Chapter 4
The β-Lactam Antibiotics: Their Future in the Face of Resistance

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Abstract The search for new β-lactam antibacterial agents is a major challenge in medicinal and pharmaceutical chemistry. Methicillin-resistant *Staphylococcus aureus* (MRSA), pan-resistant *Enterobacteriaceae,* and pan-resistant nonfermenter bacteria are present day clinical scourges. New cephalosporins and carbapenems may solve this problem, but new monosulfactams and monocarbams also show promise. High-molecular-mass PBPs are envisioned as β-lactam targets. Further research keeps revealing interesting aspects about β-lactam resistance by β-lactamases, the existence of sentinel proteins, and the complexity of the cell envelope.

4.1 Introduction

A quintessential component of modern medicine is its ability to treat injury—whether scratch or severe trauma—with confidence that the treatment will not be compromised by infection. The basis for this confidence is chemistry: the availability to the physician of an array of chemotherapeutic agents, each capable of acting in concert with the immune response so as to extinguish bacterial and fungal growth. This confidence is now threatened. While the biochemical mechanisms of resistance are ancient, the profligate use of antibiotics has resurrected and refined these resistance mechanisms. New bacterial strains with an assembly of incremental and complementary resistance mechanisms now can evade the therapeutic concentrations attainable by antibiotics. Moreover, the increasing financial costs for antibiotic discovery and clinical development, and the meager reward once regulatory approval is secured, have diminished commercial interest in new

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antibiotics. This circumstance has been correctly described (Piddock 2012; Wright 2012) as paradoxical: at a time when the collective powers of chemistry, enzymology, and cell biology are unraveling the remarkable interplay among the pathways used by bacteria to detect and perfect a response to the presence of antibiotics, we are at risk of entering a period where this understanding will not translate to improved clinical therapy (Bush et al. 2011).

A response to this paradox is the creation of new value for time-tested, and time-proven, antibiotics. The β-lactam antibacterials are preeminent in terms of efficacy and safety (Kardos and Demain 2011), notwithstanding progressive erosion of their value by the *Scylla* of diminished access to and resistance mutation within their molecular targets (Llarrull et al. 2010); and the *Charybdis* of bacterial enzymes (the β-lactamases) devoted to their molecular destruction (Bush and Fisher 2011). Here we survey the most recent developments in the chemistry, enzymology, and cell biology of the β-lactams, from the perspective of their relevance to the circumvention of bacterial resistance mechanisms and the preservation of the preeminence of the β-lactam antibiotics for future generations.

4.2 Recent Advances in the Chemistry of the β-Lactams

The β-lactam antibiotic sub-classes include the penicillins, cephalosporins, cephamycins, carbapenems, and monobactams (Testero et al. 2010). The antibiotic activity of each sub-class derives from mechanism-based inhibition of bacterial cell-wall biosynthesis. Depending on the structure, the β-lactams are efficacious against both Gram-positive and/or Gram-negative pathogens, and thus possess exceptional clinical value (see Rossolini et al.; Paitan and Ron, this volume). In addition, the weakly antibiotic clavulanate and penicillin sulfone β-lactam sub-classes (and now joined by new bicyclic lactam sub-classes) act as mechanism-based inhibitors of the serine-dependent β-lactamase enzymes, which synergize with the antibiotic β-lactams. The recent developments in the chemistry of these lactams include new cephalosporins, carbapenems, and monobactams (Bush and Pucci 2011; Butler and Cooper 2011), and new strategies for β-lactamase inactivation (Shahid et al. 2009; Drawz and Bonomo 2010; Biondi et al. 2011).

4.2.1 New Cephalosporins

4.2.1.1 Latest Generation Cephalosporins

The newest cephalosporins, each at different stages of clinical development, are ceftaroline, ceftobiprole, and CXA-101 (ceftolozane). The criteria used during the structural optimization of these (and other β-lactam) antibiotics against Gram-negative bacterial infection include the efficient inactivation of the

high-molecular-mass (biosynthetic) penicillin-binding protein (PBP) enzymes (Moya et al. 2010); minimal induction β-lactamase expression, as will result from inactivation of the low-molecular-mass penicillin-binding protein enzymes (Mark et al. 2011); intrinsic resistance to β-lactamase-catalyzed hydrolysis; and unfettered access to the enzyme targets as can be lost as a result of porin deletion (a contributory bacterial-resistance mechanism). The structural features of CXA-101 exemplify this optimization (Fig. 4.1). The aminothiazolyl-oxyimino side chain (left-hand structural segment of this CXA-101 depiction) is found in recent generations of the cephalosporins, and is used for its ability to preserve PBP affinity, while imparting stability toward many β-lactamases. A hydrophilic extension terminating in a cationic amine (seen as the right-hand structural segment of CXA-101) is also a multi-generational structural feature of the cephalosporins, and is used for its ability to enhance PBP affinity. CXA-101 is a parenteral cephalosporin with particular efficacy against *Pseudomonas aeruginosa*. The activity of CXA-101 is further potentiated against Gram-negative *Enterobacteriaceae* (such as *Escherichia coli* and *Klebsiella pneumoniae*) that express extended-spectrum β-lactamases, by co-formulation with tazobactam, a classic β-lactamase inhibitor (Livermore et al. 2010a). The CXA-101–tazobactam combination is not effective, however, against Gram-negative bacteria with more capable β-lactamases, such as the KPC carbapenemases. Indeed, the increasingly limited efficacy of all of the newest generation cephalosporins (and as well of the newest carbapenems) coincides with the increasing concern that an era of untreatable infection is near (Livermore 2012).

4.2.1.2 β-Lactam Hybrid Structures

One solution to the diminishing efficacy of the single agent β-lactam is the pairing of the β-lactam with a second therapeutic having a synergistic mechanism of action (Cottarel and Wierzbowski 2007; Ejim et al. 2011). Methods to identify synergistic pairings are further discussed in Sect. 4.4. This strategy is well exemplified—as just mentioned—by the pairing of clinically important β-lactam antibiotics with a β-lactamase inhibitor. The new pairings may correspond to co-formulation of the β-lactam with the β-lactamase inhibitor and the structural fusion of complementary antibiotics. Two examples of this latter approach are the exploratory cephalosporin-vancomycin hybrid TD-1792, and the design of antibiotic-functionalized cephalosporins for β-lactamase-catalyzed activation (Fig. 4.1).

4.2.1.3 TD-1792, A Covalent Cephalosporin-Vancomycin Hybrid

The chemical criteria for the fusion of two biologically active structures so as to attain mutual synergy are rigorous. Both the selection of the atoms for the interconnection, and the chemistry for the linker, must be perfect. Vancomycin is a

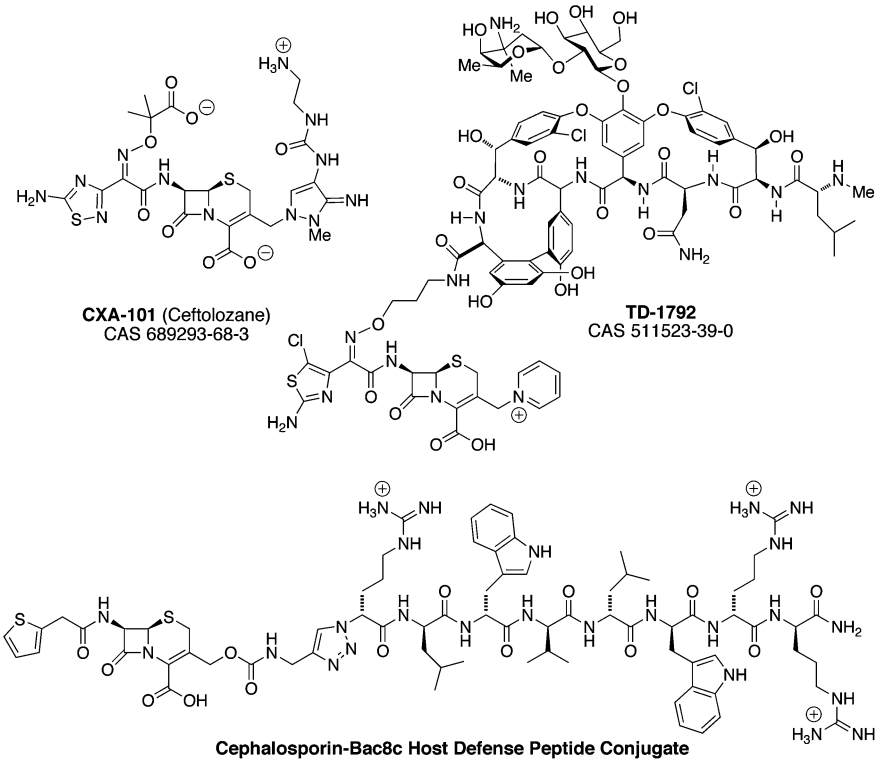

Fig. 4.1 Structures of new cephalosporins

Gram-positive antibiotic that interferes with bacterial cell-wall biosynthesis by a different, but complementary, mechanism compared to the PBP-inactivation mechanism of the β-lactams (Fisher and Mobashery 2010). Synergistic interconnection between the oxyimino segment of the cephalosporin and vancomycin yields the multivalent hybrid antibiotic TD-1792 (Long et al. 2008) having exquisite efficacy (greater than each antibiotic alone) against *Staphylococcus aureus*, including methicillin-susceptible, methicillin-resistant, and vancomycin intermediate-susceptible strains (Blais et al. 2012; Hegde et al. 2012), and as well other Gram-positive bacteria (Tyrrell et al. 2012). Given that the vancomycin structure is itself amenable to structural optimization (Allen 2011), hybrid antibiotics represent a viable strategy for the sustained efficacy of existing β-lactams against emerging pathogens. Although the gargantuan structure of TD-1792 (Fig. 4.1) may ultimately preclude this compound from progressing beyond that of an exploratory structure, it is nonetheless an innovative exemplification of the concept of multi-target synergism.

4.2.1.4 β-Lactamase-Catalyzed Antibiotic Release from Cephalosporins

The antibiotic mechanism of the β-lactams involves functionally irreversible acylation of an active-site serine residue of the PBP enzymes. Bacteria express serine-dependent, and/or metal-dependent, β-lactamase enzymes as a resistance mechanism. β-Lactamases hydrolytically open the β-lactam ring and hence abolish the antibiotic ability of the β-lactam. Cephalosporins have long been known to release their right-hand substituent—appended to C_3; this substituent is not present in the other β-lactam sub-classes—following hydrolytic opening of their β-lactam. While this ability was conceptualized as a means for secondary antibiotic release (Mobashery et al. 1986; Mobashery and Johnston 1987) to β-lactamase-expressing bacteria, its value has now expanded to include imaging of intracellular proteins (Mizukami et al. 2012). A compelling recent example which exemplifies the original conception is the cephalosporin-Bac8c peptide conjugate shown in Fig. 4.1 (Desgranges et al. 2012). The structure of the D-amino acid-derived Bac8c peptide segment used in this conjugate derives from the bovine host defense peptide, bactenecin. The antibacterial activity of the conjugate is distinct from the antibacterial activity of the separate segments, and is consistent with intrinsic activity for the conjugate itself that is abetted by β-lactamase release of the host defense peptide. Given the increasing need for antibiotics selective against emerging pathogens, the potential of this strategy to enhance the therapeutic index of synergistic antibiotic pairs is evident.

4.2.2 New Generation Carbapenems

The first carbapenem to enter into clinical practice in 1986 was imipenem. Subsequent carbapenem approvals include meropenem (1996 U.S.), ertapenem (2001 U.S.), doripenem (2007 U.S.), panipenem/betamipron (1993 Japan), and biapenem (2001 Japan). These carbapenems are categorized by their antibacterial spectrum into three classes. While the clinical impact of this class has been transformative—carbapenems are called the antibiotics of the last resort—during the past decade several experimental carbapenems have failed to progress to clinical approval. These failures suggest the possibility of structure-activity maturity within this class.

4.2.2.1 Anti-Gram-Positive Carbapenems

The continuing diffusion from hospitals to the community of methicillin-resistant *S. aureus* (MRSA) has stimulated efforts toward new anti-MRSA cephalosporin and carbapenem structures (see Rossolini et al. this volume). Carbapenems are the most potent β-lactam antibiotics, stable to hydrolysis by many β-lactamases, and

consequently effective against many cephalosporin-resistant microorganisms. Carbapenems with MRSA activity are termed as Group 3. Regrettably, several new Group 3 carbapenems have failed recently at late-stage clinical evaluation. Razupenem (Fig. 4.2) exemplifies a standard carbapenem structure, with a 1β-methyl substitution (to impart metabolic stability) and a hydrophilic, positively charged C-2 substituent found in various guises in all new carbapenems (Bassetti et al. 2009; El-Gamal and Oh 2010). This positively charged substituent is structurally similar to the substituents found at the equivalent position in ceftobiprole and ceftaroline, and embodies a structural requirement for effective inhibition of their PBP targets. Razupenem has additional antimicrobial activity against vancomycin intermediate *S. aureus* (VISA) and enterococci-like vancomycin-resistant *E. faecium* (VREF), as well as against some Gram-negative bacteria (but not *P. aeruginosa* and *A. baumannii*). Development of razupenem is now discontinued due to unacceptable levels of adverse events. The parenteral carbapenem ME1036 (Fig. 4.2) is structurally similar to razupenem, and is active against multi-drug resistant Gram-positive and Gram-negative bacteria including MRSA, VISA, and extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL). It has strong activity toward *H. influenza* and *Enterococcus faecalis*, and is more active than ceftriaxone and other broad-spectrum cephalosporins (including ceftaroline). ME1036 has potent activity against genotypic penicillin-intermediate *S. pneumonia* (gPISP) strains and genotypic penicillin-resistant *S. pneumonia* (gPRSP) strains that contain more than one mutation in their PBPs. This most recent publication on ME1036 establishes a direct correlation between its bactericidal potency against *S. pneumonia* and its PBP affinity (Hirai et al. 2011). There is no evidence that ME1036 is progressing in clinical evaluation.

The guanidine-pyrrolidine side chain found in tomopenem (Fig. 4.2), a third parenteral 1β-methyl carbapenem, provides high affinity for PBP1, PBP2, and PBP4 of *S. aureus* (Koga et al. 2009). In contrast to older carbapenems, tomopenem demonstrates activity against both MRSA and against methicillin-susceptible *S. aureus* (MSSA), as well as ESBL-producing *E. coli*, *Klebsiella* spp.,

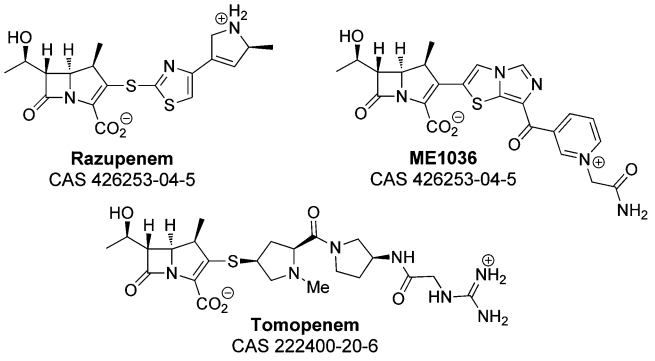

Fig. 4.2 Structures of anti Gram-positive carbapenems

imipenem-resistant and ceftazidime-resistant *P. aeruginosa*, expanded-spectrum cephalosporin-resistant *Enterobacteriaceae* and other Gram-positive and -negative bacteria (Koga et al. 2008). Tomopenem has a superior antibacterial effect against MRSA compared with vancomycin, and a longer half-life compared with other carbapenems except for ertapenem (vide infra). The development of tomopenem for countries outside of Japan is uncertain, as its development rights were returned to the innovator company.

4.2.2.2 Anti-Gram-Negative Carbapenems

A growing concern is future treatment options against pan-resistant *Enterobacteriaceae* and nonfermenter Gram-negative bacteria (see Paitan and Ron, this volume). Gram-negative bacteria acquire resistance to β-lactams by a combination of reduced permeability, active efflux, and expression of plasmid-encoded extended-spectrum β-lactamases (ESBLs, such as the CTX-M-14 and CTX-M-15 enzymes) and serine- and metal-dependent carbapenemases (Lascols et al. 2012). Often multiple β-lactamases appear in a single organism.

Imipenem (Fig. 4.3), a derivative of the natural product thienamycin, is used in combination with cilastatin (to inhibit its hydrolysis by the renal enzyme dehydropeptidase-I). It is a broad-spectrum Group 2 (nonfermenter) carbapenem. However, imipenem induces expression of the AmpC β-lactamase, a key resistance mechanism of many Gram-negatives. The more recent Group 2 meropenem (Fig. 4.3) has improved metabolic stability and reduced β-lactamase induction, and is used extensively for complicated urinary and respiratory infection. Meropenem is not as potent as imipenem or doripenem (vide infra) against *P. aeruginosa*. Biapenem (Fig. 4.3) is a broad-spectrum (active against many Gram-negative and Gram-positive aerobic and anaerobic bacteria, including β-lactamase producers) zwitterionic carbapenem, used in Japan since 2002 and more active than imipenem against ESBL-expressing *Enterobacteriaceae.*

Ertapenem (Fig. 4.3) is a Group 1 (limited non-fermenter activity) carbapenem that is structurally related to meropenem, and approved in the U.S. in 2001 for its activity against ESBL-producing *Enterobacteriaceae* (but inactive against *P. aeruginosa* and *A. baumannii*). Doripenem (Fig. 4.3), a new 1β-methyl carbapenem that was approved in Japan in 2005, has excellent Gram-positive, Gram-negative, and anaerobic coverage, including difficult to treat pathogens such as *E. coli* and *Klebsiella spp.* that produce extended-spectrum β-lactamases, penicillin-resistant *S. pneumonia*, *Pseudomonas* spp., *Citrobacter* spp., and *A. baumannii* with MIC values at least 16-fold lower than those for imipenem against the same isolates. *P. aeruginosa* isolates resistant to imipenem and meropenem may retain susceptibility to doripenem. Hydrolysis of doripenem is 2- to 150-fold slower than that of imipenem. Tebipenem-pivoxil (Fig. 4.3) is an oral carbapenem whose active metabolite shows broad-spectrum activity against Gram-positive bacteria and *Enterobacteriaceae*. Tebipenem-pivoxil is not active against MRSA, and unlike meropenem it has no activity against β-lactamase-producing *P. aeruginosa*.

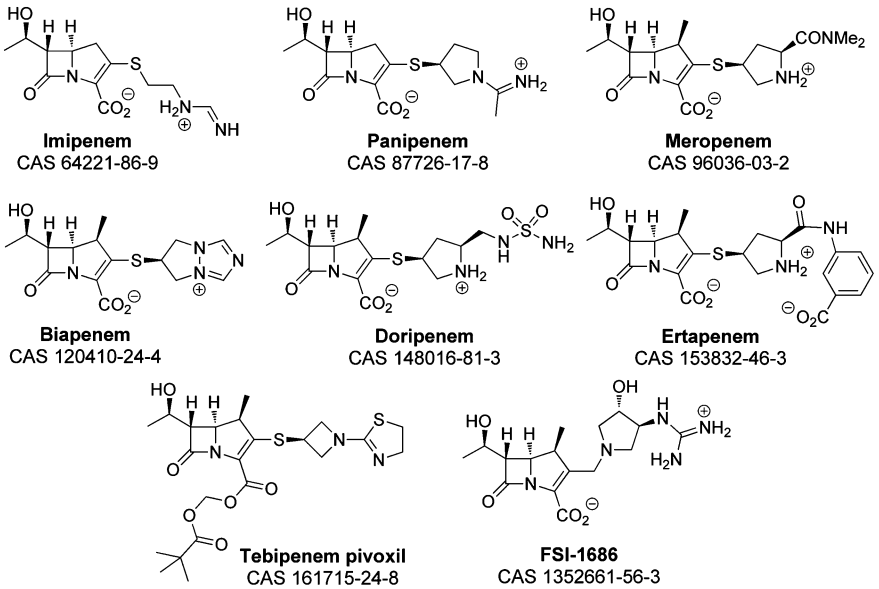

Fig. 4.3 Structures of anti Gram-negative carbapenems

It displays excellent activity against *S. pneumonia* strains, including penicillin-resistant strains. The most recent carbapenem entry is FSI-1686 (Fig. 4.3), representing a new carbapenem class that is very effective against multi-drug resistant Gram-negative bacteria including carbapenem-resistant *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae*.

4.2.2.3 The Future of the Carbapenems

As is evident from inspection of these carbapenem structures, the structural evolution of the carbapenems since imipenem coincides with the incorporation of the 1β-methyl to attain renal metabolic stability, and the iterative exploration at C-2 of hydrophilic, positively charged substitution. It may be argued that the guanidinopyrrolidine found in FSI-1686 (Fig. 4.3) represents culmination of this strategy: functionality that attains new breadth of antibacterial activity, while simultaneously presenting challenges with respect to drug synthesis, drug formulation, and patient tolerability. Whether chemical space remains for further C-2 exploration is uncertain. The newest development with the carbapenems is the co-formulation of existing carbapenems with β-lactamase inhibitors, in response to the aggressive expansion of β-lactamase catalytic ability resulting in the dramatic erosion of the β-lactamase stability of the carbapenems. For example, combination of imipenem with MK-7655 (see 4.3.2.1), a new Class C and Class A β-lactamase

inhibitor, restored the antibacterial activity of imipenem against Class A (serine) carbapenemase-producing *Enterobacteriaceae* and multi-drug-resistant *P. aeruginosa* isolates. MK-7655 is currently undergoing Phase 1 safety evaluation, in anticipation of therapeutic combination with a carbapenem (Bush and Pucci 2011). The appearance of activity against *Mycobacterium tuberculosis* by combination of meropenem with clavulanic acid suggests the possibility of a new therapeutic approach against these Gram-positive bacteria (Hugonnet et al. 2009) and previously regarded as fully β-lactam refractory due to its constitutive expression of a powerful β-lactamase (Tremblay et al. 2010).

Further structural optimization of the carbapenems will likely follow two paths. The correlation of carbapenemase structure to kinetic analyses may provide structure-based design opportunity against specific pathogens (Frase et al. 2009; Ke et al. 2012). As a further example, change to the heretofore invariant 6-(hydroxyethyl) substituent of the carbapenems may provide opportunity for optimization against specific emerging carbapenemases, as suggested by initial studies (using a penicillin core) with a Class D enzyme (Testero et al. 2009; Verma et al. 2011).

4.2.3 New Generation Monocyclic β-Lactams

The absence of antibacterial activity in the monocyclic β-lactam obtained from the reductive desulfurization of penicillin was a key observation in the structural and mechanistic elucidation of the penicillin structure. This seeming correlation—between the absence of antibacterial activity and a monocyclic β-lactam structure—was confounded by the discovery some 30 years ago of the Gram-negative monobactam antibiotics, exemplified by the monobactam aztreonam (Fig. 4.4). While the eventual clinical value of aztreonam was limited as a result of its solely Gram-negative spectrum, against the habits of antibiotic use and prevalence of Gram-positive pathogens, interest in monocylic β-lactams has increased sharply with the emergence of multi-drug resistant Gram-negative bacteria (Canton and Lumb 2011; Kollef et al. 2011; Walsh and Toleman 2012). Aztreonam, and its more recent congener structures, have intrinsic stability toward many β-lactamases (including metallo-β-lactamases) and thus potential clinical value against newer pan-resistant *Pseudomonas*, *Acinetobacter*, *Escherichia*, and *Klebsiella* species. Their β-lactamase stability is further improved by combination with β-lactamase inhibitors, as prominently exemplified by the triple combination antibiotic (coded as BAL-30376, from Basilea Pharmaceutica) comprising the monobactam BAL-19764 (Fig. 4.4) with two β-lactam-based β-lactamase inhibitors, BAL-29880 and clavulanic acid (Page et al. 2011). Perspectives on new strategies for β-lactamase inhibition are given in Sect. 4.3.2.1. Comparison of the aztreonam and BAL-19764 structures calls attention to the (1,4-dihydro-1,5-dihydroxy-4-oxo-2-pyridinyl)methoxy substitution to the imine of the monobactam side chain in the latter, and which is absent in aztreonam. This substituent is iron-chelating and acts as a bacterial siderophore mimetic. Its use was conceptualized as a means of evading

porin deletion as a resistance mechanism, a common resistance event in Gram-negative bacteria. The several-decade history of the siderophore conception is summarized concisely elsewhere (Flanagan et al. 2011). Regardless of the absence to date of experimental data demonstrating their uptake by iron transporters, the "siderophore" monocyclic β-lactams clearly demonstrate expanded antibacterial potency and a low frequency of spontaneous resistance development. Accordingly, this same siderophore substitution is retained in the newest monocyclic β-lactams, those of the monosulfactam and monocarbam sub-classes.

4.2.3.1 The Monosulfactams

Neither the monosulfactam sub-class nor the monocarbam sub-class (Sect. 4.2.3.2) is as yet fully exemplified, in terms of structure–activity study, outside of the patent literature. For this reason, and because only in vitro activity data are available for those few structures that are disclosed, it is not possible to assess the long-term clinical potential of either sub-class. Nonetheless, the initial data for both are quite promising. Examination of the structures within the three monocyclic β-lactam sub-classes suggests that a diversity of acyl activating groups on the nitrogen of the β-lactam may be used. Comparison of the monosulfactam BAL-30072 (Fig. 4.4) with the monocarbam MC-1 (Fig. 4.4) further suggests tolerance for the structural placement of the siderophore segment. If these generalizations are sustained, significant future potential for the structure–activity development of both monocyclic β-lactams sub-classes is anticipated.

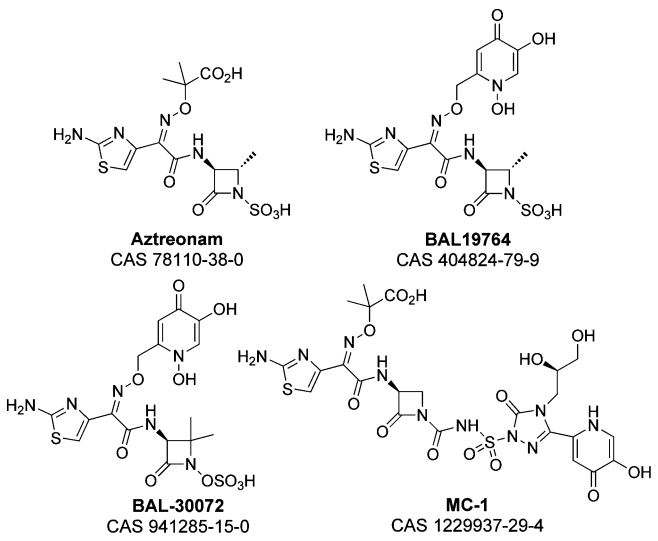

Fig. 4.4 Structures of new generation monocyclic β-lactams

The monocyclic sulfactam sub-class is exemplified thus far by BAL-30072 (Basilea) (Fig. 4.4). In vitro evaluation of BAL-30072 showed broad-spectrum Gram-negative activity, including activity against many carbapenem-resistant strains as a result of expression of Class C (AmpC) and Class D (Oxa) β-lactamases (Mushtaq et al. 2010; Page et al. 2010; Mima et al. 2011; Russo et al. 2011). For example, BAL-30072 was more active against meropenem-non-susceptible *A. baumannii* (65 % of the isolates, MIC $\leq$ 8 mg/L) when compared to other β-lactams (including imipenem, cefepime, aztreonam) and as well other antibacterials (including levofloxacin, amikacin, rifampicin) representing different antibacterial classes (Higgins et al. 2012). No correlation was found between the MIC values for those isolates with elevated BAL-30072 MIC values, with elevated MIC values for the other antibacterials.

4.2.3.2 The Monocarbams

Renewed interest in the monocarbams is evidenced by recent disclosures from Pfizer. An initial structure-activity study (Flanagan et al. 2011) has identified a prototype structure, MC-1 (Fig. 4.4). Its structure was evaluated as a point of reference for structure-based drug design, as described in the context of its crystal structure (as the acyl-enzyme) with the penicillin-binding protein targets encountered in the opportunistic Gram-negative pathogens *A. baumanii* and *P. aeruginosa* (Han et al. 2010, 2011). While numerous points for structural optimization were identified, the value of PBP acyl-enzyme structure for structure-based design toward improved kinetics for serine acylation from the Michaelis complex is uncertain (Nicola et al. 2010). These acyl-enzyme structures may provide, however, the basis for initiating computational evaluation of the key transition-state structures leading to the acyl-enzyme complex. The first QM/MM studies on PBP catalysis have been described (My et al. 2011; Shi et al. 2011).

4.3 Recent Advances in the Enzymology of the β-Lactams

The historical foci for the enzymology of the β-lactams have been their target enzymes, the penicillin-binding proteins, and the β-lactamase enzymes, as a preeminent β-lactam resistance mechanism. While the importance of these two enzyme classes is undiminished, new proteins and enzymes—both within these classes and related to them through resistance pathways—are emerging with direct relevance to the preservation of the clinical value of the β-lactams.

4.3.1 Penicillin-Binding Proteins

Cell-wall biosynthesis is required for every bacterium. Even among the "cell wall-less" bacteria a portion of the cell-wall biosynthetic pathway (exactly what portion(s) remains uncertain) is required to complete cell division (Henrichfreise et al. 2009; Gaballah et al. 2011). For those bacteria with a fully functional cell-wall biosynthetic pathway, the cell wall is assembled using several high-molecular-mass (HMM) PBP enzymes dedicated to the processes of cell-wall growth and septation. Many HMM PBPs are bifunctional, with separate transglycosylase and transpeptidase domains. The transpeptidase activity is the molecular target of the β-lactam antibiotics. Cell-wall assembly is refined using low-molecular-mass (LMM) PBP enzymes, which possess a single active site nearly identical to the transpeptidase active site of the HMM PBPs, but here with endopeptidase and/or carboxypeptidase activities. Although these enzymes are also molecular targets of the β-lactams, LMM PBP inactivation is now more clearly understood to directly connect to β-lactam resistance induction, rather than to the antibiotic activity. New revelations, within each of these two PBP sub-classes, are now influencing future thought with respect to β-lactam design.

4.3.1.1 The High-Molecular-Mass PBPs as ß-Lactam Targets

Study of the high-molecular-mass PBPs has been limited by their membrane-associated character, and by the extraordinary difficulty in accessing their substrates so as to enable meaningful evaluation of their in vitro catalytic activity. Both limitations are being addressed. Laborious, but reliable, syntheses of analogs of Lipid II—substrates for the transglycosylase activity—are now in place (Gampe et al. 2011). As membrane-associated enzymes, structural analysis of these enzymes using crystallography heretofore has used as constructs that lack the membrane-binding domain of these enzymes. This limitation is diminishing. For example, synthetic and crystallographic studies are converging with respect to understanding the interaction of the membrane-proximal transglycosylase domain of these PBPs with Lipid II, and understanding inhibitor occupancy of this same transglycosylase active site (Fuse et al. 2010; Huang et al. 2012). While the difficulty in the assay of their transpeptidase activity remains, robust screening methods for evaluating binding to the transpeptidase/β-lactamase active sites are becoming available (Bobba et al. 2011; Inglis et al. 2012).

The opportunity for structure-based optimization of β-lactam structure against Gram-negative bacteria against their specific HMM PBP target was discussed previously (Sect. 4.2.3.2). In the Gram-positive pathogen methicillin-resistant *S. aureus* (MRSA), β-lactam resistance is achieved through the acquisition of a gene encoding a new transpeptidase-specific PBP having intrinsic resistance against inactivation as a result of β-lactam antibiotic acylation (Fuda et al. 2004; Llarrull et al. 2009). While the endogenous PBP transpeptidase activity is lost as the result of β-lactam acylation of its active site serine, MRSA completes its cell

wall using the acquired PBP (termed PBP 2a). The structural basis for the intrinsic β-lactam resistance of PBP 2a is believed to be a pH-dependent steric interaction between a peptide loop guarding the active site, that is preferentially exerted against β-lactam inhibitors as opposed to the peptidoglycan substrate (Lemaire et al. 2008, 2009). Gratifyingly, experimental evidence supports allosteric modulation of this loop by the peptidoglycan itself to ameliorating the loop steric interaction, allow peptidoglycan entry (Fuda et al. 2005, 2006, 2007), and a positive correlation between β-lactam efficacy against MRSA and the ability of the β-lactam to evade this loop interaction (Villegas-Estrada et al. 2008). Future β-lactam design will be guided by the molecular structure of its PBP target (Livermore 2006). Indeed, this approach has led to unusual macrocyclic β-lactams as exploratory structures for PBP inhibition (Sliwa et al. 2012a, b).

4.3.1.2 The Connection Between the Low-Molecular-Mass PBPs and β-Lactam Resistance

The functional role of the LMM PBPs in bacteria is enigmatic (Ghosh et al. 2008). Nonetheless, key progress has been made with respect to the central roles of these enzymes in the separate events of Gram-negative bacterial growth and resistance expression. With respect to growth, select LMM PBPs "condition" the peptidoglycan polymer so as to determine cell shape, and to coordinate with the assembly of cytoskeletal proteins to enable cell division (Potluri et al. 2012). With respect to resistance development, a connection has been made recently between β-lactam structure and the differential ability of the β-lactam to induce a resistance response. While the existence of this connection is long known to the medicinal chemist, the molecular mechanisms determining this ability now are coming into focus. The key process connecting β-lactam structure to resistance is the recycling of the cell-wall peptidoglycan. During growth, new peptidoglycan is incorporated into the existing peptidoglycan. This incorporation occurs with substantial liberation of peptidoglycan components (called muropeptides). These muropeptides are liberated in the periplasmic space of the Gram-negative bacteria, proximal to the cell wall, and following enzymatic processing are internalized to the cytoplasm through dedicated permeases for further enzymatic recycling. The presumed purposes of this recycling are nutrient recovery, minimization of the innate immune response by the host—which is also muropeptide-structure-dependent (Boudreau et al. 2012)—and also control of the β-lactamase resistance response. This last purpose occurs through the involvement of LMM PBPs. Certain of these LMM PBPs alter the precise molecular composition of these muropeptides, resulting in a different "effector" pool of structures when the LMM PBP is catalytically active, compared to when it is not (as occurs upon its inactivation by β-lactams). The altered molecular composition of this effector pool is then sensed by one particular—AmpR—transcription factor (Balcewich et al. 2010). While derepression of β-lactamase expression is the notable event governed by AmpR, in some pathogenic Gram-negative bacteria the breadth of resistance and virulence

factors controlled through AmpR is extensive (Balasubramanian et al. 2012; Hennequin et al. 2012). With increasing awareness of the relationship between the muropeptide recycling pathway and resistance, the particular roles of the individual recycling enzymes in determining the effector pool are being identified: genetic deletion of one results in high-level β-lactamase expression, while co-administration of an inhibitor of another represses β-lactamase expression (Mark et al. 2011; Yamaguchi et al. 2012). The potential of synergistic drug pairings with β-lactams to forestall AmpR-dependent resistance is evident. A critical absence thus far for this understanding is the specific transformation(s) exerted on the effector pool by the LMM PBP. Notwithstanding considerable effort to identify the endogenous LMM PBP catalytic transformations through in vitro experimentation, the particular identity of the reactions they accomplish in vivo remains uncertain (Nemmara et al. 2011).

This same opportunity for synergistic combinations with the β-lactams may exist for Gram-positive pathogens. The presumption that Gram-positive bacteria cannot recycle muropeptides, since they have a peptidoglycan exoskeleton and a less-defined periplasmic space, is refuted for at least (thus far) some Gram-positives (Reith and Mayer 2011). Moreover, the BlaI transcription factor (which controls β-lactamase expression and can control that of PBP2a—a homolog of the MecI transcription factor that directly controls PBP2a expression in MRSA) is also responsive to a muropeptide effector pool (Amoroso et al. 2012). As discussed for AmpR, processes molecular strategies that alter the Gram-positive muropeptide effector pool may also forestall β-lactam-induced expression of resistance responses in these Gram-positives.

4.3.2 β-Lactamases

The separate but coincident discoveries of clavulanate and the penam sulfones (exemplified by tazobactam) as mechanism-based inhibitors of the serine β-lactamases inaugurated a three decade era of successful antibacterial therapy, that continues to this day (Shahid et al. 2009). Both inhibitors are used clinically in combination with penicillins (Drawz and Bonomo 2010). The value of these combinations is such that recent arguments favoring their use instead of carbapenems (as a carbapenem-resistance-sparing strategy) in certain infections (such as ESBL-*E. coli* bloodstream infections) have been advanced (Perez and Bonomo 2012; Rodriguez-Bano et al. 2012). Indeed, the possibility of advantageous combinations of clavulanate (or penam sulfones) with other β-lactam classes (cephalosporins, carbapenems, monobactam-type) dominates current β-lactam clinical development. The driving force behind these initiatives is the recent emergence of β-lactamases—both serine and metallo—with enhanced hydrolytic ability against the best cephalosporins and carbapenems, and impervious to the inactivation mechanisms of clavulanate and penam sulfones. This emergence, arguably more than any other event, is the basis for the concern that we are now entering an era of untreatable Gram-negative infections (Livermore 2009; Walsh and Toleman 2012).

4.3.2.1 Responding to β-Lactamase Evolution with Inhibitors

The responses against this possibility are currently the evaluation of β-lactamase nuance (the new β-lactamases are not omnipotent enzymes: as they gain one β-lactam as a substrate, they may lose this capability against another); the exploration of new β-lactamase inhibitor combinations (vide supra); and the discovery of new β-lactam-β-lactamase inhibitor combinations. The first response will have value only when clinical diagnostic methodologies are greatly advanced from where they are currently. The evaluation of new pairings of known β-lactams, the second response, is eminently sensible. The third response arguably represents the future. In this regard, the future is best described as not without hope, notwithstanding the bifurcated—metallo and serine—threat posed by these new β-lactamases.

The sudden appearance of an entirely new, and entirely capable, metallo-β-lactamase variant (the NDMs) (Nordmann et al. 2012), has underscored forcefully the absence of a useful inhibitor against these (and other) metallo-β-lactamases. Numerous structures have been premised as inhibitor leads, on the basis of millimolar-level inhibition in vitro as a result of chelation of the active site metal(s) (for example: (Faridoon et al. 2012; Schlesinger et al. 2013)). Nonetheless, none have progressed, and as a consequence the possibility of therapeutic intervention with as the simple metal chelator, EDTA, has been explored in pharmacological models (Aoki et al. 2010). A recent discovery of notable interest is the synergism (by an as yet unknown mechanism) of 2-imidazolamines with β-lactams in suppressing the resistance of certain metallo-β-lactamase-expressing strains (Worthington et al. 2012). This same 2-imidazolamine functional group class also exerts interesting anti-biofilm activity through quorum-sensing modulation (Frei et al. 2012). New β-lactam discovery in light of these emerging β-lactamases focuses on the monobactam, monosulfactam, and monocarbam β-lactam classes, in recognition of the intrinsic stability of these β-lactams to especially metallo-β-lactamase hydrolysis (Page et al. 2011).

Not surprisingly, the newest inhibitors of the serine β-lactamases incorporate similar chemical substructure as have the monobactam-type structures: the bicyclic β-lactam-sulfamate as exemplified by BAL-29880 and by MK-8712 (a congener of MK-7655), and the bicyclic imidazolidinone-*N*-sulfate exemplified by Avibactam (NXL104) (Fig. 4.5). BAL-29880 inhibits the serine Class C β-lactamases via formation of an acyl enzyme that progresses to a hydrolytically stable acyl enzyme by further reaction (Endimiani et al. 2010). BAL-29880 is formulated as one component of the triple β-lactam combination BAL-30376 (with clavulanate as a second β-lactamase inactivator and the siderophore monobactam BAL-19764), currently in clinical evaluation against β-lactam-resistant *Enterobacteriaceae* due to expression of the metallo-, AmpC, ESBL, or carbapenemase β-lactamases (Livermore et al. 2010b; Page et al. 2011). The combination of MK-7655 (Chen et al. 2011) with imipenem dramatically improved the in vitro antibacterial efficacy of imipenem against KPC-2 β-lactamase-expressing *K. pneumoniae* and *P. aeruginosa* (Hirsch et al. 2012). Avibactam (NXL-104) is a structurally

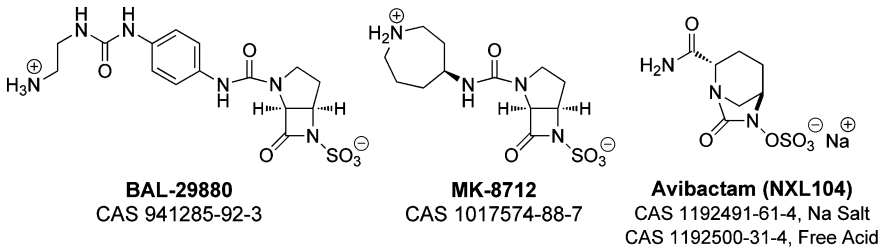

Fig. 4.5 Structures of new β-lactamase inhibitors

unprecedented β-lactamase inhibitor, active against numerous Class A (including KPC), Class C, and Class D β-lactamases, and is currently in two separate mid-stage clinical trials with two different cephalosporins, ceftazidime, and ceftaroline. Together, these two combinations could potentially encompass an impressive breadth of Gram-negative and Gram-positive bacterial pathogens, respectively (Bush and Pucci 2011). Mechanistic study shows surprising efficacy for avibactam as an irreversible acylating agent of the serine β-lactamases (Stachyra et al. 2010; Xu et al. 2012).

4.3.2.2 The Future of β-Lactamase Inhibition

The newest serine β-lactamase inhibitors represent new lactam chemotypes. While the BAL-29880/MK-7655/8712 class has been extensively explored through empirical medicinal chemistry (Chen et al. 2011), neither has yet been fully vetted by the structure-based design opportunities presented by analysis of their irreversible acylation of susceptible β-lactamases, and of the kinetic and mutational analysis of non-susceptible β-lactamases. While these structures present extraordinary challenge with respect to process-scale total chemical synthesis (Mangion et al. 2011), as was once also true for the carbapenems, chemical synthesis provides its own opportunities for structural diversification. It is entirely reasonable to contemplate, for example, appropriate modification of avibactam such that it is not just β-lactamases, but penicillin-binding proteins, that are also irreversibly acylated.

4.4 Recent Advances in the Cell Biology of the β-Lactams

For these first decades of their clinical use, the context of the molecular mechanism of the antibiotic action of the β-lactams was their mechanism-based inactivation of the PBPs. While the shortsightedness of this context was long recognized—for example, why are some β-lactams bacteriostatic, and others bactericidal?—the biological framework to more deeply understand the events

ensuing from the loss of particular PBPs was not in place. These frameworks are now emerging, and their emergence will profoundly affect the future of the β-lactams. Two emerging frameworks may be cited: the discovery that sentinel proteins are used to detect the presence of β-lactams, and secondly the imperative that peptidoglycan biosynthesis is coordinated with that of the other structural components of the cell envelope.

4.4.1 The Existence of Sentinel Proteins

While many bacterial resistance enzymes are expressed constitutively, others are not. A preeminent example of the latter is the PBP2a transpeptidase that is expressed by MRSA bacteria following the loss of the catalytic activity of the transpeptidase domain of their bifunctional PBP, as a consequence of β-lactam acylation. The reason for control of PBP2a expression is the fitness cost of this resistance mechanism. A second example is the mechanism used in many Gram-negative bacteria for β-lactamase expression. In both examples, bacteria use β-lactam-initiated signal transduction to control the resistance response. While in both examples the molecular event for β-lactam signaling is known, in neither are the details of the signaling pathways fully understood. Nevertheless, the prospect is before us that concurrent interference with the signal transduction may resensitize resistant bacteria to β-lactams.

The molecular events of these two examples are summarized. MRSA bacteria express a receptor protein for detection of β-lactams (a cognate receptor protein is used to control the expression of their β-lactamase, and is the better studied of the two). This receptor protein has cell-surface, transmembrane, and cytosolic domains (Llarrull et al. 2011). In the presence of β-lactams, a serine in the antibiotic-binding site of the cell-surface domain is irreversibly acylated (Kumarasiri et al. 2012). The occurrence of this acylation on the membrane surface propagates to the cytoplasmic domain through transduction of information entailing conformational changes, which ultimately result in derepression of the gene for PBP2a or for β-lactamase. The gene derepression takes place via the activated cytoplasmic metalloproteinase domain of the sensor protein, which degrades the gene suppressor protein. Evasion of the signaling of this resistance mechanism may be envisioned as occurring through β-lactam structure optimization to minimize receptor protein acylation, through interference with the proteolysis, or through manipulation of the muropeptide structures (Amoroso et al. 2012) that control the affinity of the repressor for its DNA. A remarkably similar pathway is used to control expression of the AmpC β-lactamase in certain Gram-negatives. Here, a low-molecular-mass PBPs acts as a sentinel enzyme for the detection of the presence of β-lactams. As a result of β-lactam-dependent loss of its catalytic activity, the composition of the muropeptide pool entering from the periplasm to the cytoplasm for recycling is altered (Boudreau et al. 2012). Structural alteration within the muropeptide pool is sensed directly by the gene regulator controlling

the β-lactamase gene, and results in transcription of the DNA. Here, inhibition of a key enzyme (nagZ) in the recycling pathway diverts the muropeptide pool so as to maintain repression of AmpC expression, and preservation of β-lactam sensitivity (Mark et al. 2011).

4.4.2 Cell-Wall Components

The second framework relates to the structural complexity of the bacterial cell envelope (Silhavy et al. 2010). During bacterial growth, a functional peptidoglycan is necessary but insufficient: the growing peptidoglycan must structurally integrate with simultaneous growth elsewhere, including the other cell-wall components, the membrane(s), and the cytoskeleton (Hanson and Neely 2012). The intuitive surmise that concurrent interference with peptidoglycan biosynthesis and a key companion event of cell growth might give profound mutual synergy is now proven (Ejim et al. 2011). In Gram-positives, coordination is required between wall teichoic acid and peptidoglycan biosynthesis (Atilano et al. 2010; Campbell et al. 2011). Inhibitors of wall teichoic acid biosynthesis that synergize β-lactam activity as a "synthetically lethal" combination have been identified (Campbell et al. 2012). Moreover, this nexus is not unique. Genomic-based technologies such as whole-genome sequencing, genotyping, and gene-expression profiling have the potential to identify similarly efficacious pairings (Roemer et al. 2012). Restoration of MRSA susceptibility to β-lactams has been demonstrated through synergy with inhibitors of other events in peptidoglycan biosynthesis (Huber et al. 2009), with other seemingly unrelated enzymes (such as glutamine synthase: Roemer et al. 2012). These synergies define the β-lactam genetic potentiation network of MRSA *S. aureus*. Remarkably, the genetic potentiation network for one β-lactam structure need not be the same as for a different β-lactam structure (Roemer et al. 2012). These same approaches can be used to define the β-lactam genetic potentiation network for specific β-lactam structures against specific bacterial pathogens.

4.4.3 Bacterial Cell Death

Further opportunity for β-lactam genetic potentiation may likewise be found through understanding of the ultimate mechanism resulting in bacterial cell death. This mechanism is just now unfolding. Regardless of their primary targets, cell death from exposure to different classes of bactericidal antibiotics results from stress-induced reactive oxygen (ROS) oxidative damage (Belenky and Collins 2011; Lee and Collins 2012; Foti et al. 2012). Accordingly, bacteria use strategies to combat the ROS produced as a result of antibiotic exposure, including exploration of mutation-conferring resistance (Kohanski et al. 2010a). The adaptive

stress responses used to detoxify the ROS may provide a framework for the development of new antibiotics (Kohanski et al. 2010b). Likewise, the use of extracellular metabolites that provoke different energetic pathways may enable the use of other antibiotics to selectively kill persister bacteria (Lewis 2010) while not affecting normal antibiotic-sensitive cells, as exemplified by even single-chemical supplementation (Kim et al. 2011). For example, Shatalin et al. found that both Gram-negative and Gram-positive bacteria were sensitized to a wide array of antibiotics by deleting (or inhibiting) enzymes that produce H_2S, implicating a direct involvement of H_2S in antibiotic tolerance (Shatalin et al. 2011).

4.5 Conclusions

Notwithstanding an interlude of 30 years since the discovery of entirely new classes of β-lactams, new opportunities will emerge within the identity of the β-lactams as natural products. These opportunities will originate from creative screening methods to empirically identify β-lactam synergy within other classes of antibacterials (Huber et al. 2009; Worthington et al. 2012), from innovative technologies that facilitate the identification of antibiotic overproducers (Charusanti et al. 2012), from epigenetic manipulation of antibiotic-producing bacteria (Wang et al. 2010), and from the ultimate dissection of the β-lactam-biosynthetic pathways (Bodner et al. 2011) coupled to robust and sensitive assays for the identification of new β-lactam structures (Phelan et al. 2012). β-Lactams are preeminent examples of natural products harnessed for the benefit of human health, and emerging discoveries and technologies within the cell networks that define β-lactam biosynthesis, resistance mechanism expression, stress response, and potentiation network will preserve this preeminence.

Antibiotic resistance is inexorable. The continuous emergence of new and powerful resistance mechanisms against the β-lactams reflects their enormous clinical value. We cannot suppress resistance development. Rather, its impact must be limited through improved clinical practice, and countered by exploiting the advantages disclosed through basic research. The challenge—and this is not a small challenge—is bringing these advantages into clinical practice in a timely, practical, and affordable manner. For a seemingly aged antibiotic class, an astonishing breadth of new discovery concerning the β-lactams is now being revealed: with respect to new β-lactam structures, new understanding of their protein targets, new understanding of their resistance pathways, new understanding of their cytotoxicity, and a better appreciation as to how all of these interrelate in terms of bacterial cellular biology. Each of these discoveries has the potential to contribute to the future of the β-lactams as an ageless antibiotic class.

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