



Review

A resurgence of β -lactamase inhibitor combinations effective against multidrug-resistant Gram-negative pathogens

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ABSTRACT

β -Lactamase inhibitors (BLIs) have played an important role in combatting β -lactam resistance in Gram-negative bacteria, but their effectiveness has diminished with the evolution of diverse and deleterious varieties of β -lactamases. In this review, a new generation of BLIs and inhibitor combinations is presented, describing epidemiological information, pharmacodynamic studies, resistance identification and current clinical status. Novel serine BLIs of major interest include the non- β -lactams of the diazabicyclo[3.2.1]octanone (DBO) series. The DBOs avibactam, relebactam and RG6080 inhibit most class A and class C β -lactamases, with selected inhibition of class D enzymes by avibactam. The novel boronic acid inhibitor RPX7009 has a similar inhibitory profile. All of these inhibitors are being developed in combinations that are targeting primarily carbapenemase-producing Gram-negative pathogens. Two BLI combinations (ceftolozane/tazobactam and ceftazidime/avibactam) were recently approved by the US Food and Drug Administration (FDA) under the designation of a Qualified Infectious Disease Product (QIDP). Other inhibitor combinations that have at least completed phase 1 clinical trials are ceftaroline fosamil/avibactam, aztreonam/avibactam, imipenem/relebactam, meropenem/RPX7009 and cefepime/AI101. Although effective inhibitor combinations are in development for the treatment of infections caused by Gram-negative bacteria with serine carbapenemases, better options are still necessary for pathogens that produce metallo- β -lactamases (MBLs). The aztreonam/avibactam combination demonstrates inhibitory activity against MBL-producing enteric bacteria owing to the stability of the monobactam to these enzymes, but resistance is still an issue for MBL-producing non-fermentative bacteria. Because all of the inhibitor combinations are being developed as parenteral drugs, an orally bioavailable combination would also be of interest.

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1. Introduction

β -Lactamases have posed a critical threat to the utility of β -lactam antibiotics ever since the introduction of penicillin [1]. Biochemical and microbiological factors both influence their role in resistance. Hydrolysis of these critical drugs may proceed at rates approaching the maximal rate for any enzymatic reaction [2]. The amount of β -lactamase also plays a role in resistance, as some enteric bacteria can produce up to 4% of their soluble protein as active β -lactamase, constitutively or following induction [3,4]. These enzymes, now known to be quite ancient in origin [5], can be categorised according to molecular structure (classes A, B, C and D) or by functionality, as shown in Table 1 [6–8]. The initial strategy to counteract the effects of these β -lactam-hydrolysing enzymes was to synthesise β -lactamase-stable antibiotics [9]. In the mid-1970s,

with the unexpected appearance of the *bla*_{TEM} gene in *Neisseria gonorrhoeae* [10–12] and *Haemophilus influenzae* [13], pharmaceutical scientists began to search for effective inhibitors of not only the TEM β -lactamases but also the penicillinases produced by staphylococci and the chromosomal AmpC cephalosporinases found in many Enterobacteriaceae [14]. Effective inhibitors of the first two sets of enzymes were identified and developed, i.e. clavulanic acid [14], sulbactam [15] and tazobactam [16], but they were not sufficiently active against the AmpC cephalosporinases to provide clinical utility against AmpC-producing bacteria.

Today, infections caused by multidrug-resistant Gram-negative bacteria are some of the most distressing challenges faced by infectious diseases clinicians. Most of these organisms harbour β -lactamase genes together with resistance determinants for other antibiotic classes, resulting in multidrug resistance or pandrug resistance [17]. Various groups such as the US Centers for Disease Control and Prevention (CDC) in the USA and the British Society for Antimicrobial Chemotherapy (BSAC) in the UK have targeted these organisms as urgent threats that require significant

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Table 1

β -Lactamase nomenclature. Representative functional groups with key enzymes relevant to this review are included in this table.

Molecular class ^a	Functional group ^b	Trivial name	Examples of families of enzymes
A	2a	Penicillinase	Staphylococcal enzymes
	2b	Broad-spectrum penicillinase	TEM, SHV
	2be	ESBL	TEM, SHV, CTX-M
	2br	Inhibitor-resistant β -lactamase	TEM, SHV
	2c	Carbenicillin-hydrolysing β -lactamase	CARB
	2e	Clavulanic acid-inhibited cephalosporinase	CepA
	2f	Serine carbapenemase	KPC, IMI, SME
B	3	Metallo- β -lactamase (MBL, carbapenemase)	IMP, NDM, SPM, VIM
C	1	Cephalosporinase	AmpC
D	2d	Cloxacillinase (may include ESBL or carbapenemase activity)	OXA

ESBL, extended-spectrum β -lactamase.

^a Structural classification according to Ambler [7].

^b Functional classification according to Bush, Jacoby and Medeiros [8].

breakthroughs in new therapeutic approaches [18,19]. Although a variety of non- β -lactam agents are being studied in attempts to address drug-resistant Gram-negative pathogens, numerous investigational approaches involve both established and new β -lactamase inhibitors (BLIs) in novel combinations [20]. The search for new inhibitor classes has been prompted in part by the increase in the number of β -lactamases, which have grown by at least an order of magnitude since the introduction of clavulanic acid [21]. During this time, new β -lactamase families have emerged with properties that make them more resistant to clavulanic acid and the sulfone inhibitors, thus encouraging the development of new approaches involving β -lactams. In this review, the most recent BLIs will be described, updating some recent reviews in this area [20,22–26] by emphasising literature from the past 2 years, including epidemiological information, pharmacodynamic studies, resistance identification and current clinical status.

2. β -Lactamase inhibitors

2.1. History

Clavulanic acid was identified in 1976 from a *Streptomyces clavuligerus* fermentation broth as a BLI that inactivated the common β -lactamases of that time: the class A TEM penicillinase; the P99 class C cephalosporinase; the class A penicillinase K1; and the class A staphylococcal PC1 penicillinase [27]. It was developed in combination with amoxicillin as an oral product and with ticarcillin to treat serious hospital infections. Penicillanic acid sulfones, semi-synthetic BLIs developed in the 1980s, included sulbactam [15] and tazobactam [16]. These suicide inactivators had an inhibitory spectrum and mechanism of inhibition that mirrored clavulanate. In comparative studies, tazobactam and clavulanic acid had comparable potencies, with sulbactam generally a weaker inhibitor [28] due in part to its greater lability to hydrolysis by the enzymes it eventually inactivated. Tazobactam was combined with piperacillin, and sulbactam was developed in combination with ampicillin in most of the world, although a cefoperazone/sulbactam combination was used in the Asia-Pacific region. None of these inhibitors had useful antibacterial activity on their own against staphylococci or the Enterobacteriaceae; however, clavulanic acid inhibited *N. gonorrhoeae* with a minimum inhibitory concentration (MIC) of ≤ 5 mg/L [29], and sulbactam unexpectedly had antibacterial activity at ≤ 4 mg/L against many strains of *Acinetobacter* spp. [30].

2.2. Non- β -lactam serine β -lactamase inhibitors

2.2.1. Diazabicyclooctane inhibitors

Avibactam (AVE1330A, NXL-104; Fig. 1a) was the first member of the bridged diazabicyclo[3.2.1]octanone (DBO) non- β -lactam

BLIs to be developed for therapeutic use [32]. Key β -lactamases such as TEM-1, KPC-2 and AmpC from *Pseudomonas aeruginosa* were inhibited more effectively by avibactam than by clavulanic acid or the sulfone inactivators, with 50% inhibitory concentrations (IC_{50} values) often at least an order of magnitude lower for avibactam [33]. IC_{50} values for avibactam were 8 nM for TEM-1 and 130 nM for AmpC, compared with values of 58 nM and $>100,000$ nM for clavulanic acid, and 32 nM and 1300 nM for tazobactam, respectively [34]. The KPC-2 β -lactamase was inhibited by avibactam with an IC_{50} of 170 nM compared with $>100,000$ nM for clavulanic acid and 50,000 nM for tazobactam.

Although several variations of the inhibitory mechanism have been presented in the literature [34–38], the consensus supports a reversible, covalent mechanism with release of intact avibactam for most class A and class C serine β -lactamases [23,35]. Partition ratios of 1:1 for most inhibited enzymes indicated no turnover of the inhibitor, supporting this mechanism [34,35]. Later studies showed that the class D OXA-10 enzyme was only slowly acylated by avibactam, but was also slowly deacylated [39]. X-ray crystallographic studies of avibactam with OXA-24 and OXA-48 further delineate this mechanism [40]. As a result of this release of intact avibactam by most serine β -lactamases, enzymes can recycle the inhibitor among multiple β -lactamases that may be present, leading to greater inhibitory efficiency than clavulanic acid or tazobactam that undergo multiple hydrolytic reactions before their enzyme targets are inactivated. In contrast, the KPC-2 serine carbapenemase provides an enzymatic exception to this reversible inhibitory reaction. KPC-2 was rapidly acylated by avibactam with a half-time for deacylation of 82 min [39], but was unique among the β -lactamases studied in that this slow deacylation of the acyl-avibactam complex resulted in hydrolysis and fragmentation of the avibactam molecule [39].

Relebactam (MK7655; Fig. 1b) is a piperidine analogue in the DBO series designed to have inhibitory activity against class A and class C β -lactamases [41]. IC_{50} values for reversible covalent inhibition were 210 nM for KPC-2, 410 nM for AmpC from *P. aeruginosa* and 4100 nM for AmpC from *Acinetobacter baumannii*. The class D carbapenemase OXA-40 (OXA-24) was not inhibited at 50 μ M. Thus, relebactam will be most effective against organisms producing class A or class C enzymes.

RG6080 (OP0595, FPI-1459; Fig. 1d) is a DBO with a unique set of mechanisms [42]. It effectively inhibited class A and class C β -lactamases, with IC_{50} values of <30 nM for class A enzymes such as TEM-1 and CTX-M-14/15 and <1 μ M for KPC-2 and *P. aeruginosa* AmpC. RG6080 was 3–13-fold less potent than avibactam against class A enzymes, but was slightly more potent against class C cephalosporinases. Weak inhibitory activity was seen against class D enzymes, with IC_{50} values ranging from 3.0 μ M for OXA-1 to 46 μ M for OXA-23. IMP-1, a class B metallo- β -lactamase (MBL),

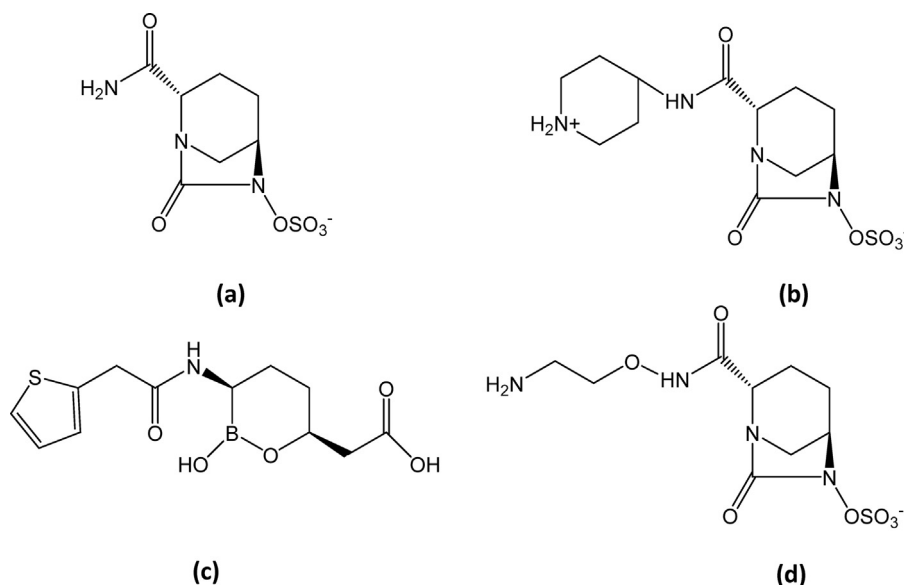


Fig. 1. Inhibitors of serine β -lactamases: (a) avibactam [33]; (b) relebactam [31]; (c) RPX7009 [131]; and (d) RG6080 [42].

was not effectively inhibited at 300 μM . When RG6080 was co-crystallised with either CTX-M-44 or AmpC, covalent binding to the active-site serine was observed [42], similar to avibactam [36].

A more intriguing aspect of RG6080 that sets it apart from avibactam is its strong binding to penicillin-binding protein 2 (PBP2) in *Escherichia coli*, with an IC_{50} value of 0.12 mg/L compared with a value of 0.49 mg/L for avibactam. The PBP binding for RG6080 results in potentially useful microbiological activity, with MICs of 1–8 mg/L for 14 enteric bacterial strains represented by eight species, compared with avibactam MICs of 8 mg/L to >32 mg/L for the same organisms [42]. Non-fermentative bacteria, some Proteae and *Serratia* strains were not inhibited at concentrations exceeding 32 mg/L. In further testing, this PBP2 inhibitor enhanced the activity of β -lactams that preferentially bind to PBP3 in enteric bacteria. This enhancer activity was also seen in strains resistant to RG6080 through non-PBP mutations and was hypothesised to be a property independent of PBP-binding activity [42].

2.2.2. Boronic acid inhibitors

Interactions of boronic acid inhibitors with serine proteases [43] provided the basis for the study of similar interactions with β -lactamases. In the 1980s, Waley's group described the inhibition of serine β -lactamases by various boronic acids [44,45]. Other investigators identified more potent non- β -lactam boronic acid inhibitors with K_i values in the nanomolar range for AmpC [46,47].

RPX7009 (Fig. 1c) is a novel cyclic boronic acid inhibitor of many class A, class C and some class D β -lactamases. This reversible inhibitor was designed in silico to interact favourably with serine β -lactamases and was shown to have high selectivity for β -lactamases compared with serine proteases [48]. RPX7009 was optimised based on its activity against the KPC class A carbapenemases with the intention of using it to potentiate the microbiological activity of carbapenems.

2.3. Metallo- β -lactamase inhibitors

2.3.1. Metal ion chelators

Selective MBL inhibitors have been an unattainable goal of many drug discovery teams. None of the current or investigational inhibitors described above can prevent β -lactam hydrolysis by the MBLs that include all β -lactam classes in their spectrum, with the exception of monobactams [8]. The most common

approaches to inhibiting these enzymes have utilised chelating groups to sequester the active-site zinc atom(s). Although effective MBL inhibitors have been identified from either natural product sources or from directed chemical synthesis, the most common feature of these inhibitors involves zinc chelation. As a result, inhibition of mammalian zinc-containing enzymes is also a common side effect, resulting in potent inhibition of enzymes such as angiotensin-converting enzyme, alcohol dehydrogenase or mammalian carboxypeptidases [49]. Three varied examples of MBL inhibitors that act as chelating agents are discussed below, although it is questionable whether any of these will be developed for clinical use, either due to lack of potency or lack of selectivity.

ME1071, a maleic acid derivative (Fig. 2a), was studied by Meiji as a means of inhibiting the IMP, NDM and VIM MBLs [50,51]. K_i values of 0.41 μM and 120 μM were determined for IMP-1 and VIM-2, respectively. At a recommended testing concentration of 32 mg/L, ME1071 could potentiate the activity of carbapenems against many MBL-producing *P. aeruginosa* strains [50] and demonstrated efficacy in a pseudomonal pneumonia model when ME1071 was combined with biapenem at doses of 100 mg/kg [52]. Because such high drug concentrations are necessary for synergy and for efficacy, potential side effects may be a concern.

Recently the natural product aspergillomarasmine A (Fig. 2b) was discovered in a cell-based screen to detect inhibitors of the NDM-1 carbapenemase [53]. Time-dependent, irreversible inhibition of the MBLs NDM-1 and VIM-2 was observed at micromolar concentrations, whilst inhibition of other MBLs (AIM, IMP and SPM-1) was poor, indicating mechanistic selectivity. When tested at 8 mg/L, the inhibitor potentiated meropenem activity against a panel of meropenem-resistant Enterobacteriaceae and *P. aeruginosa* isolates. A combination of meropenem with the inhibitor (10 mg/kg each) provided >95% protection from death in mice infected with a sublethal dose of an NDM-1-positive *Klebsiella pneumoniae* strain. Pharmacological studies that are in progress to assess toxicological properties are critical for further development, based on the history of the molecule. This Zn^{2+} chelator, and related analogues, were originally isolated from natural product screens in the 1980s as inhibitors of the angiotensin-converting enzyme [54,55]. In addition, aspergillomarasmine A is the toxin responsible for net-spot blotch disease of barley [56]. However, despite structural similarities to ethylene diamine tetra-acetic acid (EDTA),

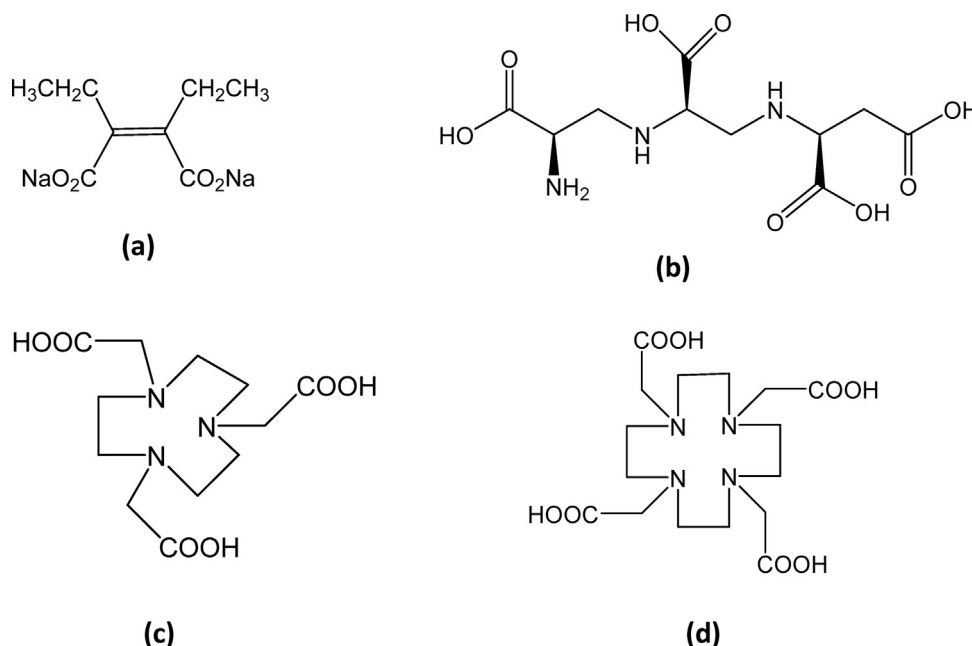


Fig. 2. Inhibitors of metallo- β -lactamases: (a) ME1071 [50]; (b) aspergillomarasmine [53]; (c) NOTA [58]; and (d) DOTA [58].

it is ca. five times less toxic [57], potentially providing a greater therapeutic window.

NOTA (Fig. 2c) and DOTA (Fig. 2d), cyclic tri- and tetra-acetic acid chelators, have been previously used to radiolabel proteins for tracking in cancer cells. They were recently reported to inhibit MBLs even more potently than aspergillomarasmine A [58] and to potentiate the activity of imipenem and meropenem. NOTA at 4 mg/L was more effective at lowering carbapenem MICs than DOTA when tested at 32 mg/L or 64 mg/L. However, no selectivity data were provided for inhibition of mammalian zinc enzymes.

3. Recently approved inhibitor combinations

3.1. Ceftolozane/tazobactam

Ceftolozane/tazobactam is a combination of a novel antipseudomonal 3'-aminopyrazolium cephalosporin and a safe, well-accepted BLI. The cephalosporin (also known as FR264205 or CXA-101) is notable for its potent activity against *P. aeruginosa*, with MIC₉₀ values initially reported as ≤ 4 mg/L for wild-type strains as well as for collections including multidrug-resistant isolates [59,60]; addition of tazobactam did not lower ceftolozane MICs against this organism [61]. More recent collections of *P. aeruginosa* demonstrated greater variation in susceptibility to the ceftolozane/tazobactam combination depending upon geographical location, with an MIC₉₀ of 2 mg/L for two sets of US *P. aeruginosa* strains ($n=3228$) [62,63] and MIC_{50/90} values of 1/ >32 mg/L for a set of 2191 European *P. aeruginosa* isolates [64]. Note that all MIC values were determined with a tazobactam concentration of 4 mg/L. Ceftolozane MICs were lowered in the presence of tazobactam when tested against ceftazidime-resistant Enterobacteriaceae, but MIC₉₀ values were ≥ 16 mg/L, indicating hydrolysis by extended-spectrum β -lactamases (ESBLs) [61]. The combination, however, retained high potency against *E. coli* isolates that produced only the CTX-M-14 or CTX-M-15 ESBL, the most common ESBLs globally, with ceftolozane MIC_{50/90} values of $\leq 0.25/0.5$ mg/L [65].

Pharmacokinetic/pharmacodynamic (PK/PD) studies in mouse thigh infection models identified the time the serum concentration exceeds the MIC ($T > \text{MIC}$) as the primary pharmacodynamic

index driving efficacy for ceftolozane. For wild-type Enterobacteriaceae producing ESBLs, $T > \text{MIC}$ was 31.1% for a static effect and 34.8% for a 1 log₁₀ kill. For *P. aeruginosa* a static effect was seen when $T > \text{MIC}$ was 24.0% and a 1 log₁₀ kill when $T > \text{MIC}$ was 31.5% [66]. When the pharmacodynamics of the ceftolozane/tazobactam combination was studied in vitro, a threshold concentration was identified for tazobactam as 0.25 mg/L for organisms with low and moderate β -lactamase production and 0.5 mg/L for organisms with high enzyme production levels [67]. When this threshold was used to study three CTX-M-15-producing *E. coli* strains, the magnitudes of % Time $>$ threshold associated with bacterial growth inhibition were 35% for a stasis dose and 50% for a dose that resulted in a 1 log₁₀ decrease in bacterial count.

Ceftolozane/tazobactam was studied to determine its penetration into lung tissue in a murine pharmacokinetic study as a preliminary evaluation of the combination for treatment of experimental pneumonia. Pharmacokinetic studies demonstrated higher relative concentrations of tazobactam in the epithelial lining fluid (ELF) than for ceftolozane. When the drugs were dosed at 8 mg/kg or 32 mg/kg in the mouse model, ELF levels as ratio of serum concentrations were 0.34 and 0.31 for ceftolozane and 0.60 and 0.83 for tazobactam, respectively, suggesting that sufficient drug levels may be present to be able to treat bacterial lung infections [68].

The ceftolozane/tazobactam combination was recently approved in the USA for the treatment of complicated intra-abdominal infections (cIAIs) when used in combination with metronidazole and for complicated urinary tract infections (cUTIs), including pyelonephritis [69]. The combination was non-inferior to meropenem in the primary endpoints for the randomised, double-blind phase 3 cIAI trial [70]. In this trial, cure rates were 100% (13/13) and 72.7% (8/11) for infections caused by Enterobacteriaceae producing CTX-M-14 and CTX-M-15 ESBLs, respectively. In the phase 3 cUTI trial, the combination was superior to levofloxacin for composite cure [71]. Ceftolozane/tazobactam may be expected to show efficacy in the treatment of pneumonia based on its ca. 2-fold better penetration into pulmonary tissue compared with piperacillin/tazobactam [72]. Phase 3 clinical studies are currently in progress comparing ceftolozane/tazobactam to meropenem in the treatment of ventilator-associated pneumonia [73].

Resistance to the ceftolozane/tazobactam combination has been identified in several in vitro studies. Unsurprisingly, resistance to ceftolozane in clinical isolates may occur through hyperproduction of the pseudomonal AmpC cephalosporinase that confers resistance to other antipseudomonal cephalosporins [74,75]. In one study, 20 variants of the AmpC enzyme were inserted into an AmpC-negative *P. aeruginosa* strain, resulting in ceftolozane MICs that increased up to 64-fold [74]. Mutations that affected the cephalosporin activity were mapped to the Ω loop, the R2 domain and the C-terminal end of the protein, as well as two new mutations (F121L and P154L) that may result in increasing the size of the substrate binding pocket. Resistance selection studies in vitro showed that ceftolozane/tazobactam-resistant mutants of *P. aeruginosa* arose more slowly than mutants resistant to ceftazidime, meropenem or ciprofloxacin [76]. High-level resistance to ceftolozane (MICs > 16 mg/L) was observed only when the selecting strain was a mismatch-repair-deficient ($\Delta mutS$) mutator derivative of *P. aeruginosa* PAO1. Strains with moderate resistance carried a high fitness cost; the mutations were not associated with common β -lactam resistance mechanisms but global pleiotropic mutations. As seen in previous studies, mutations associated with *ampC* were found in the highly resistant strains selected from the mutator strain [76]. Further clinical experience will determine whether these laboratory observations will be observed in clinical isolates.

3.2. Ceftazidime/avibactam

Ceftazidime/avibactam has demonstrated potent inhibitory activity against most Enterobacteriaceae that produce class A and class C β -lactamases, with some inhibitory capabilities against organisms producing class D enzymes [77]. Recent surveillance studies support the earlier observations of synergistic activity, whereby the addition of 4 mg/L avibactam to ceftazidime can lower MICs as much as 2048-fold against enteric bacteria [78]. In vitro testing of all avibactam combinations discussed below utilised 4 mg/L of the inhibitor. When avibactam was added to ceftazidime, ceftazidime MIC₉₀ values were ≤ 4 mg/L for collections of isolates that included ceftazidime-resistant ESBL-producing isolates, serine carbapenemase-producing strains and cephalosporinase-producing enteric bacteria from the USA, the European and Mediterranean region, Latin America and the Asia-Pacific/South Africa regions [78–86]. Against multiple collections of recent *P. aeruginosa* isolates, MIC₉₀ values were usually ≤ 8 mg/L for strains from a variety of body sites and infections from Chinese, European and US patients [82–88]. An exception was a study that reported 81% of the *P. aeruginosa* UTI isolates collected in 2011 from patients in Europe and the Mediterranean region had ceftazidime/avibactam MICs of ≤ 8 mg/L [84]. *Acinetobacter* spp. remained uninhibited by the combination, with MICs generally exceeding 16 mg/L [82]. Notably, a retrospective study of clinical isolates from 2009 showed that avibactam restored ceftazidime MICs to their epidemiological cut-offs (ECOFFs) both for Enterobacteriaceae and *P. aeruginosa* [89].

Because KPC-producing enteric bacteria pose an extreme clinical threat, studies with avibactam have emphasised the microbiology of the avibactam combinations against a variety of KPC-producing strains. The Kreiswirth laboratory recently reported that the ceftazidime/avibactam combination lowered ceftazidime MICs to ≤ 4 mg/L [one dilution less than the Clinical and Laboratory Standards Institute (CLSI) breakpoint for susceptibility] against 72 KPC-producing *K. pneumoniae* strains with diverse mechanisms of resistance, including various combinations of KPC subtypes with ESBLs and/or OmpK36 mutations [90]. Notably, the median ceftazidime MICs were higher for KPC-3-producing strains than for KPC-2-producing strains ($P=0.02$).

Resistance to ceftazidime/avibactam has been studied both in historical isolates and in laboratory mutants, with multiple mechanisms responsible for resistance, based on the organism and enzyme that was studied. Resistance due to production of MBLs is the most common mechanism among natural populations of Enterobacteriaceae [80]. In laboratory studies, a set of isogenic *E. coli* strains containing engineered KPC variants showed that mutations at S130 as well as K234 and R220 could contribute to resistance to inhibition by avibactam, but that the mutations S140G, K234R and R220M would be unlikely to be clinically relevant because those enzymes did not confer resistance to ceftazidime [91]. Interestingly, a similar set of isogenic *E. coli* strains producing SHV variants showed that the SHV-1 enzyme with S130G exhibited the greatest resistance to avibactam inhibition, thus implicating Ser130 in the avibactam inhibitory mechanism for class A β -lactamases [92]. The rate of resistance selection among Gram-negative organisms, including *P. aeruginosa*, has generally been low when the combination was used as the selecting agent [93]. When the ceftazidime/avibactam combination was used to select resistance in KPC-3-producing *K. pneumoniae* or *Enterobacter cloacae*, the most frequent KPC-3 variants had mutations in the Ω loop (Asp179Tyr). When resistance to the combination was observed in historical pseudomonal strains, the most common mechanisms were related to efflux and permeability issues [94]. In *P. aeruginosa* strains with derepressed AmpC production, ceftazidime/avibactam selected for resistance primarily due to AmpC mutations, frequently as a result of deletions in the Ω loop region [93]. In *K. pneumoniae* KPC-3 variants selected by the combination, ceftazidime/avibactam resistance was often accompanied by increased susceptibility to cefepime and the carbapenems (meropenem MICs as low as 0.06 mg/L) in strains with Ω loop mutations [95]. Because ceftaroline/avibactam MICs were not affected to as great an extent as ceftazidime/avibactam, it was suggested that resistance was due to increased ceftazidime hydrolysis by the mutant enzymes rather than decreased affinity to avibactam.

Resistance to ceftazidime/avibactam has now been reported for a limited number of KPC-producing clinical isolates [80,96]. In two reports, *K. pneumoniae* with KPC-2 had elevated ceftazidime MICs when the strain also contained both ESBLs and porin mutations [90,96]. A highly resistant *K. pneumoniae* isolate with a ceftazidime/avibactam MIC > 32 mg/L produced not only KPC-2 but also CMY-2, TEM and SHV β -lactamases, together with the MBL VIM-4; in addition, the strain overexpressed the AcrAB–TolC efflux pump and had reduced expression of two porins (OmpK35 and OmpK37), thus explaining the extreme resistance [80]. In a recent KPC-producing isolate, wild-type KPC-3 alone was identified from a hospitalised patient who harboured multiple strains of *K. pneumoniae* with varying antibiotic susceptibilities; one strain had ceftazidime/avibactam MICs of 4/4 mg/L, whilst another strain had MICs for the combination of 32/4 mg/L [96]. The resistant isolate had no mutations in *bla*_{KPC}, nor was efflux the apparent cause for ceftazidime resistance. Further investigation is necessary to determine the cause for the elevated ceftazidime MICs.

In an unusual move in the antibacterial world, the US Food and Drug Administration (FDA) approved the ceftazidime/avibactam combination on the basis of phase 2 clinical trial data for treatment of cIAI used in combination with metronidazole and for treatment of cUTI, including pyelonephritis [96]. Although the approval was based on the medical need to treat resistant Gram-negative infections, the approval came with restrictions. Edward Cox, the director of the Office of Antimicrobial Products in the FDA Center for Drug Evaluation and Research, stated, 'It is important that the use of [ceftazidime–avibactam] be reserved to situations when there are limited or no alternative antibacterial drugs for treating a patient's infection.' The combination had been reviewed under the designation of a Qualified Infectious Disease Product (QIDP), a classification

resulting from the Generating Antibiotic Incentives Now (GAIN) Act that allows expedited review for antibacterial products that will potentially treat serious or life-threatening infections [97,98]. In addition to the limited clinical data available, the FDA approval was also based on the contribution of avibactam to ceftazidime efficacy in animal model infections and in vitro studies [98]. The combination has recently completed phase 3 clinical trials and is continuing to be studied in a trial comparing ceftazidime/avibactam versus meropenem in hospitalised adults with nosocomial pneumonia [73].

4. Combinations in clinical development

4.1. Avibactam combinations

4.1.1. Ceftaroline/avibactam

Ceftaroline fosamil, the prodrug of ceftaroline, is an agent approved by the FDA to treat acute bacterial skin and skin-structure infections and community-acquired bacterial pneumonia caused by susceptible bacteria, including methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) [99]. Because of potent binding to PBP2a in MRSA and other critical PBPs in MSSA and streptococci [100], ceftaroline has retained an MIC₉₀ against MRSA of ≤ 2 mg/L over the past decade [101,102]. However, it is susceptible to hydrolysis by many β -lactamases in Gram-negative pathogens [103,104]. Thus, a combination of ceftaroline with avibactam might provide an anti-MRSA cephalosporin that could be used to treat mixed infections caused by various β -lactamase-producing Gram-negative organisms. In vitro susceptibility testing of ceftaroline with avibactam has shown significant enhancement of ceftaroline microbiological activity against enteric bacteria, with ceftaroline MICs lowered as much as 4096-fold, with MIC_{50/90} values typically 0.06/0.12 mg/L and 0.12/1 mg/L for ESBL-positive and KPC-producing Enterobacteriaceae, respectively [78,88,105]. However, the addition of avibactam did not synergise ceftaroline activity in *P. aeruginosa* or *Acinetobacter* spp. where ceftaroline MIC₉₀ values exceed 32 g/L [106]. Ceftaroline fosamil in combination with avibactam has been studied in phase 1 trials for evaluation of the pharmacokinetics and safety of the two components [107,108], determination of mass balance [109], assessment of the impact of supratherapeutic doses on the QT/QTc interval of healthy individuals [110] and evaluation of the effect of the two components on normal human intestinal flora [111]. No unexpected adverse events were observed when avibactam was added to ceftaroline fosamil compared with the cephalosporin alone. A phase 2 trial comparing co-administered ceftaroline fosamil and avibactam to intravenous doripenem in adult subjects with cUTIs was completed in 2014 [73].

4.1.2. Aztreonam/avibactam

Aztreonam, the only monobactam approved for therapeutic use by the FDA, represents a unique opportunity for combination with a BLI. Although this monobactam is inherently stable to hydrolysis by broad spectrum β -lactamases (group 2b enzymes) and MBLs [8,112,113], it is readily hydrolysed by most ESBLs and KPC carbapenemases [114,115]. Thus, aztreonam MICs were often ≥ 16 mg/L when tested against enteric bacteria that produced ESBLs or KPC carbapenemases [78,116]. When avibactam was added to aztreonam against these same enteric strains, 99.7% of the aztreonam MICs were ≤ 4 mg/L, including strains producing a MBL [78,82,116,117]. Against a global collection of 23,516 Enterobacteriaceae isolates from 2012–2013, the aztreonam MIC_{50/90} values were 0.06/0.12 mg/L for the combination. Five Enterobacteriaceae isolates producing both a MBL and a KPC enzyme responded to the combination with MICs of ≤ 0.5 mg/L [78,116]. One *E. coli*

isolate producing VIM-1 with KPC-3 and TEM-1 was less susceptible to the combination, with an aztreonam MIC of 8 mg/L [78]. Aztreonam/avibactam was somewhat less effective when tested against resistant isolates of *P. aeruginosa*; the addition of avibactam caused at most a 2-fold decrease in aztreonam MIC₉₀ values to 32 mg/L or 64 mg/L [82,116]. Testing of the combination showed that 44% of 118 MBL-positive *P. aeruginosa* isolates were susceptible to aztreonam using CLSI breakpoints [116]. As seen with the other avibactam combinations, the aztreonam combination was ineffective against *A. baumannii* isolates, with aztreonam MIC_{50/90} values of 64 mg/L and >128 mg/L [116].

Aztreonam/avibactam phase 1 clinical trials have been completed [73]. Formation of a public–private partnership to develop the combination further in Europe was the topic of a call for proposals from the Innovative Medicines Initiative COMBACTE-CARE project New Drugs 4 Bad Bugs (ND4BB) in 2013 [118,119]. The purpose was to study the pharmacokinetics, safety, efficacy and tolerability of the combination in a phase 2 study for the treatment of cIAls and to conduct a prospective, randomised, comparative phase 3 study for the treatment of serious infections due to MBL-producing Gram-negative pathogens by aztreonam/avibactam [120].

4.1.3. Preclinical pharmacokinetics/pharmacodynamics of avibactam combinations

Pharmacodynamic studies have been conducted recently for all three avibactam combinations in various model systems. Avibactam in combination with either ceftazidime or aztreonam was studied in hollow-fibre models using various novel dosing regimens [121–123].

Ceftazidime:avibactam at a 4:1 ratio (1 g or 2 g of ceftazidime) was effective in suppressing the growth of eight strains of ceftazidime-resistant Enterobacteriaceae [122]; unexpectedly, all strains were rapidly killed with growth suppression for ≥ 10 h when ceftazidime was dosed as a continuous infusion and avibactam was given as a single bolus dose. In a hollow-fibre study with aztreonam and avibactam, fixed doses of each component were studied as a function of varying doses of the other component; the percentage of time avibactam exceeded a threshold concentration of 2–2.5 mg/L correlated with efficacy for all of the β -lactamase-producing strains studied [123]. Additional PK/PD studies in murine lung infection models have demonstrated comparable pharmacokinetic characteristics for ceftazidime and avibactam entry into ELF, supporting the study of the combination for the treatment of lung infections [124,125].

4.2. Imipenem/relebactam

The imipenem/relebactam combination has been studied primarily against Gram-negative pathogens, with an emphasis on carbapenemase-producing *E. coli*, *K. pneumoniae* and *Enterobacter* spp. [126,127]. Imipenem MICs were lowered as much as 64-fold when tested with relebactam at 4 mg/L against KPC-producing *K. pneumoniae* [126,127]. Potentiation of imipenem by relebactam was seen, albeit to a smaller extent, among Enterobacteriaceae that were imipenem-resistant due to AmpC or ESBL production in strains with permeability defects [126]. Little or no reduction in imipenem MICs was seen in OXA-48-producing *K. pneumoniae* [126] or OXA-23-producing *A. baumannii* [127] when 4 mg/L relebactam was added. Like the avibactam/cephalosporin combination, no synergy was observed with the combination when tested against MBL-producing pathogens. However, imipenem MICs were reduced from ≥ 16 mg/L to 2 mg/L in the presence of relebactam when imipenem-resistant *P. aeruginosa* isolates were tested [127], suggesting that the relebactam combination may serve to recover some of the utility of imipenem against this important pathogen.

Two pharmacodynamic studies have been conducted for the imipenem/relebactam combination. Although the efficacy of imipenem is related to the time the drug concentration exceeds a defined threshold [128], efficacy of the combination with relebactam (and cilastatin) is less well established. Hollow-fibre studies based on the results from mathematical models and computer simulations defined a novel pharmacodynamic index, the time above instantaneous MIC, or $T > MIC_i$ [129]. This study showed that different imipenem/relebactam dosing regimens correlated with comparable bacterial killing when $T > MIC_i$ was 69%. In a murine thigh model, the index defining the effect of relebactam was poorly related to the maximum serum drug concentration, but showed correlations that were similar for both $T > MIC$ (when relebactam was dosed at 4 mg/L) and area under the concentration–time curve (AUC)/MIC ratio [130]. Efficacy in dose-escalating studies in the thigh model was related to the imipenem MIC of the strain, the dose of imipenem/cilastatin and the AUC for the unbound fraction of relebactam (mean $fAUC$ of 26 mg h/L).

Clinical development has utilised the imipenem/cilastatin combination together with relebactam for phase 2 and phase 3 trials. This triple combination completed a phase 2 dose-ranging trial that studied the safety, tolerability and efficacy of the combination to treat cIAI [73], utilising two relebactam doses (125 mg and 250 mg) with a standard dose of 500 mg imipenem/cilastatin dosed intravenously every 6 h. Two randomised, double-blind, active comparator-controlled phase 3 clinical trials are being initiated. One is studying the efficacy and safety of the triple combination to treat imipenem-resistant bacterial infection compared with imipenem/cilastatin plus colistin, and the second is comparing the safety and efficacy of the combination against piperacillin/tazobactam for the treatment of pneumonia [73]. In these studies, imipenem/cilastatin and relebactam will be dosed in a 2:1 ratio. In the first study there is an option for an open-label arm for patients to be treated with the relebactam triple combination if pathogens are deemed to be resistant to both sets of study drugs [73].

4.3. Meropenem/RPX7009 (Carbavance™)

RPX7009 was first studied in combination with biapenem, a carbapenem that was never submitted for regulatory approval in the USA, although is currently being marketed in Japan [131]. An early study by Livermore and Mushtaq focused on the activity of the biapenem/RPX7009 combination against carbapenem-resistant Enterobacteriaceae [131]. In vitro studies using the inhibitor at concentrations of 4 mg/L or 8 mg/L showed that biapenem $MIC_{50/90}$ values could be lowered from 16/64 mg/L to 0.12/1 mg/L for organisms producing KPC carbapenemases [131,132]. Although good inhibitory activity was demonstrated against organisms with serine carbapenemases, RPX7009 did not further enhance the carbapenem activity against MBLs or OXA carbapenemases. More recent isolates from New York City were studied with the meropenem/RPX7009 combination [133]. In confirmation of the biapenem data, meropenem activity was considerably potentiated by RPX7009 against KPC-producing Enterobacteriaceae, with meropenem MICs lowered as much 512-fold by the addition of 4 mg/L or 8 mg/L of the inhibitor. However, there was little enhancement of the meropenem activity when tested against carbapenem-resistant *A. baumannii* or *P. aeruginosa*; only 2 of 84 and 6 of 98 meropenem-non-susceptible isolates, respectively, demonstrated a ≥ 4 -fold decrease in meropenem MICs when tested with 8 mg/L RPX7009 [133]. Of the *Acinetobacter* isolates, 58 of 84 produced an OXA-23-type carbapenemase, indicating that RPX7009 has little effect on this set of class D β -lactamases.

Clinical development is moving rapidly for the meropenem/RPX7009 combination. Following the successful

completion of phase 1 clinical trials for biapenem/RPX7009, the meropenem/RPX7009 combination proceeded directly into phase 3 clinical trials to evaluate the safety, efficacy and tolerability for treatment of (i) adults with cUTIs or acute pyelonephritis compared with piperacillin/tazobactam, with a follow-up of oral levofloxacin and (ii) adults with selected serious infections due to carbapenem-resistant Enterobacteriaceae compared with the best available therapy [73].

4.4. Cefepime/AAI101

Limited information is available about the combination of cefepime with the novel BLI AAI101 [134,135]. This penicillanic acid sulfone is described as an ESBL inhibitor with activity against selected class A and class D enzymes, including KPC and OXA-48 carbapenemases [136,137]. The addition of AAI101 (8 mg/L) to cefepime when tested against a set of 223 cefepime-non-susceptible Enterobacteriaceae containing ESBLs and carbapenemases resulted in a shift of the cefepime $MIC_{50/90}$ values from $>64/>64$ mg/L to 0.12/32 mg/L [134,135]. The cefepime/AAI101 combination has entered clinical development [135].

5. Other inhibitors and inhibitor combinations

5.1. RG6080

The PBP2-binding DBO RG6080, which has successfully completed phase 1 clinical trials by Fedora/Meiji, is being developed by Roche for entry into phase 2 studies. At the time of writing, no companion β -lactam had been identified to serve as the partner in this BLI combination. MICs for piperacillin, cefepime and ceftazidime were lowered as much as 16,000-fold when tested with 4 mg/L RG6080 against a panel of Gram-negative bacteria. MICs for these drugs were generally lowered 2- to 8-fold against *P. aeruginosa*. No enhancer activity was observed against Gram-positive bacteria or *A. baumannii* [42]. Little synergistic activity was seen when RG6080 was combined with the PBP2-binding meropenem, except when a serine carbapenemase was present. Based on the preliminary data that have been presented, it would appear scientifically justified to combine this inhibitor with an agent that binds to PBP3 such as piperacillin or the cephalosporins.

5.2. Cefepime/tazobactam

Tazobactam in combination with cefepime is being used primarily in India as a treatment for infections caused by Enterobacteriaceae [138,139]. In a recent Indian study of 500 consecutive non-duplicative isolates of *E. coli*, *K. pneumoniae*, *Enterobacter aerogenes* and *Proteus mirabilis*, a cefepime/tazobactam combination (inhibitor concentration was not specified) was more effective than piperacillin/tazobactam or cefoperazone/sulbactam, but was less effective than a carbapenem, especially when tested against *K. pneumoniae* [139].

6. Future perspectives

BLI combinations have provided major contributions to the anti-infective armamentarium. Amoxicillin/clavulanic acid has been relied upon in the paediatric oral antibacterial arena for years [140,141], whilst piperacillin/tazobactam has been a drug of choice for many nosocomial infections [142]. However, their usage has been compromised by the increase in the number of resistant Gram-negative pathogens that do not respond well to any β -lactam-containing molecules. Now that the number of unique,

naturally occurring β -lactamases exceeds 1800 (Bush, personal communication), it has become impossible to find a β -lactam molecule that will either inhibit or exhibit stability to every β -lactamase. Thus, the identification of novel BLIs with a broader spectrum of activity has been welcomed by the anti-infective community. The regulatory approval of ceftolozane/tazobactam allows a potent antipseudomonal cephalosporin to be used in mixed infections where the CTX-M ESBLs could compromise its antimicrobial activity. Perhaps more significant was the rapid approval of ceftazidime/avibactam that can be used to treat infections caused by many of the multidrug-resistant enteric bacteria that had become untouchable by the β -lactam drugs. As a result of the GAIN Act

and QIDP designations, approval of this combination on the basis of limited phase 2 clinical data signals a positive change in the regulatory environment, where an urgent medical need is weighted heavily in risk:benefit evaluations, and bodes well for future investigational antibacterial agents.

Ceftazidime/avibactam is just the first of a series of investigational non- β -lactam inhibitor combinations with potent inhibitory activity against carbapenem-resistant Enterobacteriaceae. Each new combination in clinical development has distinctive qualities that may allow all of them to proceed through full development (Table 2). Although many promising inhibitor combinations are in development to treat carbapenem-resistant Gram-negative

Table 2
 β -Lactamase inhibitor combinations of interest.

Inhibitor	Accompanying β -lactam	Targeted β -lactamases ^a	Development phase	QIDP ^b designation?	Comments	References
Clavulanic acid	Amoxicillin	Group 2a, 2b	Approved by FDA, EMA	NA ^c	Oral combination frequently used for paediatric infections.	[141]
	Ticarcillin	Group 2a, 2b	Approved by FDA, EMA	NA	Discontinued in many parts of the world.	[143]
Sulbactam	Ampicillin	Group 2a, 2b	Approved by FDA, EMA	NA	Useful to treat <i>Acinetobacter</i> that are susceptible to sulbactam.	[30]
Tazobactam	Piperacillin	Group 2a, 2b	Approved by FDA, EMA	NA	Currently used to treat susceptible nosocomial bacterial infections.	[142]
	Ceftolozane	Group 2b, some ESBLs	Approved by FDA	Yes	Increasing resistance. Potent activity against ceftolozane-susceptible <i>Pseudomonas aeruginosa</i> . Susceptibility against <i>Escherichia coli</i> producing CTX-M-14 and CTX-M-15.	[62–65,70]
	Cefepime	Group 2a, 2b	Used in Asia	NA	Limited utility against ESBL- and carbapenemase-producing Enterobacteriaceae.	[139]
Avibactam	Ceftazidime	ESBLs, KPC	Approved by FDA (cUTI, cIAI). Phase 3 (NP/VAP)	Yes	Broad inhibitory activity against class A, class C and some class D β -lactamases, including OXA-48. Antipseudomonal activity due to ceftazidime activity.	[40,78–88]
	Aztreonam	ESBLs, KPC	Phase 2 (IMI, in progress)	Yes	Similar inhibitory activity as ceftazidime/avibactam against enteric bacteria. Stability of aztreonam to MBLs may provide utility against MBL-producing enteric bacteria. Weaker antipseudomonal activity than other combinations, except when MBLs are present.	[78,82,116,117]
	Ceftaroline	ESBLs, KPC	Phase 2	Yes	Similar inhibitory activity as the ceftazidime/avibactam combination. Anti-MRSA activity due to ceftaroline, but no useful antibacterial activity observed against non-fermenters.	[78,88,102,105]
Relebactam (MK7655)	Imipenem (+ cilastatin)		Phase 3, cUTI (in progress) Phase 2 cIAI (completed)	Yes	A DBO combination with a similar profile as ceftazidime/avibactam. Limited inhibition of Class D-producing bacteria. Antipseudomonal activity furnished primarily by imipenem.	[41,42]
RPX7009	Meropenem		Phase 3, CRE (in progress). Phase 3, cUTI (in progress)	Yes	Novel boronic acid inhibitor of class A carbapenemases. Rapid advancement into phase 3 trials.	[133]
AAI101	Cefepime	ESBLs	Phase 1 complete	No	Little published data are available.	[134,135]
RG6080 (OP0595, FPI-1459)	Unknown	ESBLs, KPC	Phase 1 complete	NA	A DBO that inhibits PBP2 in Gram-negative bacteria providing growth inhibition in addition to inhibitory activity against class A and class C β -lactamases. The partner β -lactam has not been identified.	[42]

FDA, US Food and Drug Administration; EMA, European Medicines Agency; ESBL, extended-spectrum β -lactamase; cUTI, complicated urinary tract infection; cIAI, complicated intra-abdominal infection; NP, nosocomial pneumonia; VAP, ventilator-associated pneumonia; IMI, Innovative Medicines Initiative; MBL, metallo- β -lactamase; MRSA, methicillin-resistant *Staphylococcus aureus*; DBO, diazabicyclo[3.2.1]octanone; CRE, carbapenem-resistant Enterobacteriaceae; PBP, penicillin-binding protein.

^a β -Lactamases that hydrolyse the accompanying β -lactam.

^b QIDP, Qualified Infectious Disease Product, as defined by the Generating Antibiotic Incentives Now (GAIN) Act in the USA. Yes, indicates the combination has been so designated.

^c NA, not applicable. Drug does not qualify or an application has not been filed.

pathogens, all of the drugs described in this review are parenteral agents. There is still a serious medical need to identify new combinations, or other drug classes, that can be dosed orally to eradicate these multidrug-resistant organisms. In addition, resistance to all these new combinations must be tracked carefully in the future. The fact that many of the inhibitors do not contain a β -lactam ring may mean that novel resistance mechanisms will arise. The effects on the partner β -lactam must be monitored closely, as early laboratory results suggest that both increased and decreased susceptibility to other β -lactams may occur. Only through additional clinical experience will the medical community be able to determine how rapidly the new inhibitors select for resistance in the clinical setting, and what the long-term consequences will be.

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