New β-Lactamase Inhibitors: a Therapeutic Renaissance in an MDR World

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As the incidence of Gram-negative bacterial infections for which few effective treatments remain increases, so does the contribution of drug-hydrolyzing β-lactamase enzymes to this serious clinical problem. This review highlights recent advances in β-lactamase inhibitors and focuses on agents with novel mechanisms of action against a wide range of enzymes. To this end, we review the β-lactamase inhibitors currently in clinical trials, select agents still in preclinical development, and older therapeutic approaches that are being revisited. Particular emphasis is placed on the activity of compounds at the forefront of the developmental pipeline, including the diazabicyclooctane inhibitors (avibactam and MK-7655) and the boronate RPX7009. With its novel reversible mechanism, avibactam stands to be the first new β-lactamase inhibitor brought into clinical use in the past 2 decades. Our discussion includes the importance of selecting the appropriate partner β-lactam and dosing regimens for these promising agents. This "renaissance" of β-lactamase inhibitors offers new hope in a world plagued by multidrug-resistant (MDR) Gram-negative bacteria.

The production of β-lactam-hydrolyzing enzymes, i.e., β-lactamases, by Gram-negative and -positive bacteria remains one of the most significant threats to the efficacy of this life-saving class of antimicrobial agents (1). Drug discovery and development since the mid-to-late 1980s led to the introduction of β-lactamase inhibitors which provided "new approaches" for combating this clinical challenge. However, bacteria continue to evolve, as they are amazingly capable of responding to environmental pressure via selection of existing mutations and acquisition of new genes (2, 3). The currently available β-lactamase inhibitors, clavulanic acid, tazobactam, and sulbactam (Fig. 1a), are now met with an increasingly prevalent panel of inhibitor-resistant bacterial strains (4). Regrettably, we are faced with the daunting challenge of designing effective inhibitors for an ever-increasing number of diverse β-lactamases.

The clinically available inhibitors share a β-lactam backbone. Sulbactam and tazobactam are penicillanic acid sulfones, while clavulanic acid is a clavam. β-Lactamase inhibitors take advantage of conserved active-site residues to interact with their target (Fig. 1b). However, inhibitors differ from substrates in their abilities to assume long-lived, stable intermediates with β-lactamases, thus "tying up" the enzymes, while the partner β-lactam inhibits the penicillin binding protein target. As more catalytically versatile β-lactamases (e.g., *Klebsiella pneumoniae* carbapenemases [KPCs]) continue to emerge and acquire the ability to hydrolyze inhibitors faster, a new approach is required (5).

This review focuses on the recent studies illustrating the exceptional promise of agents with novel mechanisms of inhibition that are on the threshold of clinical application. Specifically, we summarize the growing body of data supporting the potential use of avibactam as the first clinically available β-lactamase inhibitor introduced in the United States since the piperacillin-tazobactam combination in 1993. We advance the claim that this novel inhibitor illustrates several important features which serve as lessons for improved therapeutic design. Next, we discuss a compound of the same chemical class as avibactam, MK-7655, as well as other non-β-lactams, such as the RPX7009 boronate which recently completed phase 1 trials and cyclobutanones that offer additional new approaches for Ambler class A and C β-lactamases. Lastly, we highlight some of the specific challenges of inhibiting class B and D enzymes. Although the quest for a "universal" β-lactamase inhibitor continues, it is becoming concerning that this notion may be unrealistic. Fundamental research is still required to decipher the basic mechanisms of catalysis of each β-lactamase class.

AVIBACTAM AND MK-7655: "NON-β-LACTAM INHIBITORS"

(i) Avibactam. Avibactam, known formerly as both AVE1330A and NXL104, is a bridged diazabicyclo[3.2.1]octanone non-β-lactam inhibitor (Fig. 2a and c) (6–8). Compared to clavulanic acid, sulbactam, and tazobactam, this non-β-lactam achieved both lower 50% inhibitory concentrations (IC_{50}s) (range, 3 to 170 nM) and decreased reactivation rates for the clinically relevant class A and C β-lactamases such as TEM-1, KPC-2, and P99 and the AmpC from *Pseudomonas aeruginosa* (7, 9, 10). Comparable IC_{50}s were observed for the extended-spectrum β-lactamases (ESBLs) CTX-M-15 and SHV-4.

The success of avibactam may be owing first to its structural similarity to β-lactams at the electrophilic carbonyl group. This molecular mimicry is important for rapid recognition and formation of a stable adduct by β-lactamases, as indicated by rapid enzyme "on" rates (i.e., 370,000 $M^{-1} s^{-1}$ for TEM-1) (9, 11).

The second defining feature of this promising compound is the stable acyl enzyme formed by the carbamoyl link between the inhibitor and the enzyme active-site serine residue. Current analyses reveal that the inhibitor comes off (k_{off}) of class A, C, and D
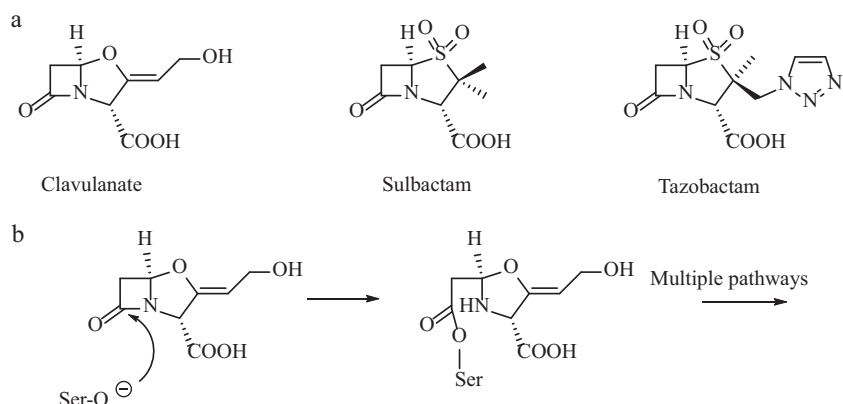
FIG 1 (a) Chemical structures of current clinically available β-lactamase inhibitors. (b) Acylation step in general mechanism of inhibition of a class A β-lactamase by a β-lactamase inhibitor, illustrated here for clavulanic acid (4).

β-lactamases at a very low rate; measured for TEM-1, CTX-M-15, KPC-2, P99, *P. aeruginosa* AmpC, OXA-10, and OXA-48, values ranged from 0.0019 s^{-1} to <0.0000016 s^{-1}, yielding enzyme re-activation half-lives of 6 to >7,200 min (11, 12). In contrast, the kinetic details of avibactam interactions with OXA-10 merit consideration (12). Against OXA-10, acylation (1.1×10^1 M^{-1} s^{-1}) and deacylation (1.6×10^{-6} M^{-1} s^{-1}) were significantly slowed, resulting in an enzyme that is relatively resistant to inactivation yet slow to reactivate. In contrast, the acylation rate for OXA-48 was 100-fold higher. Similarly to class D β-lactamases, BlaC, a class A

β-lactamase from *Mycobacterium tuberculosis*, also demonstrated slow acylation and deacylation by avibactam (13).

Further, avibactam's inhibition is believed to be reversible and the active inhibitor is regenerated via deacylation and recyclization of the 5-membered urea ring. Notably, such cyclic regeneration is not observed with sulfones and clavulanic acid, presumably because the four-member β-lactam ring is too constrained (i.e., after inhibitors are hydrolyzed, the energy required to close and form the original β-lactam ring is too great). Detailed kinetic studies of TEM-1, combined with nuclear magnetic resonance (NMR) analysis and mass spectroscopy, did not yield evidence for irreversible deacylation pathways through hydrolysis or chemical rearrangements (11). Acyl enzyme transfer experiments added support to the idea of the reversible mechanism, where deacylated avibactam was released from a donor enzyme-avibactam mixture and acylated a second enzyme. The mixtures of these apo and acyl enzyme species showed proportions of acyl enzyme that reflected avibactam's affinity for each β-lactamase (11). However, with KPC-2, avibactam hydrolysis was observed after 24 h (only 10% of the enzyme remained acylated with avibactam, as shown by mass spectrometry) (12). Several intermediates that resulted from loss of SO$_3$, loss of a water molecule, and imine hydrolysis were observed using mass spectrometry. The carbamate linkage was subsequently hydrolyzed, and a decarboxylation reaction regenerated free KPC-2.

The recently defined crystal structures of avibactam in complex with CTX-M-15, *P. aeruginosa* AmpC, and *M. tuberculosis* BlaC have offered important insight into the structural bases of the inhibitor's activity (13, 14). Avibactam adopts very similar active-site conformations in class A and C enzymes, making contact with key conserved residues with limited molecular flexibility. Additionally, the sulfate group has more polarity than the C$_3$/C$_4$ carboxylate β-lactams, forming multiple hydrogen bonds in the active site (14). The opened avibactam ring retains a conformation similar to that of the native form, which aids in the recyclization mechanism. Deacylation over hydrolysis is likely explained by the stability of the carbamoyl bond and the lack of an appropriately positioned and activated water molecule, i.e., the latter due to the charges created by the protonated glutamic acid at position 166 (Glu166) in CTX-M-15 (14). These mechanistic details have important implications not just for avibactam but also as possible strategies for additional inhibitor compounds.
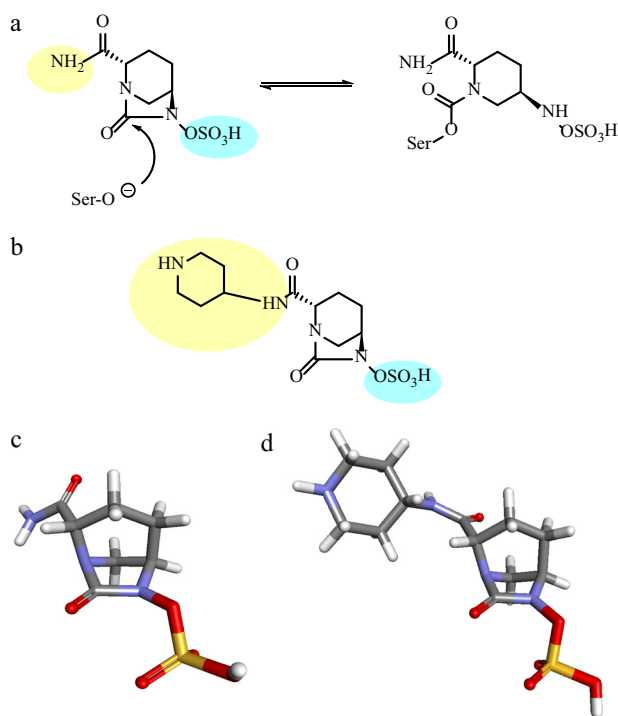

FIG 2 (a) Hypothesized mechanism of avibactam acylation and regeneration with a class A β-lactamase; the amine and sulfate are highlighted in yellow and blue, respectively (11). (b) Structure of MK-7655; the piperidine ring and sulfate are highlighted in yellow and blue, respectively (40). (c and d) Three-dimensional structures of avibactam (c) and MK-7655 (d), constructed using Fragment Builder tools and minimized using a Standard Dynamics Cascade protocol in Discovery Studio 3.1.

TABLE 1 MICs of β-lactam and β-lactam-avibactam combinations against select pathogens[a]

Pathogen	MIC (μg/ml)[b]					
	CAZ	CAZ-AVI	CPT	CPT-AVI	ATM	ATM-AVI
K. pneumoniae with OXA-48	256/512	0.25/0.5				
K. pneumoniae with CTX-M-15	8/64	0.06/0.25				
K. pneumoniae with KPC-2	≥512/≥512	0.25/1			≥512/≥512	≤0.06/≤0.06
E. coli with ESBL	16/64	0.12/0.25				
E. coli with AmpC	16/64	0.12/0.5				
E. coli with OXA-48	4	<0.008				
E. coli with IMP-1	256	64				
Enterobacteriaceae with multiple β-lactamases, including KPC-2			>64/>64	0.5/2		
Enterobacteriaceae with multiple β-lactamases, including AmpC			256/>256	0.5/2		
Enterobacteriaceae with VIM	64–512	64–512			0.25–256	0.12–0.5
P. aeruginosa	8/64	4/8	>64/>64	16/>32	16/32	8/32
P. aeruginosa with ESBL PER-1	128/128	4/16				
A. baumannii			>64/>64	32/>32		
A. baumannii with PER-1, OXA-51, and OXA-58	128/≥512	32/256				
S. aureus			1/2	1/2		

[a] Data were adapted from references 15, 16, 19, 20, 21, and 24. Avibactam was added at 4 μg/ml. Abbreviations: CAZ, ceftazidime; AVI, avibactam; CPT, ceftaroline; ATM, aztreonam.

[b] Numbers separated by a forward slash indicate MIC$_{50}$/MIC$_{90}$ values. Empty cells indicate that values were not reported.

As stated above, release of intact avibactam allows the compound to acylate another β-lactamase, in contrast to the β-lactam inhibitors which follow hydrolytic routes that yield molecules without inhibitory activity. Not only is the active inhibitor regenerated, but so is the active enzyme. As shown by acyl enzyme exchange experiments, this can result in "shuffling" of the inhibitor to higher-affinity enzymes (11). The outcome of this partitioning, and possible selective inhibition of certain β-lactamases within a strain producing multiple enzymes, is not clear and awaits further clinical data. The k_{off} rates and the amount of enzymes present likely have some bearing on the proportion of enzymes inactivated.

The importance of complementing enzyme kinetic and structural studies with whole-cell and *in vivo* assays is central to drug development. Fortunately, the studies with avibactam are also promising. Avibactam has been studied primarily with two partner cephalosporins, ceftazidime and ceftaroline (the active metabolite of ceftaroline-fosamil). These combinations restored activity against *Enterobacteriaceae*, most strains of *P. aeruginosa*, and *Burkholderia cepacia* complex as well as isolates producing one or multiple KPCs (a serine carbapenemase) and ESBL and AmpC (cephalosporinases) enzymes (see Table 1 for representative MIC values) (10, 15–23). Ceftazidime-avibactam lacked activity against bacteria carrying class B metallo-β-lactamases (MBLs) as well as against *Acinetobacter baumannii*, which was most likely due to coexpression of OXA β-lactamases (i.e., OXA-23, -24/40, -51, and -58). *Enterobacteriaceae* expressing the carbapenemase OXA-48 were susceptible to ceftazidime with avibactam, while the alternative combination of aztreonam and avibactam was very effective *in vitro* against strains carrying MBLs (see MBL section below) (19, 24). The lack of activity versus the carbapenem-hydrolyzing OXA enzymes (i.e., OXA-23 and OXA 24/40) remains to be explained.

The addition of avibactam to ceftaroline may be particularly useful for treatment of infections with a presumptively high burden of Gram-positive organisms, such as *Staphylococcus aureus* in diabetic foot wounds (25). The potent activity of ceftaroline against most aerobic organisms is complemented by the ability of avibactam to inhibit β-lactamases from anaerobes, including *Bacteroides fragilis*. Murine sepsis models demonstrated that avibactam added to ceftazidime and ceftaroline regimens increased the susceptibility of highly resistant ESBL- and KPC-producing *K. pneumoniae* isolates (26, 27). Depending on the partner β-lactam, avibactam combinations have the potential to be highly effective against many multidrug-resistant (MDR) pathogens.

Optimal dosing regimens for these novel combinations have been studied in multiple hollow-fiber models, assays which exposed a bacterial suspension to clinically relevant and fluctuating drug concentrations (22, 23, 28, 29). The increasing sophistication of pharmacodynamic modeling may help refine regimens for potential inhibitors to prevent mislabeling a drug as ineffective due to dosing failures (30). Investigations of dosing schedules for both the ceftazidime-avibactam and ceftaroline-avibactam combinations argued that time above a critical concentration (time > MIC) was the essential parameter for suppressing bacterial growth, as has been shown for imipenem (23, 28, 29, 31). For example, for *K. pneumoniae* 27-908M, the ceftaroline-avibactam MIC was 0.75 μg/ml for ceftaroline and 4 μg/ml for avibactam. The ceftaroline-avibactam concentrations needed to be above the MICs for 62% to 80% of the dosing interval for treatment success with ceftaroline-avibactam (administered as ceftaroline at 600 mg every 8 h and avibactam at a daily dose as a continuous infusion or as divided doses every 8 h) (23). The concentrations differed depending on the organism, inoculum, partner antibiotic, and β-lactamase expression profile. Louie et al., who examined KPC-2-, CTX-M-15-producing *K. pneumoniae*, and AmpC-overproducing *Enterobacter cloacae*, concluded that avibactam trough levels should be at least 2.5 μg/ml and that patients should be dosed with ceftaroline every 8 h for infections with highly resistant pathogens (23). In contrast, Nichols et al. determined that the required avibactam trough concentration was closer to 0.3 μg/ml

when the drug was partnered with ceftazidime in a model with *E. cloacae* and *K. pneumoniae* at lower inocula (29).

These hollow-fiber models and pharmacokinetic analyses have helped inform dosing regimens for clinical trials, and avibactam is well on its way in moving from "bench to bedside." The first study to report avibactam data from a phase 2 trial showed that a ceftazidime-avibactam combination (500 mg/125 mg, every 8 h) is comparable in efficacy and safety to imipenem-cilastin in hospitalized patients with complicated urinary tract infections (UTIs) (32). That study did not elucidate particular resistance mechanisms, but it is likely that the most common uropathogen isolated from these patients, *Escherichia coli*, harbored only class A enzymes (33). More recently published phase 2 results of complicated intra-abdominal infections (IAIs) treated with ceftazidime-avibactam-metronidazole (2,000 mg/500 mg/500 mg, every 8 h) versus meropenem demonstrated comparable clinical responses (91.2% and 93.4%, respectively) and similar rates of treatment-emergent adverse events (64.4% and 57.8%, respectively) (34). That investigation nicely translates *in vitro* data of the potency of the triple combination against anaerobes, making this formulation well-suited for polymicrobial IAIs (of note, the anaerobic activity of ceftazidime-avibactam was limited without the addition of metronidazole) (35, 36). With these promising results, multiple phase 3 trials are currently recruiting for ceftazidime-avibactam in complicated cases of UTIs and IAIs (www.clinicaltrials.gov), as well as for one for nosocomial pneumonia (registration no. NCT01808092). The ceftaroline-avibactam combination is not yet as thoroughly tested, although one recently completed phase 2 study compared the combination to doripenem for complicated UTIs (NCT01281462). Finally, a phase 1 trial of aztreonam-avibactam safety has suspended recruitment following a change in dosing regimens, totaling a second suspension for this study (NCT01689207). While it is unclear what types of problems underlie these suspensions, aztreonam-avibactam is likely to receive further attention. In latter half of 2013, the European Innovative Medicines Initiative called for proposals for development of phase IIa pharmacokinetic/pharmacodynamic and phase III efficacy and safety studies of Gram-negative pathogens, with particular attention to MBL producers (www.imi.europa.eu/sites/default/files/uploads/documents/9th_Call/Calll_9_Text.pdf) (37).

Clinicians and researchers need to be aware that, as with all agents, bacteria with mechanisms of resistance to these novel inhibitors can emerge. Passage of *E. coli* and *E. cloacae* in the presence of ceftaroline-avibactam selected for organisms harboring AmpC and CTX-M-15 enzymes with deletions or mutations that conferred resistance (38). In addition, CMY-2 variants (i.e., enzymes with amino acid substitutions of Asn152 to alanine, threonine, and serine) demonstrated higher IC_{50}s for avibactam (i.e., 0.5 to 1.0 μM) than their wild-type counterpart (i.e., 0.06 μM) (39). The clinical impact of these findings is being monitored. Nevertheless, we can optimistically project that with appropriate antibiotic stewardship, any one β-lactamase conferring resistance to a particular inhibitor may remain susceptible to inactivators that work by different mechanisms. As such, introduction of agents employing diverse mechanisms may allow successful therapy. For example, those strains selected by ceftaroline-avibactam pressure were relatively "unstable," lost significant ESBL activity, and were susceptible to tazobactam (38).

(ii) MK-7655. MK-7655 is similar to avibactam's diazabicyclooctane core with the addition of a piperidine ring (Fig. 2b and

d). MK-7655 is predicted to function through a similar mechanism (40). MK-7655 exhibits synergistic activity in combination with imipenem against carbapenem-resistant *Enterobacteriaceae* and *P. aeruginosa* with diverse resistance mechanisms, including KPC production and impermeability due to porin loss and class A and C β-lactamase production (41–43). For example, imipenem MICs for KPC-producing *K. pneumoniae* and *Enterobacter* spp. fell from a range of 16 to 64 μg/ml to a range of 0.12 to 1 μg/ml with the addition of 4 μg/ml of MK-7655 (42). Additional data from another study showed that imipenem and MK-7655 were synergistic for *K. pneumoniae* and *Enterobacter* strains with imipenem resistance due to both impermeability and ESBL or AmpC activity, lowering MICs from a range of 2 to 16 μg/ml to ≤1 μg/ml. The combination was not effective in restoring susceptibility to the *Enterobacteriaceae* expressing OXA-48 carbapenemase or IMP, NDM, or VIM MBLs, and similarly, MK-7655 did not lower MICs for imipenem-susceptible isolates with ESBLs or AmpCs. Against *P. aeruginosa*, MK-7655 was successful in lowering imipenem MICs for imipenem-susceptible strains, likely by inhibiting endogenous AmpC-mediated imipenem protection (42). OprD porin-deficient *P. aeruginosa* required 8 μg/ml of MK-7655 to lower imipenem MICs from a range of 16 to 64 μg/ml to ≤2 μg/ml for most (7/8) isolates. MK-7655 was unable to lower imipenem MICs for MBL-producing *P. aeruginosa* and lowered them only to 4 to 8 μg/ml for MDR strains, including those from patients with cystic fibrosis.

MK-7655 dosing regimens were examined in a hollow-fiber model (41). Five hundred milligrams of both imipenem and MK-7655 suppressed growth of a KPC-2-producing *K. pneumoniae* strain and an OprD-deficient, AmpC-overexpressing *P. aeruginosa* strain at 72 h. Increasing MK-7655 to 1,000 mg achieved growth suppression in an additional *P. aeruginosa* strain. In a related hollow-fiber model, a unique parameter, time above instantaneous MIC ($T>MIC_i$), was derived to reflect changing *in vivo* susceptibility (44). Provided the MK-7655 $T>MIC_i$ was greater than 69% in the presence of 500 mg imipenem, similar levels of 48-h killing were observed for the KPC-2-producing *K. pneumoniae* strain regardless of escalating inhibitor doses. These data suggest that time over a susceptibility threshold is essential to bacterial killing for this novel inhibitor as well.

A phase 1 trial of the pharmacokinetics of MK-7655 in patients with impaired renal function was completed in March 2012 (www.clinicaltrials.gov; NCT01275170), and reported data suggested that the required dosage reduction was unchanged by the addition of the β-lactamase inhibitor (45). Phase 2 clinical trials are presently examining the imipenem-cilastatin-MK-7655 combination for treatment of complicated UTIs and IAIs, with doses of 125 mg or 250 mg of MK-7655 combined with 500 mg of imipenem and cilastatin every 6 h (NCT01505634 and NCT01506271).

Taking a reductionist view, avibactam and MK-7655 are "similar." Although the clinical development of avibactam is ahead of that of MK-7655 based upon the number of clinical trials, it is very likely that in controlled studies, both will perform well, especially as they are effective against ESBLs, AmpCs, and serine carbapenemases (KPCs). That property alone is a truly welcome addition to our therapeutic armamentarium and a significant advance with respect to what we currently have available to us. As a result, barring unforeseen complications or regulatory problems, avibactam and MK-7655 should proceed to market. Once in use, clinicians will witness a "natural experiment" and observe what ends up
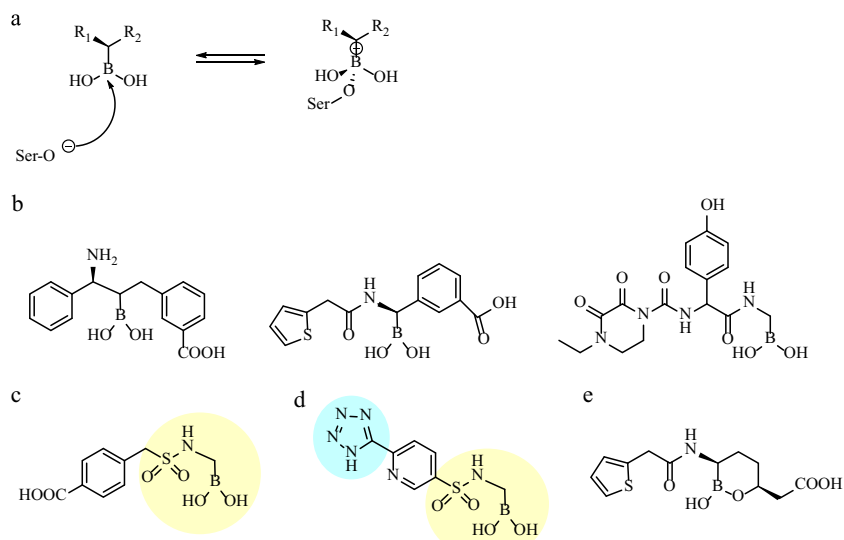
FIG 3 (a) General mechanism of reversible inhibition of a class A β-lactamase by a boronic acid compound. (b) Structure of β-lactam substrate analog boronic acid inhibitors (ampicillin, cephalothin, and cefoperazone mimics). (c) Structure of sulfonamide boronic acid (tested as described in reference 57); the sulfonamide boronic acid group is highlighted in yellow. (d) Compound 5 developed from FBLD optimization of sulfonamide boronate structure (59); the sulfonamide boronic acid group and tetrazole are highlighted in yellow and blue, respectively. (e) Structure of RPX7009 (65).

being the "better partner" β-lactam. Is a "carbapenem-sparing" combination less likely to foster more resistance? The cephalosporins have well-established clinical safety records, and yet carbapenems may be less susceptible to efflux in *P. aeruginosa* (46). Ultimately, clinical use and the emergence of resistance will answer this vexing question. We must keep in mind, however, that resistance is inevitable, and although these inhibitors promise "a battlefield victory" over certain MDR organisms, we have not yet "won the war" and the struggle between "bug and drug" will likely continue.

BORONIC ACID β-LACTAMASE INHIBITORS

Since the late 1970s, boronates have been documented as effective inhibitors of serine β-lactamases *in vitro* (47–49). Working via a novel mechanism compared to that of the clinically available β-lactamase inhibitors, boron forms a reversible dative bond with the β-lactamase, is not hydrolyzed by the enzyme, and serves as a competitive inhibitor (Fig. 3a) (48). Recently, the functional groups bound to the boronate core have been modified extensively based on hypothesized and structurally confirmed interactions with β-lactamase active-site residues (50–52).

Glycylboronates are based on β-lactam substrate homology and contain side chains of penicillins and cephalosporins. Studies have shown analogs of ampicillin, cephalothin, and cefoperazone to be inhibitors of clinically relevant class A and C β-lactamases in the nM range (i.e., 9.3 nM, 420 nM, and 11 nM for *K. pneumoniae* SHV and for AmpCs from *P. aeruginosa* and *Acinetobacter* spp., respectively) (Fig. 3b) (53–56). These compounds are still preclinical.

More recently, new modifications to the β-lactam analog boronate were added by replacing the carboxamide group, conserved in all penicillin and cephalosporins, with a sulfonamide (Fig. 3c) (57, 58). These novel derivatives result in very potent *E. coli* AmpC inhibitors with affinities approaching 0.025 μM, 23 times more potent than their carboxamide analogs. Combined with ceftazidime, the lead compounds lowered MICs up to 64-fold

(from 64 μg/ml to 1 μg/ml) against microorganisms expressing class A and class C β-lactamases.

Shortly thereafter, using a boronic acid scaffold, fragment-based lead discovery (FBLD) compounds were optimized to better target β-lactamases (59). FBLD is a powerful new method for building drug leads from "fragments of molecules" (this approach has recently yielded important therapeutic candidates for treatment of Alzheimer's disease and cancer) (60). The strategy here lies in the ability to identify multiple small molecules with favorable β-lactamase binding properties and then to apply structural and chemical information to combine the components into drug leads. In this regard, FBLD is used to refine the highest-affinity boronates with the aim of yielding compounds with significant *in vivo* antimicrobial activity, as previous generations of boronates have demonstrated favorable *in vitro* kinetics but failed to improve susceptibility (e.g., affinities in the nM range but poor MICs) (59). Examination of binding models using FBLD revealed subtle changes such as reorientation of functional groups and ring structures that improved affinity by 500-fold. Lead compound 5, in Eidam et al., when combined with cefotaxime, reduced *E. coli* MICs from a range of 8 to 128 μg/ml to a range of 0.5 to 1 μg/ml (Fig. 3d). Mice challenged with AmpC-overproducing *E. coli* had a significant 65% improved survival when treated with cefotaxime combined with compound 5 at 50 and 200 mg/kg of body weight compared to 50 mg/kg cefotaxime alone ($P \leq 0.0005$). Despite these promising *in vivo* animal studies, the FBLD approach remains a work in progress that has not yet promoted any candidates into anti-infective preclinical assessment.

At the 2012 Interscience Conference on Antimicrobial Agents and Chemotherapy, a novel boronic acid-based β-lactamase inhibitor, RPX7009 (Fig. 3e), was presented in combination with a carbapenem antibiotic (RPX2003; biapenem) (61–64). Alone, RPX7009 did not exhibit antibacterial activity, but the combination showed strong potentiation of biapenem against class A carbapenemase-producing *Enterobacteriaceae* (e.g., KPC, SME, and
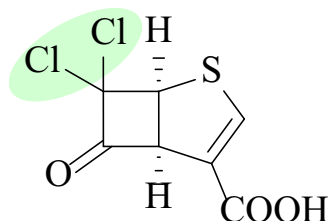
FIG 4 Structure of cyclobutanone inhibitor with high affinity for class C enzymes; the chlorine atoms are highlighted in green (68).

IMI) (65). Of a panel of 145 KPC-positive isolates, 89.7% were inhibited by ≤1 µg/ml of biapenem with 2 µg/ml of RPX7009 and 94.4% were inhibited when RPX7009 was increased to 8 µg/ml. Biapenem with RPX7009 at 4 µg/ml demonstrated MIC_{50} values of 0.12 µg/ml for KPC-producing *Enterobacteriaceae* that coexpressed either one to four additional β-lactamases (including class A [narrow- and extended-spectrum], CTX-M, and noncarbapenemase OXA) or hyperexpressed chromosomal AmpC enzymes (62). However, against isolates with impermeability and either AmpC or ESBL expression (*Enterobacter* and *Klebsiella* spp., respectively), 8 µg/ml of RPX7009 showed only weak potentiation of biapenem (50% and 33% of isolates, respectively, had MICs > 1 µg/ml) (65). Potentiation was not seen with *Enterobacteriaceae* expressing IMP, VIM, or NDM MBLs or OXA-48.

Biapenem-RPX7009 activity for the nonfermenters *P. aeruginosa* and *A. baumannii* was similar to that of meropenem and imipenem (66). Testing of a panel of anaerobic pathogens, including more than 25 *Bacteroides* spp., demonstrated that biapenem and RPX7009 were comparable to meropenem alone. However, biapenem given without RPX7009 showed similar results, suggesting that RPX7009 lacked anaerobic activity (67). Animal models (neutropenic thigh models of infection) with KPC-producing strains also demonstrated promising results for this boronate inhibitor (63). Biapenem-RPX7009 recently completed a phase 1 clinical trial without current reports of toxicity (NCT01772836). Interestingly, an additional trial of RPX7009 in combination with a novel carbapenem, RPX2014, is currently in recruitment phase (NCT01897779). The structure of RPX2014 is unknown at this time, and insights as to efficacy are awaited.

CYCLOBUTANONE AND PENAM SULFONE β-LACTAMASE INHIBITORS

Cyclobutanone-based inhibitors, as mimetics of β-lactams, demonstrate activity against all four classes of β-lactamases; however, they appear most effective against class C enzymes. One lead compound (Fig. 4) inhibited KPC-2, IMP-1, *E. cloacae* GC1, and OXA-10 with IC_{50}s of 26, 213, 4.5, and 370 µM, respectively (68). These compounds are reversible inhibitors with a bicyclic system, and particular substituents help force the 5-membered ring into conformations that enhance broad-spectrum-β-lactamase binding. While this lead inhibitor reduced meropenem MICs from 64 µg/ml to 16 µg/ml for MBL-producing strains of *Chryseobacterium meningosepticum* and *Stenotrophomonas maltophilia*, this limited potentiation may not be clinically useful.

Derivatized penam sulfone inhibitors are another group of inhibitors with a β-lactam core that are being rationally designed to help address specific clinical problems. For example, functional groups taking advantage of active-site residues have led to compounds with high affinities for KPC-2 and *P. aeruginosa* AmpC (e.g., 3.9 µM and 180 nM, respectively) (69, 70). Despite what may be promising prototypes, there are no current reports suggesting the promotion of cyclobutanone and penam sulfone inhibitors beyond the preclinical stage.

THE UNIQUE CHALLENGE OF CLASS B MBLS; CLOSER THAN WE THINK?

This "class apart" of β-lactamases presents a particular challenge for clinicians and medicinal chemists (71). The spread of these metalloenzymes is often facilitated by mobile genetic elements, and the rapid emergence of the NDM-1 β-lactamase demonstrates how an MDR strain from one part of the world can quickly become a global problem (72). The hydrolytic mechanisms of MBLs are substantially different from those of the other classes, requiring one or two zinc atoms depending on subclass (i.e., B1, B2, or B3). The amino acid or primary sequence diversity is extremely broad, and the substrate profile includes all known β-lactams (including the available β-lactamase inhibitors), with the exception of monobactams (73).

While none of the promising inhibitors mentioned have significant activity against class B enzymes, there are two important exceptions, the combinations of cyclobutanone with meropenem (see above) and avibactam with aztreonam (19, 68). The aztreonam-avibactam combination, with MICs of ≤4 µg/ml for carbapenem-resistant *Enterobacteriaceae*, including MBL-containing pathogens, may offer great promise for this clinical challenge (19). Most likely, avibactam inhibits coproduced ESBL and AmpC enzymes as aztreonam evades the MBLs and exerts its antimicrobial effect. Aztreonam is hypothesized to bind poorly and unproductively to MBLs; molecular modeling reveals that the β-lactam moiety is too far from the nucleophile at the first Zn^{2+} binding site due to the interactions of the sulfonate group of aztreonam with the second Zn^{2+} binding site (74). As discussed above, the phase 1 trial of aztreonam-avibactam (NCT01689207) has been suspended for the second time in the study's history, but further work fostered by the European Innovative Medicines Initiative may provide important data for further therapeutic development.

This review focuses on the merits of inhibitor design which move beyond the β-lactam core structure for class A and C β-lactamases. Because of the special characteristics of the class B enzymes, this approach has already been applied and yielded candidate agents such as thiols and succinate derivatives, pyridine dicarboxylates, and tricyclic natural products (4). Of these, the thiol derivatives have shown the most promise for meeting the need for broad-spectrum activity against each of the MBL subclasses. Thiols, including the clinically available antihypertension agent L-captopril, have shown effective inhibition of NDM-1 and subclass B1, B2, and B3 enzymes (75–78). For example, synthesis informed by binding mode structural analyses led to a thiol compound with 0.019, 5.7, and 1.8 µM affinity values for MBLs from each class, IMP-1, CphA, and L1, respectively (Fig. 5a) (76). A single thiol compound can adopt unique binding conformations in different enzymes and yet still achieve inhibition through the common mechanisms of zinc chelation and/or displacement of the hydrolytic water.

Much of the recent literature on identifying possible inhibitors for MBLs describes *in vitro* testing of compounds based on insights gained from solved crystal structures. This trend may be partly explained by our relatively rudimentary understanding of
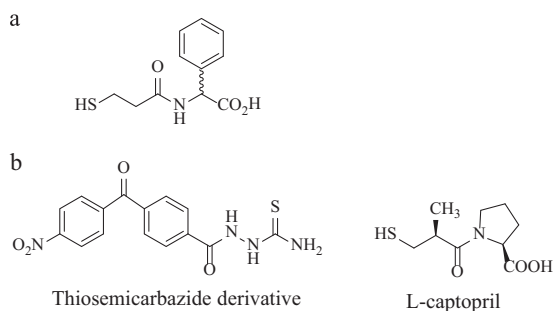
FIG 5 (a) Thiol compound with inhibitory activity for the three MBL classes (76). (b) Structure of thiosemicarbazide derivative and L-captopril, metallo-β-lactamase IMP-1 inhibitors (85, 87).

FIG 6 (a) Structure of EDTA. (b) Structure of LN-1-255 (108). (c) Structure of DSABA (109).

this group of enzymes compared to the better studied class A and C β-lactamases. Not until the last couple decades have MBLs presented in pathogenic Gram-negative organisms such as *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* spp. (79–81). As these enzymes have "grown up" during this technological age, multiple computer-based approaches, such as computer modeling of target compounds, integrating protein shape and surface charge profiles into *in silico* screening, and compiling crystal structures in automated databases for future studies, have been described for drug discovery (82–84).

To illustrate, a modified form of fragment screening was employed to identify lead molecules for inhibition of the increasingly common IMP-1 enzyme, followed by *in silico* studies of the binding mechanisms of 1,2,4-triazole-3-thiols (85, 86). While optimization of the initial thiols yielded compounds with only mid-μM affinities, derivatives of thiosemicarbazides achieved low-μM affinity (range of 11 to 75 μM) (87). Interestingly, the final affinity value of the lead compound was comparable to that of L-captopril for IMP-1 (11 and 12.5 μM, respectively) (85) (Fig. 5b).

Few papers describing animal models of potential MBL inhibitors have been published. Two studies showed inhibitory effects of calcium-ethylenediamine-N,N,N′,N′-tetraacetic acid (CaEDTA) in whole-cell assays and murine models of MBL-producing *P. aeruginosa* and *E. coli* (Fig. 6a) (88, 89). In combination with imipenem, CaEDTA improved survival and decreased bacterial burden in cases of pneumonia caused by IMP- and VIM-producing *P. aeruginosa* (88). Furthermore, CaEDTA inhibited the activity of the elastase-type *P. aeruginosa* metalloprotease virulence factor. A murine neutropenic sepsis model of NDM-1-producing *E. coli* also showed reduced bacterial liver and blood counts after administration of imipenem-CaEDTA combinations, although the effects on overall survival were less clear (89).

Whether these *in vivo* studies offer real clinical promise is unclear. The chelating properties of EDTA for metals present in MBLs have been known for decades such that the agent is used to screen for MBL-producing organisms in clinical laboratories (90–93). In addition to chelating the zinc ions required for MBL hydrolysis, ion chelation may disrupt bacterial cell membranes and help disperse bacterial biofilms (94, 95). Literature also exists supporting the potential benefits of Na$_2$EDTA, including decalcifying atherosclerotic plaque; however, recently published data from a clinical trial (NCT00044213) found an only modestly reduced risk of adverse cardiovascular outcomes from Na$_2$EDTA therapy in patients with a history of myocardial infarction (96).

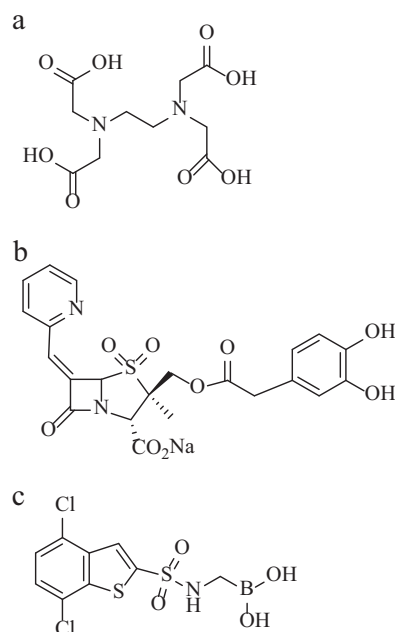Presently, clinicians often have to resort to treatment regimens that include relatively toxic antimicrobials, such as colistin, and/or those likely to foster further resistance, such as tigecycline, for MBL-producing organisms (97). However, serious concerns exist about the possible toxicities of EDTA therapy, including deaths after documented infusions of Na$_2$EDTA (98). In fact, Na$_2$EDTA was withdrawn from the market in 2008 because of safety concerns. Approximately one-third of human proteins are metalloproteins, and nonspecific chelation of essential cofactors could have significant biological effects (99). While pharmaceutical companies are unlikely to select EDTA for development as an MBL inhibitor, the concept of chelator fragments with improved specificity may still be worth attention. This notion of selective inhibition has been applied to the design of inhibitors for the metalloenzyme HIV integrase which inhibited viral replication in cell culture assays (100). Many important issues about a chelation strategy for MBLs, including whether these agents can penetrate bacterial cell walls to reach their targets and whether modification is possible to create safe therapeutic options, remain unresolved.

CLASS D β-LACTAMASES; ANTICIPATING A GROWING NEED

The class D β-lactamases, named the OXA type for their oxacillin-hydrolyzing properties, comprise a very diverse group of enzymes with substrate profiles ranging from narrow to extended spectrum and more recently including the carbapenems (101, 102). As a class, these OXA β-lactamases are not effectively inactivated by the available β-lactam-type inhibitors; however, *in vitro*, concentrations of NaCl > 100 mM inactivate most class D enzymes (103, 104). This inhibitory property is not well understood, although substitution of a specific active-site Tyr144 residue can produce a resistant phenotype (103); of note, this mechanistic insight may someday provide a focus for inhibitor design. The OXA enzymes can be spread through mobile elements such as insertion sequences and transposons and have recently been increasingly de-
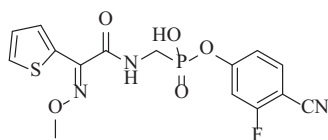
FIG 7 Structure of thiophenyl oxime-derived phosphonate compound with strong class C inhibitory activity (110).

tected worldwide in *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii* (105, 106).

There is a relative dearth of literature addressing the challenge of inhibiting class D enzymes. With regard to β-lactam derivatives, substituted penicillin sulfones have turnover numbers (i.e., partitioning of the initial enzyme inhibitor complex between hydrolysis and enzyme inactivation) ranging from 0 to 8 for narrow- and extended-spectrum and carbapenemase OXA enzymes (107, 108). Bou et al. showed that select compounds from this series demonstrate exceptional potency (nM affinity versus OXA-24/40) (108). In addition, LN-1-255, compound 1, lowered meropenem MICs for *A. baumannii* possessing OXA-24/40 from 32 μg/ml to 4 μg/ml (Fig. 6b).

While the studies mentioned above describe penicillin sulfones with promising inhibition of class A and C enzymes, boronates with activity against OXA β-lactamases had not been described until recently. In 2010, medicinal chemists reported on a 4,7-dichloro-1-benzothien-2-yl sulfonylaminomethyl (DSABA) compound with a 5.6 μM IC$_{50}$ against OXA-24/40 (Fig. 6c) (109). DSABA also inhibits class A and C β-lactamases with low-μM IC$_{50}$s (i.e., 0.57 and 1.1 μM for SHV-5 and TEM-1 and 0.62 and 1.2 μM for P99 and *P. aeruginosa* AmpC, respectively). The whole-cell data were less impressive, as 100 μM DSABA reduced imipenem MICs only from 256 μg/ml to 128 μg/ml for *A. baumannii* expressing class A, C, and D β-lactamases.

Phosphonates, which share functional similarities with boronates and have been similarly derivatized with side chains to screen for high-affinity active-site binding, may offer a better ability to bridge the gap to class D enzymes. The same medicinal chemistry group working on DBASA evaluated a panel of thiophenyl oxime-derived phosphonate compounds among which a lead compound showed strong nM IC$_{50}$ data against class C β-lactamases (i.e., 7.5 to 32 nM for *P. aeruginosa* AmpC and P99) and 17 to 22 μM values against class A and D enzymes (Fig. 7) (110). This compound potentiated imipenem against clinical strains of AmpC-producing *P. aeruginosa* in a dose-dependent manner and, despite its relatively weaker class D activity, lowered imipenem MICs from 128 to 16 μg/ml in OXA-24/40-expressing *A. baumannii*.

While we recognize that the impact of OXA-23 and -48 is significant, the clinical contribution of other carbapenem-hydrolyzing members of the class has been debated (111, 112). The carbapenem inactivation rate is relatively low; however, the affinities are high, and recent *in vitro* data suggest that OXA-23, OXA-24/40, and OXA-58 add significantly to decreased carbapenem susceptibilities, particularly when combined with other resistance mechanisms such as decreased membrane permeability (113). Owing likely to the significant diversity in substrate spectra of this class of β-lactamases, laboratory detection of these enzymes is quite challenging (105). Specific class-wide phenotypic markers have not been identified, and this difficulty in detection may

contribute to the spread of OXA-expressing organisms as their presence goes unidentified.

The search for novel mechanisms again presents promising data for avibactam combinations, specifically with ceftazidime or ceftaroline, restoring susceptibility in *K. pneumoniae* expressing OXA-48 (Table 1) (19). In contrast, ceftazidime-avibactam did not lower MICs for *A. baumannii* strains harboring carbapenemases OXA-23, -24/40, -51, and -58 (18). This failure may reflect problems with membrane permeability or an intrinsic lack of activity against these enzymes. The ceftazidime-avibactam combination is also inactive against the structurally distinct extended-spectrum-type enzymes in *P. aeruginosa*, such as those derived from OXA-2 and OXA-10 (18, 114). These extended-spectrum-type class D enzymes generally maintain susceptibility to carbapenems, although broad inhibition certainly remains a paramount goal in this era of antibiotic stewardship. The further dissemination of OXA-48-type enzyme expressers in *Enterobacteriaceae* was foreshadowed, coining the phrase "the phantom menace" for this β-lactamase (111). Treatment of *Acinetobacter* spp. expressing OXA carbapenemases may also become an increasingly relevant clinical problem (115, 116). None of the discussed inhibitors are likely solutions for carbapenem- and aztreonam-resistant strains; however, the novel siderophore monosulfactam BAL30072 restores susceptibility in many MDR strains (117).

PERSPECTIVE

This paper reviews the current state of research and development for β-lactamase inhibitors of novel or non-β-lactam-based mechanisms (for a full review of the existing patent literature, see reference 118). Since our last review on β-lactamase inhibitors 4 years ago (4), we have noted a growing focus on computation-based design methods. Such approaches are yielding inhibitors with low-nM affinities rarely before reported. We are eager to see how these agents will survive thorough *in vivo* testing and if they will continue to adapt as the boronates have.

However, the reader must appreciate that the majority of the compounds we discuss, with the exceptions of avibactam, MK-7756, and RPX7009, are years from clinical testing (116, 119). This "gap" is a call to action and, fortunately, one that has recently seen an increase in attention and momentum in a world with ever-rising rates of MDR pathogens. In much the same way that we need novel inhibition mechanisms, researchers, clinicians, and policy makers alike need to embrace a diverse and multifaceted approach to this problem. The Infectious Disease Society of America outlined in their 2012 white paper a new approach to streamline and expedite trials for treatment of highly resistant bacteria (120). Additionally, authorities have advocated focusing not just on incentivizing new drug development but also on interventions such as requiring transparency through public reporting of antibiotic use tied to reimbursement, harnessing molecular techniques for diagnostic confirmation of antibiotic indications, and exploring agents that modify host immune responses to pathogens to circumvent resistance selection (121). The role of regulatory agencies in this process is also highlighted (122). We need to pay closer attention to the variables over which clinicians do have control, such as dosing regimens. A recent double-blind randomized controlled trial showed improved clinical cure for critically ill septic patients receiving continuous infusion versus intermittent boluses of β-lactams (123). Why have we been reticent to embrace these approaches? A better understanding of the

relationships between bacterial burden, patient factors, and pharmacodynamics has the potential to revitalize our β-lactam armamentarium (124). In this context, we can answer that novel mechanisms are indeed an advantage. The renaissance of interest is unlikely to provide a "magic bullet" that effectively inhibits all β-lactamase classes but will hopefully engender a collaborative solution.

In closing, a position that has some merit is to design optimal β-lactamase inhibitors for specific applications, based upon the pathogen detected. This would require a level of expertise beyond the scope of our current clinical microbiology laboratory. Yet, with the advent of molecular diagnostics, we may be able to define the β-lactamase background present and target appropriately. The current clinical threats should force decisions about antibiotic treatment to become more deliberate. We hope this prudence will allow agents with novel inhibitory mechanisms to succeed.

ACKNOWLEDGMENTS

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