Activity of Imipenem, Meropenem, Cefepime, and Sulbactam in Combination with the β-Lactamase Inhibitor LN-1-255 against Acinetobacter spp.

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Abstract: Treatment of infections caused by Acinetobacter spp., particularly A. baumannii, is a major clinical problem due to its high rates of antibiotic resistance. New strategies must be developed; therefore, restoration of β-lactam efficacy through the use of β-lactamase inhibitors is paramount. Activities of the antibiotics imipenem, meropenem, cefepime, and sulbactam in combination with the penicillin-sulfone inhibitor LN-1-255 were tested by microdilution against 148 isolates of Acinetobacter spp. collected in 14 hospitals in Spain in 2020. Relevantly, the MIC90 (i.e., minimum concentration at which 90% of isolates were inhibited) of antibiotics in combination with LN-1-255 decreased 4- to 8-fold for all of the Acinetobacter isolates. Considering only the carbapenem-resistant A. baumannii isolates, which produce carbapenem-hydrolyzing class D β-lactamases, the addition of LN-1-255 decreased the resistance rates from 95.1% to 0% for imipenem, from 100% to 9.8% for meropenem, from 70.7% to 7.3% for cefepime, and sulbactam resistance rates from 9.8% to 0% and intermediate susceptibility rates from 53.7% to 2.4%. The inhibitor also decreased the minimum inhibitory concentrations (MICs) when tested against non-carbapenem-resistant Acinetobacter spp. isolates. In conclusion, combining LN-1-255 with imipenem, meropenem, cefepime, and sulbactam to target A. baumannii, and especially carbapenem-resistant isolates, represents an attractive option that should be developed for the treatment of infections caused by this pathogen.

Keywords: Acinetobacter spp.; Acinetobacter baumannii; β-lactam antibiotic resistance; β-lactamase inhibitors; LN-1-255; imipenem; meropenem; cefepime; sulbactam; carbapenem-hydrolyzing class D β-lactamases (CHDLs)

1. Introduction

Acinetobacter is a highly diverse genus comprising both human pathogens and environmental microorganisms. Regarding human pathogens, the Acinetobacter calcoaceticus-Acinetobacter baumannii (ACB) complex (A. calcoaceticus, A. baumannii, A. nosocomialis, and A. pittii) is the group of most concern in terms of clinical importance. A. baumannii...
is currently one of the most important nosocomial pathogens [1], known to be associated with life-threatening infections in immunocompromised hosts and in patients with severe underlying diseases. Treatment and eradication are increasingly challenging not only because of the intrinsically high resistance of this pathogen but also because of its natural propensity to develop multidrug resistance, including resistance to carbapenems, via the horizontal acquisition of broad-spectrum resistance mechanisms [2].

Carbapenems, like imipenem and meropenem, are the main therapeutic options available to treat serious infections caused by *A. baumannii*, partly due to the ability of these antibiotics to withstand modifications by the naturally produced β-lactamases of *A. baumannii* (OXA-51 and ADC-type cephalosporinases), as well as their good target-binding properties and favorable safety profile [3]. Cephalosporins such as cefepime, a zwitterionic cephalosporin with some degree of stability to hydrolysis mediated by ADC β-lactamases [4] and with enhanced bacterial cell penetration, may still remain useful. Likewise, sulbactam, a class A β-lactamase inhibitor with a high affinity for the *A. baumannii* penicillin-binding protein 2 (PBP2) [5] and intrinsically bactericidal against this pathogen, is another possible treatment option.

Treatment of infections caused by *A. baumannii* is particularly challenging because of the propensity of this species to develop antimicrobial resistance through horizontal acquisition and/or upregulation of intrinsic mechanisms, and very few therapeutic options are currently available. Thus, acquired carbapenem-hydrolyzing class D β-lactamases (CHDLs), such as OXA-23, OXA-24/40, OXA-58, OXA-143, or OXA-235, are of great concern as they represent the main threat to the use of carbapenems, the first-line antibiotics available for managing *A. baumannii* infections [6]. Reduced permeability of the outer membrane and active efflux are also involved in carbapenem resistance [7]. Resistance to cefepime in *A. baumannii* can be mediated by alteration of the outer membrane properties, the presence of horizontally acquired β-lactamases, and the production of extended-spectrum AmpC β-lactamases, such as ADC-56 [8–10]. Finally, sulbactam resistance in *A. baumannii* is probably multifactorial and has been related to the expression of *bla*OXA-23 [11], *bla*TEM-1D, increased production of ADC (fISAba1-ampC) [12], and reduced expression of PBP2 [13].

To date, β-lactamase inhibitors have been successfully used to restore the efficacy of β-lactam antibiotics for treatment of infections caused by β-lactamase-producing Gram-negative pathogens [14] (Figure 1). However, CHDLs produced by *A. baumannii* are recalcitrant to inhibition by classical (e.g., tazobactam, sulbactam, and clavulanate) or recent commercially available inhibitors (e.g., avibactam, relebactam, and vaborbactam) [15–17]. As a consequence, inhibition of CHDLs remains an unmet challenge regarding the use of β-lactams to treat severe *A. baumannii* infections. In this regard, the emergence of new broad-spectrum inhibitors, mainly durlobactam (formerly ETX2514), a 1,6-diazabicyclo[3.2.1]octane [18]; QPX7728, a cyclic boronate [19]; and LN-1-255, a penicillin sulfone derivative (all of which are able to block the most widespread CHDLs produced by *A. baumannii*) may represent a step forward in the fight against infections caused by β-lactam-resistant and, in particular, carbapenem-resistant *A. baumannii*.

LN-1-255 is a 6-alkyldiene-2′-substituted penicillin sulfone inhibitor with demonstrated activity against class A, class C, and class D β-lactamases [20,21] and against the carbapenem-hydrolyzing oxacillinases produced by *A. baumannii*. This inhibitor presents a catechol moiety responsible for effective internalization via bacterial iron uptake pathways. LN-1-255 is anchored in the active site of β-lactamases by strong electrostatic and hydrogen-bonding interactions between the sulfinate group of the inhibitor and diverse polar residues within the pocket of the enzymes [17].

In previous research, we observed that relative to tazobactam and avibactam, LN-1-255 yields significant in vitro inhibitory activity against isogenic *A. baumannii* strains carrying OXA-23, OXA-24/40, OXA-58, OXA-143, and OXA-235 CHDLs enzymes, displaying LN-1-255 approximately three logs higher affinity for CHDLs (*Kᵢ*) than comparators [22]. Murine pneumonia models were likewise used to test the in vivo performance of this penicillin
sulfone inhibitor, with promising results being obtained in terms of toxicity and reduction of the bacterial burden relative to imipenem monotherapy in mice [23].

1, 6-Diazabicyclo[3.2.1]octanes

![Images of Avibactam, Relebactam, and Durlobactam (ETX2514)]

Cyclic Boronates

![Images of Vaborbactam, Taniborbactam (VNRX-5133), and QPX7728]

Penicillin-based sulfones

![Images of Sulbactam, Tazobactam, and LN-1-255]

Figure 1. Selected examples of relevant β-lactamase inhibitors.

National and international surveillance studies are important for determining the in vitro activity of newly developed antimicrobials. These traditional approaches are useful to evaluate and control antimicrobial resistance trends and for guiding decisions regarding appropriate treatments. Nevertheless, LN-1-255 activity has not been tested with large collections of Acinetobacter spp. clinical isolates. Therefore, the aim of the present study was to evaluate whether LN-1-255 enhances (restores) the activity of imipenem, meropenem, cefepime, and sulbactam against a collection of Acinetobacter spp. clinical isolates recovered from 14 hospitals across Spain in 2020, in order to confirm the therapeutic potential of this β-lactamase inhibitor and to determine the best LN-1-255/antibiotic combination.
2. Results and Discussion

Antimicrobial susceptibility testing by reference broth microdilution was performed to determine the minimum inhibitory concentrations (MICs) for imipenem, meropenem, cefepime, and sulbactam alone or in combination with the inhibitor LN-1-255. Important differences in rates of resistance to the antibiotics tested were observed in relation to the *Acinetobacter* species and CHDLs production, as might be expected. Resistance rates to the four antibiotics were higher in *A. baumannii* strains carrying acquired CHDLs. For this reason, and for the purpose of simplicity, the results of this study are presented for the whole collection of isolates and separately for CHDL-producing *A. baumannii* isolates, non-CHDL-producing *A. baumannii*, and all of the non-*A. baumannii* isolates (which included isolates of *A. calcoaceticus*, *A. dispersus*, *A. nosocomialis*, *A. dijkshoorniae*, *A. ursingii*, *A. pittii*, *A. guillouiae*, *A. johnsonii*, and *A. bereziniae*).

2.1. Carbapenems/LN-1-255

Carbapenem resistance rates in the whole set of 148 clinical isolates of *Acinetobacter* spp. were 28.4% for imipenem and 35.1% for meropenem (following CLSI clinical breakpoints) and no important differences in MIC\(_{50/90}\) values were observed (MIC\(_{50}\) ≤ 0.5/16 and ≤ 0.5/32, respectively) (Tables 1 and 2).

### Table 1. Susceptibility of *Acinetobacter* spp. isolates to imipenem and imipenem/LN-1-255.

| Isolates                      | MIC (mg/L) | CLSI Category | MIC (mg/L) | CLSI Category |
|------------------------------|------------|---------------|------------|---------------|
|                              | IMIC\(_{50}\) | IMIC\(_{90}\) | Range      | %S | %I | %R | IMIC\(_{50}\) | IMIC\(_{90}\) | Range | %S | %I | %R |
| All isolates (n = 148)       | ≤0.5       | 16             | ≤0.5 to 32 | 64.9 | 3.8 | 28.4 | ≤0.5 | 2 | ≤0.5 to 4 | 91.2 | 8.1 | 0.0 |
| CHDL-producing *A. baumannii* (n = 41) | 16 | 32 | 4 to 32 | 0.0 | 4.9 | 95.1 | 2 | 4 | ≤0.5 to 4 | 68.3 | 31.7 | 0.0 |
| Non-CHDL-producing *A. baumannii* (n = 48) | ≤0.5 | 4 | ≤0.5 to 16 | 77.1 | 16.7 | 6.3 | ≤0.5 | 1 | ≤0.5 to 2 | 100 | 0.0 | 0.0 |
| Non-*A. baumannii* (n = 59) | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0.0 | 0.0 | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0.0 | 0.0 |

S, susceptible; I, intermediate; R, resistant.

### Table 2. Susceptibility of *Acinetobacter* spp. isolates to meropenem and meropenem/LN-1-255.

| Isolates                      | MIC (mg/L) | CLSI Category | MIC (mg/L) | CLSI Category |
|------------------------------|------------|---------------|------------|---------------|
|                              | IMIC\(_{50}\) | IMIC\(_{90}\) | Range      | %S | %I | %R | IMIC\(_{50}\) | IMIC\(_{90}\) | Range | %S | %I | %R |
| All isolates (n = 148)       | ≤0.5       | 32             | ≤0.5 to 64 | 64.9 | 0.0 | 35.1 | ≤0.5 | 4 | ≤0.5 to 16 | 81.1 | 16.2 | 2.7 |
| CHDL-producing *A. baumannii* (n = 41) | 32 | ≥64 | 8 to 64 | 0.0 | 0.0 | 100 | 4 | 4 | ≤0.5 to 16 | 41.5 | 48.7 | 9.8 |
| Non-CHDL-producing *A. baumannii* (n = 48) | ≤0.5 | 16 | ≤0.5 to 16 | 77.1 | 0.0 | 22.9 | ≤0.5 | 2 | ≤0.5 to 4 | 91.7 | 8.3 | 0.0 |
| Non-*A. baumannii* (n = 59) | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0.0 | 0.0 | ≤0.5 | ≤0.5 | ≤0.5 | 100 | 0.0 | 0.0 |

S, susceptible; I, intermediate; R, resistant.
Susceptibility to carbapenems was very different in the CHDL-producing A. baumannii subgroup \((n = 41)\), which mainly harbored \(\text{bla}_{\text{OXA-23-like}}\) \((n = 34, 82.92\%)\) (Table 3), than in the A. baumannii isolates lacking acquired CHDLs \((n = 48)\) and the non-A. baumannii isolates \((n = 59)\). None of the non-A. baumannii isolates were CHDL producers. None of the A. baumannii strains with acquired CHDLs were considered fully susceptible to imipenem or meropenem, to which 95.1% and 100% of the isolates were resistant, respectively. Among the non-CHDL-producing A. baumannii isolates, resistance rates decreased to 6.3% and 22.9% for imipenem and meropenem, respectively. No carbapenem-resistant isolates were detected in the non-A. baumannii species (Figures 2 and 3).

### Table 3. Identification of CHDLs between 41 CHDLs-producing A. baumannii.

| CHDLs     | Number of A. baumannii Isolates Producing CHDLs (%) |
|-----------|-----------------------------------------------------|
| OXA-23-like | 34 (82.92%)                                           |
| OXA-24-like | 0                                                    |
| OXA-58-like | 7 (17.08%)                                            |
| OXA-148-like| 0                                                    |
| OXA-235-like| 0                                                    |

![Figure 2](image-url) **Figure 2.** Comparative analysis of imipenem and imipenem/LN-1-255 MICs (mg/L): (A) All isolates \((n = 148)\), (B) CHDL-producing A. baumannii \((n = 41)\), (C) A. baumannii producing only OXA-51-like \((n = 48)\), and (D) non-A. baumannii \((n = 59)\).

Addition of LN-1-255 at a fixed concentration of 8 mg/L decreased the carbapenem resistance rates in all the A. baumannii strains tested (Tables 1 and 2). For the group of acquired CHDL-producing A. baumannii, imipenem and meropenem decreased the resistance rates from 95.1% to 0% and from 100% to 9.8%, respectively, decreasing the MIC\(_{50}\) and MIC\(_{90}\) 8-fold for imipenem and 8- and \(\geq 16\)-fold for meropenem, and thus indicating strong potentiation of the in vitro activity (Figures 2B and 3B). Importantly, in
the presence of the inhibitor, no carbapenem-resistant strains were detected among the group of CHDL-non-producing *A. baumannii*, with imipenem and meropenem MIC<sub>50/90</sub> values of ≤0.5/1 and ≤0.5/2 mg/L, i.e. 4- and 8-fold decreases in MIC<sub>90</sub>, respectively. The absence of resistance in the presence of LN-1-255 in the latter subset, without acquired CHDLs, could be explained by inhibition of the chromosomal OXA-51, which exhibits weak carbapenemase activity but can contribute to carbapenem resistance to some extent via overexpression mechanisms [8]. Finally, susceptibility to carbapenems was not greatly modified by the combination with the inhibitor in the 59 non-*A. baumannii* isolates as, in all cases, these strains were already fully susceptible to these antibiotics. Of note, the MICs of LN-1-255 alone (MIC > 512 mg/L) indicated that this compound did not exert antimicrobial activity against any of the *Acinetobacter* spp. strains evaluated.

![Figure 2](#)

**Figure 2.** Comparative analysis of imipenem and imipenem/LN-1-255 MICs (mg/L): (A) All isolates (n = 148), (B) CHDL-producing *A. baumannii* (n = 41), (C) *A. baumannii* producing only OXA-51-like (n = 48), and (D) non-*A. baumannii* (n = 59).

Studies showing the efficacy of inhibitors in recovering the susceptibility to carbapenems in a collection of CHDL-producing carbapenem-resistant *A. baumannii* are scarce. In a recent similar approach to restoring meropenem efficacy against carbapenem-resistant *Acinetobacter* spp., Nelson et al. studied the activity of the combination of the inhibitor QPX7728 (Qpex Biopharma, San Diego, CA, USA) and meropenem against a collection of carbapenem-resistant *A. baumannii* isolates. In a subset of genetically characterized *Acinetobacter* spp. expressing OXA-23 (MIC<sub>50/90</sub> of 64/64 mg/L for meropenem), the meropenem MIC<sub>50/90</sub> decreased 32- and 8-fold (i.e., 2/8 mg/L) after the addition of 8 mg/L of QPX7728 [24]. Our findings for the group of acquired CHDL-producing isolates displayed a similar increase in susceptibility, as the addition of LN-1-255 at 8 mg/L decreased the MIC<sub>50/90</sub> values 8- and ≥16-fold (i.e., 4/4 mg/L), respectively, relative to the values rendered by meropenem alone (Table 2).

Combinations of carbapenems and new β-lactamase inhibitors already in clinical use have also been evaluated in previous studies. Lod et al. and, later, Karlowsky et al.
published the results of research aimed at ascertaining the activity of imipenem/relebactam against Gram-negative ESKAPE pathogens isolated from patients in North American and European hospitals [25,26]. The findings showed promising results for both Enterobacterales and Pseudomonas aeruginosa, but failed to tackle resistance in A. baumannii. Similarly, a study of the new meropenem/vaborbactam combination revealed that it was very active against carbapenem-resistant Enterobacterales but that the activity was similar to that of meropenem alone against Acinetobacter spp. isolates [27]. Thus, the patent inability of these two recent commercially available β-lactamase inhibitors to overcome carbapenem resistance in Acinetobacter is underwhelming and, regrettably, exacerbates the urgent clinical need for effective compounds.

Our findings are consistent with those of previous studies testing the inhibition potential of LN-1-255 against A. baumannii. Susceptibility assays involving strains harboring the most common CHDLs in this species demonstrated the effectiveness of LN-1-255, successfully placing the carbapenem MIC below the resistance clinical breakpoints and, together with inhibition kinetics and docking assays, identifying LN-1-255 as a pan-inhibitor of all A. baumannii CHDLs [22]. Moreover, LN-1-255 was able to significantly reduce the bacterial load in the lungs of mice infected with carbapenem-resistant A. baumannii strains (carrying either OXA-23 or OXA-24/40) relative to imipenem monotherapy when administered at a dose of 50 mg/kg q3h [23]. Therefore, our results, not only restoring the susceptibility of imipenem but also meropenem in a collection of Acinetobacter spp. clinical isolates, add further evidence regarding the suitability of using LN-1-255 in the fight against Acinetobacter spp. and specifically carbapenem-resistant A. baumannii.

2.2. Cefepime/LN-1-255

In susceptibility assays, the cefepime MIC$_{50}$ and MIC$_{90}$ values for the 148 Acinetobacter spp. isolates were 2 and 32 mg/L, respectively (Table 4). As expected, A. baumannii harboring acquired CHDLs yielded the highest MIC$_{50/90}$ values (32/64 mg/L) and the highest rate of resistance (70.7%) to cefepime, followed by the group of A. baumannii isolates without CHDLs (2/16 mg/L, 6.3% of resistance) and the non-A. baumannii group ($\leq$1/4 mg/L), which did not include any cefepime-resistant isolates (Figure 4). The activity of the cefepime/LN-1-255 combination against the CHDLs-producing A. baumannii subset showed the greatest leap, with an 8-fold decrease in MIC$_{50/90}$ (4/8 mg/L) and a considerable reduction in resistance rates (from 70.3% to 7.3% in the presence of the inhibitor; Figure 4B). These parameters also decreased in the other two groups of isolates, although less dramatically (MIC$_{50/90}$ of 2/8 mg/L for A. baumannii without acquired CHDLs and $\leq$1/2 mg/L for non-A. baumannii isolates).

Table 4. Susceptibility of Acinetobacter spp. isolates to cefepime and cefepime/LN-1-255.

| Isolates | Cefepime | Cefepime/LN-1-255 |
|----------|-----------|------------------|
|          | MIC (mg/L) | CLSI Category | MIC (mg/L) | CLSI Category |
|          | MIC$_{50}$ | MIC$_{90}$ | %S | %I | %R | MIC$_{50}$ | MIC$_{90}$ | %S | %I | %R |
| All isolates ($n = 148$) | | | | | | | | | | |
| CHDL-producing A. baumannii ($n = 41$) | 2 | 32 | $\leq$1 to $\geq$128 | 66.2 | 12.2 | 21.6 | 2 | 8 | $\leq$1 to $\geq$128 | 95.9 | 1.4 | 2.7 |
| Non-CHDL-producing A. baumannii ($n = 48$) | 32 | 64 | 4 to $\geq$128 | 7.3 | 22.0 | 70.7 | 4 | 8 | $\leq$1 to $\geq$128 | 92.7 | 0.0 | 7.3 |
| Non-A. baumannii ($n = 59$) | 2 | 16 | $\leq$1 to $\geq$128 | 79.2 | 14.6 | 6.3 | 2 | 8 | $\leq$1 to 32 | 95.8 | 2.1 | 2.1 |

S, susceptible; I, intermediate; R, resistant.
Table 4. Susceptibility of Acinetobacter spp. isolates to cefepime and cefepime/LN-1-255.

| Isolates               | Cefepime | Cefepime/LN-1-255 |
|------------------------|----------|-------------------|
| All isolates (n = 148) | 2 to 32  | ≤1 to ≥128        |
|                        |          | 66.2%             |
|                        |          | 12.2%             |
|                        |          | 21.6%             |
|                        | 2 to 8   | ≤1 to ≥128        |
|                        |          | 95.9%             |
|                        |          | 1.4%              |
|                        |          | 2.7%              |
| CHDL-producing A. baumannii (n = 41) | 32 to 64 | 4 to ≥128        |
|                        |          | 7.3%              |
|                        |          | 22.0%             |
|                        |          | 70.7%             |
| Non-CHDL-producing A. baumannii (n = 48) | 2 to 16 | ≤1 to ≥128        |
|                        |          | 79.2%             |
|                        |          | 14.6%             |
|                        |          | 6.3%              |
| Non-A. baumannii (n = 59) | ≤1 to 4  | ≤1 to 1          |
|                        |          | 96.6%             |
|                        |          | 3.4%              |
|                        |          | 0.0%              |

S, susceptible; I, intermediate; R, resistant.

Figure 4. Comparative analysis of cefepime and cefepime/LN-1-255 MICs (mg/L): (A) All isolates (n = 148), (B) CHDL-producing A. baumannii (n = 41), (C) A. baumannii producing only OXA-51-like (n = 48), and (D) non-A. baumannii (n = 59).

Hyperproduction of chromosomal OXA-51-like, activation of efflux pumps, and changes in outer membrane porins hinder the efficacy of cefepime against Acinetobacter. In addition, the potential acquisition of other CHDLs, extended-spectrum β-lactamases (ESBLs), or metallo-β-lactamases (MBLs) [9,10] a priori rule out cefepime as a treatment option for these pathogens. Interestingly, the MIC of cefepime/LN-1-255 was ≤8 mg/L (the susceptibility breakpoint according to CLSI guidelines) for 38 out of 41 CHDL-producing A. baumannii and for 46 out of 48 A. baumannii isolates without acquired CHDLs (Figure 4). These findings thus suggest that inhibition of chromosomal OXA-51-like, acquired OXA-type carbapenemases, and, potentially, ESBLs [28] by LN-1-255 may lead to reappraisal of the use of cefepime for A. baumannii. Whole-genome sequencing studies will be conducted with the strains of this collection to determine the specific antimicrobial resistance mechanisms carried by isolates.

2.3. Sulbactam/LN-1-255

Differences in sulbactam MICs were also observed in the various Acinetobacter groups, as found during testing of other antibiotics. As Acinetobacter spp. are intrinsically resistant to ampicillin, mainly due to the chromosomal cephalosporinase, the CLSI breakpoints for the ampicillin/sulbactam combination are based on the bactericidal activity of sulbactam against this pathogen. For the whole set of isolates, the sulbactam MIC50/90 value was 0.5/8 mg/L. The highest MIC50/90 for the A. baumannii isolates was observed within the CHDL-producing subset (8/16 mg/L) relative to those lacking CHDLs (0.5/2 mg/L). However, the resistance rates for sulbactam were the lowest among the β-lactam antibiotics.
tested. Non-\textit{A. baumannii} representatives rendered MIC$_{50}$ and MIC$_{90}$ values of 0.5 and 1 mg/L, respectively (Table 5). When LN-1-255 was combined with sulbactam, the greatest impact on the MIC$_{50/90}$ was again observed in the group of CHDL-producing \textit{A. baumannii} (1/4 mg/L), with 8- and 4-fold decreases in the sulbactam MIC$_{50}$ and MIC$_{90}$ values, respectively. Thus, the rate of susceptibility to sulbactam increased from 36.6% to 97.6% when tested in the presence of the inhibitor (MIC susceptibility breakpoint \(\leq 4\) mg/L). No apparent improvement over the use of LN-1-255 was detected among the other \textit{Acinetobacter} spp. included in this study, for which low MICs of sulbactam (alone) were obtained, in all cases, below the clinical susceptibility breakpoints (Table 5).

Table 5. Susceptibility of \textit{Acinetobacter} spp. isolates to sulbactam and sulbactam/LN-1-255.

| Isolates               | Sulbactam | Sulbactam/LN-1-255 |
|------------------------|-----------|---------------------|
|                        | MIC (mg/L)| CLSI Category       | MIC (mg/L)   | CLSI Category |
|                        | MIC$_{50}$| MIC$_{90}$ Range    | %S | %I | %R | MIC$_{50}$| MIC$_{90}$ Range | %S | %I | %R |
| All isolates \((n = 148)\) | 0.5       | 8                   | \(\leq 0.25\) to \(\geq 32\) | 82.4 | 14.9 | 2.7 | 0.5       | 2                   | \(\leq 0.25\) to 8 | 99.3 | 0.7 | 0.0 |
| CHDL-producing \textit{A. baumannii} \((n = 41)\) | 8         | 16                  | 2 to \(\geq 32\) | 36.6 | 53.7 | 9.8 | 1         | 4                   | 0.5 to 8 | 97.6 | 2.4 | 0.0 |
| Non-CHDL-producing \textit{A. baumannii} \((n = 48)\) | 0.5       | 2                   | \(\leq 0.25\) to 4 | 100  | 0.0  | 0.0 | 0.5       | 2                   | \(\leq 0.25\) to 2 | 100  | 0.0  | 0.0 |
| Non-\textit{A. baumannii} \((n = 59)\) | 0.5       | 1                   | \(\leq 0.25\) to 1 | 100  | 0.0  | 0.0 | 0.5       | 0.5                 | \(\leq 0.25\) to 1 | 100  | 0.0  | 0.0 |

S, susceptible; I, intermediate; R, resistant.

As sulbactam remains of potential use, various attempts to assess its therapeutic value have been carried out: in non-life-threatening \textit{A. baumannii} [29] and \textit{A. calcoaceticus} infections [30], in \textit{A. baumannii} borne meningitis [31], and in pharmacodynamic in vitro modeling using human-simulated exposure [32]. In the present study, we observed that in the presence of LN-1-255, the sulbactam MIC values for \textit{Acinetobacter} spp. can probably be reduced to values that would yield therapeutic success in vivo, which would be particularly valuable against carbapenem-resistant isolates (Figure 5).

Studies of any new inhibitors that effectively decrease sulbactam resistance in strains of \textit{A. baumannii} are, again, very scarce. Probably the most remarkable example is the new 1,6-diazabicyclo[3.2.1]octane \(\beta\)-lactamase inhibitor durlobactam (formerly ETX2514, Entasis Therapeutics, Waltham, MA, USA) [33]. The sulbactam/durlobactam combination was tested to exploit the properties of sulbactam, thus bypassing resistance mediated by \(\beta\)-lactamasers through the addition of durlobactam. In a study using isolates of carbapenem-resistant \textit{A. baumannii} with different genetic backgrounds, addition of durlobactam at a concentration of 4 mg/L lowered the sulbactam MIC$_{50}$ and MIC$_{90}$ values 16- and 32-fold, respectively [34], placing them below the susceptibility breakpoint (\(\leq 4\) mg/L). Similar approaches were used to assess the susceptibility of \textit{A. baumannii} to sulbactam/durlobactam in mainland China, with similar results [35].

Our findings highlight the strong in vitro activity of the inhibitor LN-1-255 in combination with different \(\beta\)-lactams against the difficult-to-treat carbapenem-resistant CHDL-producing \textit{A. baumannii} isolates (Figure 6). When tested alone, the \(\beta\)-lactam antibiotics (imipenem, meropenem, cefepime, and sulbactam) were weakly active, but when combined with the inhibitor, the MICs decreased greatly. The best results were observed for carbapenems; the susceptible and intermediate rates for imipenem alone increased from 4.9% to 100% in the presence of LN-1-255, and for meropenem, it increased from 0% to 90.2%. However, LN-1-255 also greatly improved the activity of sulbactam, whose susceptibility
rates increased from 36.6% to 97.6% when in combination with the inhibitor, and cefepime, increasing the susceptibility from 7.3% to 92.7%.

Figure 5. Comparative analysis of sulbactam and sulbactam/LN-1-255 MICs (mg/L): (A) All isolates ($n = 148$), (B) CHDL-producing $A. \text{baumannii}$ ($n = 41$), (C) $A. \text{baumannii}$ producing only OXA-51-like ($n = 48$), and (D) non-$A. \text{baumannii}$ ($n = 59$).

Figure 6. Comparative analysis of the activity of imipenem, meropenem, cefepime, and sulbactam alone (A) or in combination with LN-1-255 (B) against the set of CHDL-producing $A. \text{baumannii}$ isolates.
3. Materials and Methods

3.1. Bacterial Isolates

Public hospitals in Spain were invited to participate in a nationwide survey of Acinetobacter spp. isolates, either from infected patients or as part of colonization studies, for prospective recovery during a 6-month period in 2020. Finally, 14 participated in the survey. Bacterial strains were frozen in Luria-Bertani (LB) broth with 15% glycerol and were maintained at –80 °C until analysis. The clinical microbiological laboratory in the Complexo Hospitalario Universitario A Coruña (A Coruña, Spain), a third-level academic hospital, acted as the reference laboratory.

3.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by broth microdilution in 96-well microdilution plates, with Mueller-Hinton II broth (Becton, Dickinson and Company, Sparks, MD, USA) and according to the CLSI reference guidelines. Minimum inhibitory concentrations (MICs) were determined for imipenem, meropenem, ceftazime, and sulbactam (Sigma, St. Louis, MO, USA) alone or in combination with the inhibitor LN-1-255 at a fixed concentration of 8 mg/L. LN-1-255 was synthesized at the Center for Research in Biological Chemistry and Molecular Materials (CIQUS, University of Santiago de Compostela, Santiago de Compostela, Spain), as previously reported [36]. The 2020 CLSI clinical breakpoints and guidelines (CLSI M100 ED30:2020) were used for interpretation [37]. We adopted a breakpoint of ≤4 mg/L for susceptibility (S), 8 mg/L for intermediate susceptibility (I), and ≥16 mg/L for resistance (R) to sulbactam alone (no breakpoints available), based on that on the ampicillin/sulbactam combination, reported by the CLSI (≤8/4 mg/L, S; 16/8 mg/L, I; and ≥32/16 mg/L, R).

3.3. Multiplex PCR Assay

Multiplex PCR was used to identify the oxacillinase-encoding genes expressed by the entire set of isolates, as previously described [38]. The primers used to identify \( \text{bla}_{\text{oxa-23-like}} \), \( \text{bla}_{\text{OXA-24/40-like}} \), \( \text{bla}_{\text{OXA-51-like}} \), \( \text{bla}_{\text{OXA-58-like}} \), \( \text{bla}_{\text{OXA-143-like}} \), and \( \text{bla}_{\text{OXA-235-like}} \) are listed in Table 6.

| Primer          | Sequence (5′-3′)                           | Product Size (pb) | Reference |
|-----------------|--------------------------------------------|-------------------|-----------|
| OXA-23likeFw    | GATCGGATTGGAGAACCAGA                      | 501               | [38]      |
| OXA-23likeRv    | ATTTCTGGACCCATTTCCAT                     |                   |           |
| OXA-24/40likeFw | GGTATGTTGCCCCCTTAAA                      | 246               | [38]      |
| OXA-24/40likeRv | AGTTGACGGGAAGGGGATAT                     |                   |           |
| OXA-51likeFw    | TAATGCTTTGATCCGCTTG                      | 353               | [38]      |
| OXA-51likeRv    | TGGATTGCACCTCATCTTG                      |                   |           |
| OXA-58likeFw    | AAGTATGCGGCTTTGGCTG                      | 599               | [38]      |
| OXA-58likeRv    | CCCCTCTGGCCGCTCATAAC                     |                   |           |
| OXA-143likeFw   | TACAAACTGAGATTTTCA                      | 390               | This study |
| OXA-143likeRv   | GGCTTACATCCATCC                        |                   |           |
| OXA-235likeFw   | ATGGGATGCGAAGAAGC                      | 239               | This study |
| OXA-235likeRv   | GAGGCAATTGACCTCT                      |                   |           |

4. Conclusions

In this study, we evaluated for the first time the efficacy of the penicillin sulfone LN-1-255 in combination with several β-lactam antibiotics against a collection of clinical isolates of Acinetobacter spp. isolated in a multicenter study. Our findings highlight the ability of the β-lactamase inhibitor LN-1-255 to restore the efficacy of imipenem and meropenem as first-line antibiotics in the fight against A. baumannii infections and identify this inhibitor as one of the very few in development that is able to block CHDLs produced by this bacterium.
Use of LN-1-255 also brings compounds such as cefepime and (notably) sulbactam back into play in the effort to diminish the selective pressure derived from overuse of carbapenems.

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