needed for pathogen identification is reduced, as simplified assay technology will support point-of-care diagnostics and as broader application will reduce the costs per assay, antibiotics with limited spectrum coverage will probably become a treatment option [134].

Another principal option is the combination of two active agents with the aim to exploit their synergistic activity or in order to protect them from rapid resistance development. Although the free combination of two or more active antibiotics from different classes is daily business for physicians in the treatment of severe bacterial infections in the clinical setting or in tuberculosis therapy, fixed combinations of two active ingredients are still the exception in the field. Prototypical examples are the combination of diaminopyrimidines (e.g., trimethoprim) with sulfonamides (e.g., sulfamethoxazole) to inhibit two consecutive enzymes in tetrahydrofolate biosynthesis [137] or the streptogramins quinupristin and dalfopristin binding synergistically to adjacent sites of the ribosomal P-site. So far, companies have been rather reluctant of developing combination products of two novel chemical entities due to the additional risks attached to and development efforts required for such an approach (e.g., the necessity of matched pharmacokinetics, the demonstration of superior efficacy of the combination product versus the single ingredients, the increased cost of goods and the increased risk of adverse effects). However, in the light of the shortage of novel broad-spectrum antibiotic classes the commercial values of such combination approaches will probably increase.

Commercial & regulatory implications

So far, this article has discussed the scientific challenges and chances of antibacterial drug discovery, including the target selection procedure, the compound sources and the screening process. Economical and regulatory aspects that triggered considerable fluctuations among the companies active in the field are discussed below. Bacterial diseases are predominantly acute illnesses and the corresponding treatment is generally prescribed for a short course therapy. Several highly successful broad-spectrum antibiotics have demonstrated that blockbuster status can also be reached in this field and that it can even persist after patent expiry (e.g., ciprofloxacin and amoxicillin/clavulanic acid). However, it is comparably difficult for antibiotics to reach sales volumes that match those of long-term therapeutics, especially in the light of the reasonable attempts to reduce overall antibiotic usage and with regard to narrow-spectrum agents. On the other hand, the overall costs for the development of an antibacterial agent are in the same range as for drugs for other indications and have increased tremendously over the last two decades (from US\$231 million in 1987 to US\$800 million in 2001, including expenditures for failed programs) [138]. In addition, the antibacterial market is mature and new drugs are confronted with substantial generic competition. The recommendation to preserve novel resistance-breaking agents for the treatment of serious cases, together with the policy of health insurance companies to fund the cheapest available treatment, reduces return on investment further. These factors combined make the risk-adjusted net present value, as a measure of the commercial potential of a product, about ten times less for an antibiotic compared with a muscular-skeletal pain killer [205]. Political and regulatory authorities together with medical and pharmaceutical alliances have been engaged in lively and controversial debates about this dilemma for years [139,140,206] and it bodes well that recent discussions aim at finding reasonable and feasible economic incentives for investors in order to attenuate this commercial imbalance [205].

The design of the clinical studies and the efficacy and safety that a new resistance-breaking antibiotic has to demonstrate in order to obtain regulatory approval is another point to consider. Regulatory requirements have increased continuously and pharmaceutical companies experience uncertainty with respect to the goals that they actually have to meet in order

to obtain approval of their drugs [141]. The sum of these challenges together with a process of pharmaceutical industry concentration by mergers and acquisitions had led many big players to retreat from antibacterial drug discovery and to redirect their effort to the more profitable therapies of chronic diseases. The field has been left to a handful of big-pharma competitors and a substantial number of biotechnology enterprises [142–144]. As a result, the number of patent applications in the antibacterial field declined substantially [138] along with the number of approved antibiotics [139,141,145]. If we do not want to lose our good therapeutic standard for the treatment of bacterial infections, it is important that we introduce new potent and resistance-breaking drugs into therapy *before* resistance prevalence of bacteria against the current standard-of-care reaches substantial values and it is currently high time for that. Clear and lasting regulatory guidelines that companies can rely on in their study design must be formulated, the requirements regarding efficacy and safety need to be balanced and achievable and the expenditures necessary to obtain the requested results must be reasonable in order to attract companies back to the antibacterial field. However, it is promising that first big-pharma companies are now showing renewed interest in antibacterial research and are in the process of building up such structures again. They seem to have realized that the development of antibiotics also carries substantial advantages in relation to other therapeutic areas [139,145,146]. The overall profiling cascade for assessing the potential of an investigational antibacterial agent is well established and includes standardized *in vitro* assays for determining antibacterial activity, highly predictive animal models for a broad range of infections and elaborate techniques for metabolic, pharmacokinetic and pharmacodynamic investigations. As a result, in most cases it is possible to judge as early as the pre-clinical phase whether an investigational antibiotic will have sufficient therapeutic efficacy and tolerability in clinical studies and to predict suitable treatment doses, which even take into account mutation-prevention concentrations. While this early identification of pitfalls and the high predictability cause a comparably high attrition rate in exploratory antibacterial research, the advantage shows at later stages, because the success rate from Investigational New Drug applications (the documentation required to obtain regulatory approval for first studies in man) to market authorization, is

in the range of 30% for anti-infectives compared with 15% for cardiovascular, cancer and central nervous system therapeutics [139,146]. Furthermore, clinical studies for antimicrobials are generally shorter than for most other therapeutic areas due to the shorter treatment courses and corresponding omission of long-term safety studies. Accordingly, regulatory authorities usually require less time for data analysis and for conclusion of the approval process.

Future perspective

Although the genomics and functional techniques did not directly pay off by providing the naively expected wealth of novel antibacterial lead structures, they have added much to our understanding of bacterial physiology, pathogenicity, pathogen–host interaction, mechanisms of antibiotic action and bacterial resistance development. As a result, our knowledge about the characteristics of a successful antibiotic has considerably expanded and our technical capabilities for determining these requirements have improved in parallel. Thus, in principle, our intellectual and methodological position for discovering novel antibiotics is better than ever and there is no reason to believe that this should not yield new antibacterial classes in the medium-term even against Gram-negative problem pathogens. It is probably also an advantage that the screening strategies employed and the therapeutic options evaluated have noticeably increased in diversity, providing more chances of success. Regarding the sources of antibiotics, it is important to note that the past has shown us that successful antibiotics can, in principle, be found among natural products as well as among synthetic molecules and therefore we should continue to search within both pools of compounds. However, we will need access to new natural products as sources for new leads and we will have to consider the particular physicochemical and structural features required for penetration into bacterial cells and for antibiotic action during synthetic library design and during optimization programs.

On the other hand, there is no reason to hope that we will enter a second ‘golden age of antibiotics’, where we will discover novel potent and tolerable broad-spectrum antibiotics easily. In addition, some antibiotic resistance mechanisms that have been selected in the bacterial population during the decades of antibiotic treatment, such as efflux pump overexpression or porin reduction, affect available bacterial classes broadly and there is risk that they also impact on novel classes with unrelated mechanisms of action. Therefore, we

should make the best of the already established classes, even in the light of increasing resistance against these agents. Recent examples have demonstrated that it is still possible to design resistance-breaking properties into well-known antibiotic classes and this option will be further exploited in the future. The need to cover the required bacterial spectrum despite increasing resistance prevalence will probably also lead to a rising necessity for, and acceptance of, narrow-spectrum drugs and antibacterial combination therapies, either as combinations of:

- Two active antibiotics that support each other with respect to mechanism of action, spectrum coverage or resistance prevention;
- One antibacterially active ingredient plus one agent without its own growth inhibitory activity that interferes with a corresponding resistance factor;
- An antibiotic together with an immune-stimulating or virulence-reducing agent.

Improvements in diagnostics for identification of the causative agent can be expected to increase the number of agents considered suitable

for these approaches. In summary, there are still multiple options for discovering novel antibiotics and further antibacterial treatment principles in the future, which together with their rising commercial value in light of increasing medical need will probably attract more of the bigger pharmaceutical companies back to the antibacterial drug discovery field.

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Executive summary

New antibiotic classes are rare in progressed stages of antibacterial drug development

- During the next 5 years no new Gram-negative antibiotic class can be expected to enter the market.
- The Gram-positive pipeline is better filled, but several agents are currently facing problems during late-stage development and registration.

Target-based high-throughput screening proved unsuccessful for antibacterial drug discovery in the way it had been conducted

- Reasons for this lack of success are related to target selection, screening strategies and compound collections.
- Established targets and metabolic pathways are especially valuable and new antibacterial classes can be found that inhibit these targets/pathways without cross-resistance with currently used drugs. Screening strategies that select for cell penetration in addition to target inhibition yield improved hits. Physicochemistry of antibiotics differs from that of drugs in other therapeutic sectors and natural products are favorable in this respect.
- The short timelines assigned to projects in the high-throughput screening era were inappropriate in relation to the substantial optimization requirements of antibacterial leads.

Postgenomic discovery strategies for antibiotics are diverse

- Interesting starting points for antibacterial drug discovery have been revealed by re-visiting previously abandoned microbial extracts and underexploited natural product classes using modern technologies.
- Rational inhibitor design on the basis of high-resolution target structures and focused compound libraries has yielded new agents with promising antibacterial activity *in vitro* as well as in infection models.
- Novel representatives of known antibiotic classes demonstrate therapeutic efficiencies even against resistant isolates and form the majority of current late stage development compounds.

Alternative strategies have potential to support the activity of antibiotics

- Species-specific therapeutic monoclonal antibodies directed against bacterial virulence factors and the first mimics of host defense peptides involved in innate immunity are being evaluated in clinical trials.
- Exploratory approaches include small molecule inhibitors of virulence and resistance factors as well as phage endolysins.

Small spectrum drugs & combination approaches will gain increased value

- Current pharmaceutical pipelines contain very few broad-spectrum agents of novel structural classes.
- Medical need due to rising resistance against existing therapies and technical progress in bacterial diagnostics can together be expected to promote acceptance for, and commercial value of, narrow-spectrum agents and combination approaches.

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