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SUSCEPTIBILITY

Assessment of Activity and Resistance Mechanisms to Cefepime in Combination with the Novel β-Lactamase Inhibitors Zidebactam, Taniborbactam, and Enmetazobactam against a Multicenter Collection of Carbapenemase-Producing *Enterobacterales*

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ABSTRACT The global distribution of carbapenemases such as KPC, OXA-48, and metallo-β-lactamases (MBLs) gives cause for concern, as these enzymes are not inhibited by classical β-lactamase inhibitors (BLIs). The current development of new inhibitors is one of the most promising highlights for the treatment of multidrug-resistant bacteria. The activity of cefepime in combination with the novel BLIs zidebactam, taniborbactam, and enmetazobactam was studied in a collection of 400 carbapenemase-producing *Enterobacterales* (CPE). The genomes were fully sequenced and potential mechanisms of resistance to cefepime/BLI combinations were characterized. Cefepime resistance in the whole set of isolates was 79.5% (MIC50/90 64/≥128mg/L). The cefepime/zidebactam and cefepime/taniborbactam combinations showed the highest activity ($MIC_{50/90}$ ≤0.5/1 and ≤0.5/2 mg/L, respectively). Cefepime/zidebactam displayed high activity, regardless of the carbapenemase or extended-spectrum β-lactamase (ESBL) considered (99% of isolates displayed MIC ≤2 mg/L). Cefepime/taniborbactam displayed excellent activity against OXA-48- and KPC-producing *Enterobacterales* and lower activity against MBL-producing isolates (four strains yielded MICs ≥16 mg/L: 2 NDM producers with an insertion in PBP3, one VIM-1 producer with nonfunctional OmpK35, and one IMP-8 producer). Cefepime/enmetazobactam displayed the lowest activity ($MIC_{50/90}$ 1/≥128 mg/L), with MICs ≥16 mg/L for 49 MBL producers, 40 OXA-48 producers (13 with amino acid changes in OmpK35/36, 4 in PBPs and 11 in RamR) and 25 KPC producers (most with an insertion in OmpK36). These results confirm the therapeutic potential of the new β-lactamase inhibitors, shedding light on the activity of cefepime and BLIs against CPE and resistance mechanisms. The cefepime/zidebactam and cefepime/taniborbactam combinations are particularly highlighted as promising alternatives to penicillin-based inhibitors for the treatment of CPE.

KEYWORDS β-lactamase inhibitors, antimicrobial resistance, carbapenemase-producing *Enterobacterales*, zidebactam, taniborbactam, enmetazobactam, cefepime, restoring antimicrobial activity, restoring antimicrobial efficacy

The main strategy to restore the effectiveness of β-lactam antibiotics is the use of β-lactamase inhibitors (BLIs). The global distribution of class A carbapenemases such as KPC, class B β-lactamases (metallo-β-lactamases [MBLs]) such as VIM, IMP, and NDM,

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and class D β-lactamases such as OXA-48, is a cause for concern because they are not inhibited by the classical inhibitors (1). After a period of no new significant advances in this area, several families of broad-spectrum inhibitors have emerged in recent years in the fight against bacterial multidrug resistance (2–4).

Three main families of compounds with inhibitory ability are attracting the attention of scientific and clinical societies: (i) the diazabicyclooctanes (DBOs), approved inhibitors in this group are relebactam and avibactam (5); (ii) boronic acid derivatives, within this group, vaborbactam has been approved (6, 7); and (iii) penicillin-based sulfones, such as the classical inhibitors (8). The next-generation β-lactamase inhibitors belonging to these families are zidebactam (WCK 5107, DBO), taniborbactam (VNRX-5133, boronate), and enmetazobactam (AAI101, penicillanic acid sulfone) (Fig. S1). The efficacy of these three compounds is currently being evaluated in combination with cefepime in phase III clinical trials with very promising results (9).

Cefepime's twice-a-day dosage schedule, enhanced activity against *Enterobacterales* and some Gram-positive organisms, and stability against AmpC give it several advantages over other cephalosporins and penicillins and allow its widespread use by physicians (10). However, cefepime can be hydrolyzed by extended-spectrum β-lactamases (ESBLs) and carbapenemases (with moderate resistance to hydrolysis by OXA-48). This important limitation calls for the search and development of new cefepime/BLIs combinations for use as carbapenem-sparing alternatives and also against carbapenemase-producing *Enterobacterales* (CPE) (9).

In recent years, several studies have evaluated the activity of new inhibitors in combination with cefepime against multidrug-resistant (MDR) pathogens (see review of Isler et al.) (9); however, the experimental comparison of the *in vitro* activity of the new β-lactamase inhibitors zidebactam, taniborbactam, and enmetazobactam has not yet been performed.

The objective of this study was to evaluate these novel combinations of cefepime with inhibitors, in phase III clinical trials, against a collection of 400 CPEs (304 OXA-48-producing, 44-KPC-producing, and 56-MBL-producing *Enterobacterales*), collected in a multicenter study of Spanish hospitals in 2018, in which treatment with carbapenems would not be the choice. Carbapenem-resistant *Enterobacterales* without carbapenemases were not included in the collection in order to evaluate the activity of new inhibitors against these enzymes. The genomes of whole collection have been fully sequenced and their mechanisms of resistance to β-lactam antibiotics characterized. The results of this study will help us to reaffirm the therapeutic potential of these new alternatives and the activity of cefepime combinations with zidebactam, taniborbactam, and enmetazobactam against CPE.

RESULTS AND DISCUSSION

Activity of cefepime/BLI combinations against carbapenemase-producing *Enterobacterales*. Given that cefepime/BLI breakpoints have not yet been established, to facilitate comparison and to evaluate the activity of new combinations, the cefepime breakpoints of ≤2 mg/L for susceptibility and ≥16 mg/L for resistance were adopted in this study. The cefepime resistance rate for the complete set of isolates evaluated in this study was 79.5%, $MIC_{50/90}$ 64/≥128 mg/L. Cefepime/zidebactam was the most active combination (99.0% inhibited at MIC ≤2 mg/L, $MIC_{50/90}$ ≤0.5/1 mg/L), followed by cefepime/taniborbactam and cefepime/enmetazobactam (90.0% and 61.8% inhibited at MIC ≤2 mg/L, $MIC_{50/90}$ ≤0.5/2 and 1/≥128 mg/L, respectively) (Table 1 and Table S1). The antimicrobial activity of the inhibitors alone also was determined. Taniborbactam and enmetazobactam alone did not show antimicrobial activity and all isolates showed MICs ≥256 mg/L (data not shown). In contrast, significant activity of zidebactam alone, which shows affinity for PBP2, was observed against most of the strains tested. In total, 73.3% of strains displayed a zidebactam MIC ≤1 mg/L with $MIC_{50/90}$ of ≤0.5/≥128 mg/L (Table 1).

We observed that the activity of the cefepime/BLI antimicrobial combinations varied significantly depending on the carbapenemase produced, as well as the presence/absence of ESBLs, as shown in Table 2 and Fig. S2. Cefepime/zidebactam was able to decrease MICs

TABLE 1 Cumulative MIC distribution of cefepime in the presence and absence of zidebactam, taniborbactam, and enmetazobactam in 400 carbapenemase-producing *Enterobacterales* strains

Isolate type	Cefepime/BLIs combinations[a]	Cumulative % of isolates at MIC (mg/L)									% isolates with MICs values	
		≤0.5	1	2	4	8	16	32	64	≥128	≤2 mg/L	≥16 mg/L
All isolates (n = 400)												
	Cefepime	5.5	12.3	15.0	18.8	20.5	25.5	38.3	52.5	100	15.0	79.5
	Cefepime/zidebactam	87.5	96.0	99.0	99.5	100					99.0	0
	Zidebactam	64.3	73.3	77.5	78.0	78.0	78.0	79.3	79.3	100	77.5	22.0
	Cefepime/taniborbactam	55.0	77.8	90.0	97.0	99.0	99.5	99.5	99.5	100	90.0	1.0
	Cefepime/enmetazobactam	36.0	50.3	61.8	67.8	72.5	79.3	83.5	86.5	100	61.8	27.5
All isolates												
No producing ESBLs (n = 106)	Cefepime	15.1	34.9	41.5	47.2	49.1	53.8	59.4	66.0	100	41.5	50.9
	Cefepime/zidebactam	76.4	95.3	100							100	0
	Zidebactam	63.2	78.3	84.9	84.9	84.9	84.9	85.8	85.8	100	84.9	15.1
	Cefepime/taniborbactam	51.9	73.6	88.7	96.2	99.1	100				88.7	0.9
	Cefepime/enmetazobactam	34.9	44.3	50.9	53.8	56.6	62.3	65.1	69.8	100	50.9	43.4
Producing ESBLs (n = 294)	Cefepime	2.0	4.1	5.4	8.5	10.2	15.3	30.6	47.6	100	5.4	89.8
	Cefepime/zidebactam	91.5	96.3	98.6	99.3	100					98.6	0
	Zidebactam	64.6	71.4	74.8	75.5	75.5	75.5	76.9	76.9	100	74.8	24.5
	Cefepime/taniborbactam	56.1	79.3	90.5	97.3	99.0	99.3	99.3	99.3	100	90.5	1.0
	Cefepime/enmetazobactam	36.4	52.4	65.6	72.8	78.2	85.4	90.1	92.5	100	65.6	21.8

[a]Taniborbactam and enmetazobactam did not show antimicrobial activity, displaying MICs ≥256 mg/L for all isolates.

to levels below the cefepime resistance breakpoint against all strains, regardless of the carbapenemase involved or the presence/absence of ESBLs. Cefepime/taniborbactam showed excellent activity against OXA-48-producing *Enterobacterales*; however, the rates of KPC- and- MBL-producing isolates showing low MICs (≤2 mg/L) to cefepime/taniborbactam were lower than those obtained with cefepime/zidebactam. Finally, cefepime/enmetazobactam showed less ability overall to increase susceptibility to cefepime; ESBL- and KPC-producing isolates and OXA-48-producing isolates without ESBLs displayed the lowest MICs to this combination.

In a more detailed analysis of the OXA-48-producing group of isolates (n = 304), the resistance rate to cefepime was 74.7%, and the most active combinations were cefepime/zidebactam and cefepime/taniborbactam (99.3% and 93.1% showed a MIC ≤2 mg/L, respectively, Table 2). When the presence or absence of ESBLs in this group of OXA-48-producing strains was analyzed, we observed, as expected, that the low resistance rates to cefepime in the absence of ESBLs hardly varied in combination with BLIs. In ESBL-producing strains, however, the high cefepime resistance levels (only 6.1% of isolates displayed a MIC ≤2 mg/L) decreased relevantly in the presence of zidebactam, taniborbactam, and enmetazobactam (99.2%, 93.1%, and 71.7% showed MIC ≤2 mg/L, respectively).

On the other hand, in the group of KPC-producing *Enterobacterales* (n = 44), all strains were resistant to cefepime and 56.8% were inhibited at ≥16 mg/L of cefepime/enmetazobactam (resistance breakpoint for cefepime). Cefepime/zidebactam and cefepime/taniborbactam displayed the highest activity, being 100% and 84.1% of isolates inhibited at MIC ≤2 mg/L, respectively (Table 2). In the KPC- and ESBL-producing subgroup of strains, the three cefepime/BLI combinations showed high activity (>85% inhibited at MIC ≤2 mg/L). In the subgroup without ESBLs, 92.6% of isolates showed a MIC ≥16 mg/L to cefepime/enmetazobactam, while the cefepime/taniborbactam and cefepime/zidebactam combinations were able to completely overcome resistance to cefepime.

Lastly, in the MBL-producing group of strains (n = 56), cefepime displayed high resistance rates (92.9%), while cefepime/enmetazobactam was unable to inhibit MBLs, with 87.5% showing a MIC ≥16 mg/L. However, cefepime/zidebactam and cefepime/taniborbactam showed high activity (96.4% and 75.0% showed a MIC ≤2 mg/L, respectively, Table 2). As expected, the presence/absence of ESBLs in MBL-producing strains did not substantially affect the elevated MICs to cefepime. Thus, high rates of resistance to cefepime were observed in both the non-ESBL- and ESBL-producing subgroups of strains (83.3% and 100%, respectively). Cefepime/enmetazobactam also showed

TABLE 2 MIC_{50}, MIC_{90}, and % isolates with MICs values ≤2 and ≥16 mg/L to cefepime in the presence and absence of zidebactam, taniborbactam, and enmetazobactam in OXA-48-, KPC-, and MBL-producing *Enterobacterales* strains[a]

Isolate type	Cefepime/BLIs combinations	MIC		% isolates with MICs values	
		MIC_{50}	MIC_{90}	≤2 mg/L	≥16 mg/L
OXA-48-producing isolates					
All isolates (*n* = 304)	Cefepime	64	≥128	18.8	74.7
	Cefepime/zidebactam	≤0.5	≤0.5	99.3	0
	Zidebactam	≤0.5	≥128	75.7	24.0
	Cefepime/taniborbactam	≤0.5	2	93.1	0
	Cefepime/enmetazobactam	1	16	74.7	13.2
No producing ESBLs (*n* = 57)	Cefepime	1	16	73.7	15.8
	Cefepime/zidebactam	≤0.5	≤0.5	100	0
	Zidebactam	≤0.5	≥128	82.5	17.5
	Cefepime/taniborbactam	≤0.5	1	93.0	0
	Cefepime/enmetazobactam	≤0.5	4	87.7	5.3
Producing ESBLs (*n* = 247)	Cefepime	≥128	≥128	6.1	88.3
	Cefepime/zidebactam	≤0.5	≤0.5	99.2	0
	Zidebactam	≤0.5	≥128	74.1	25.5
	Cefepime/taniborbactam	≤0.5	2	93.1	0
	Cefepime/enmetazobactam	1	16	71.7	15.0
KPC-producing isolates					
All isolates (*n* = 44)	Cefepime	≥128	≥128	0	100
	Cefepime/zidebactam	≤0.5	1	100	0
	Zidebactam	1	2	90.9	6.8
	Cefepime/taniborbactam	1	4	84.1	0
	Cefepime/enmetazobactam	64	≥128	40.9	56.8
No producing ESBLs (*n* = 27)	Cefepime	≥128	≥128	0	100
	Cefepime/zidebactam	1	1	100	0
	Zidebactam	1	2	92.6	7.4
	Cefepime/taniborbactam	2	4	81.5	0
	Cefepime/enmetazobactam	≥128	≥128	7.4	92.6
Producing ESBLs (*n* = 17)	Cefepime	32	≥128	0	100
	Cefepime/zidebactam	≤0.5	≤0.5	100	0
	Zidebactam	≤0.5	2	88.2	5.9
	Cefepime/taniborbactam	≤0.5	2	88.2	0
	Cefepime/enmetazobactam	≤0.5	1	94.1	0
MBL-producing isolates					
All isolates (*n* = 56)	Cefepime	≥128	≥128	3.6	92.9
	Cefepime/zidebactam	≤0.5	1	96.4	0
	Zidebactam	≤0.5	≥128	75.0	25.0
	Cefepime/taniborbactam	1	8	75.0	7.1
	Cefepime/enmetazobactam	64	≥128	3.6	87.5
No producing ESBLs (*n* = 24)	Cefepime	64	≥128	8.3	83.3
	Cefepime/zidebactam	≤0.5	≤0.5	100	0
	Zidebactam	≤0.5	≥128	79.2	20.8
	Cefepime/taniborbactam	1	4	79.2	4.2
	Cefepime/enmetazobactam	32	≥128	8.3	83.3
Producing ESBLs (*n* = 32)	Cefepime	≥128	≥128	0	100
	Cefepime/zidebactam	≤0.5	2	93.8	0
	Zidebactam	≤0.5	≥128	71.9	28.1
	Cefepime/taniborbactam	1	8	71.9	9.4
	Cefepime/enmetazobactam	64	≥128	0	90.6

[a]Five strains produced two carbapenemases: OXA-48 + KPC-3, OXA-48 + IMP-13, OXA-48 + NDM-1, OXA-48 + VIM-1, and KPC-2+ IMP-22.

high MICs in these groups (83.3% and 90.6% inhibited at MIC ≥16 mg/L), while cefepime/zidebactam and cefepime/taniborbactam were highly active against the same subgroups (<10% with a MIC ≥16 mg/L).

In a previous study with the same isolates from this multicenter collection, the imipenem/relebactam and ceftazidime/avibactam combinations, recently approved, were evaluated (11). In that study, following CLSI criteria, 16.2% of strains were resistant to ceftazidime/avibactam ($MIC_{50/90}$ 1/≥256 mg/L) and 14.2% to imipenem/relebactam

($MIC_{50/90}$ 0.5/4 mg/L), thus presenting less activity than cefepime/zidebactam and cefepime/taniborbactam.

With respect to the literature assessing the activity of cefepime/zidebactam, $MIC_{50/90}$ values of 0.5/2, 0.5/2, and 0.5/4 mg/L, were observed in the groups of OXA-48-, KPC-, and MBL-producing *Enterobacterales*, respectively (12), which are consistent with those obtained in our study ($\leq$0.5/$\leq$0.5, $\leq$0.5/1, and $\leq$0.5/1 mg/L, respectively).

Cefepime/taniborbactam has recently shown high activity against a collection of carbapenemase-producing *Enterobacterales* (13). Similarly, in other study, KPC-producing strains showed $MIC_{50/90}$ values of 16/>128 and 0.12/1 mg/L for cefepime and cefepime/taniborbactam, respectively (14), while another collection of KPC-producing *Enterobacterales* showed $MIC_{50/90}$ values of >256/>256 and 2/8 mg/L, respectively (15), highlighting the good activity of taniborbactam. In line with those results, we found cefepime and cefepime/taniborbactam $MIC_{50/90}$ values of $\geq$128/$\geq$128 and 1/4 mg/L, respectively for KPC-producing *Enterobacterales*. For OXA-48-producing *Enterobacterales*, a previous study showed $MIC_{50/90}$ of 2/128 and 0.25/1 mg/L for cefepime and cefepime/taniborbactam, respectively (14), while in our study, for this group were 64/$\geq$128 and $\leq$0.5/2 mg/L, respectively.

One of the most relevant aspects of taniborbactam is its activity against MBLs, so that it is of interest to examine this class of β-lactamases in detail. In this study, we determined a $MIC_{50/90}$ of 1/8 mg/L for all MBL-producing strains, although MIC values varied considerably according to MBL subclass, which was previously observed (14). In our study, in 42 VIM-producing strains, we observed a $MIC_{50/90}$ for cefepime/taniborbactam of 1/4 mg/L. In the other two MBL groups, with four IMP- and 10 NDM- producing strains, $MIC_{50/90}$ values for cefepime/taniborbactam were 8/16 mg/L and 2/16 mg/L, respectively (Fig. S3). Consequently, cefepime/taniborbactam shows high activity against VIM-producing *Enterobacterales* strains, although as previously stated, taniborbactam did not show significant activity against IMP. Lastly, the NDM-producing subgroup is of particular interest. Taniborbactam has previously shown high activity against NDM-1 and NDM-1 variants (16–18), although other NDM-like enzymes were not analyzed. However, Wang et al. found NDM-5-producing *Escherichia coli* strains with MICs $\geq$16 mg/L to cefepime/taniborbactam carrying a mutation in PBP3, which may be involved in resistance (15). In our study, 10 NDM-producing isolates were tested (only one NDM-5-producing *E. coli*), which displayed low MICs to cefepime/taniborbactam. For all the above reasons, the activity of cefepime/taniborbactam against NDM-producing strains needs to be investigated further.

Finally, with respect to the third combination, several studies have shown excellent cefepime/enmetazobactam activity against different collections of *Enterobacterales* strains, especially ESBL-producing *Enterobacterales*, with similar or better activity than other approved combinations, such as ceftazidime/avibactam, ceftolozane/tazobactam, and even carbapenems (8, 19, 20). Other studies have shown good cefepime/enmetazobactam activity against carbapenemase-producing *Enterobacterales* (21, 22). Considering the low activity of enmetazobactam against OXA-48 (23), and that most OXA-48-producing strains also produce ESBLs, the activity of cefepime/enmetazobactam is probably due more to ESBL inhibition than to carbapenemase inhibition. KPC-producing strains deserve a special mention, because the activity of cefepime/enmetazobactam against KPC-producing *Enterobacterales* is more controversial, and has not yet been clarified in the literature (8, 19, 23). Although enmetazobactam showed good activity against KPC enzymes (23), recent studies showed that the cefepime/enmetazobactam combination had limited microbiological activity against KPC-producing strains, showing $MIC_{50/90}$ of 32/64 mg/L or higher (8, 19) and did not improve the $MIC_{50/90}$ of cefepime by more than 4-fold. In our study, including 44 KPC-producing strains, we found high $MIC_{50/90}$ values (64/$\geq$128 mg/L) for cefepime/enmetazobactam (Table S2). Analyzing these isolates more closely, we observed a relationship between MIC and the clonality of the isolates studied; all but one ST512 isolate (n = 22) showed MICs $\geq$128 mg/L for cefepime/enmetazobactam, while isolates belonging to ST307 (n = 15) showed MICs $\leq$1 mg/L, therefore the variability of the activity of cefepime/enmetazobactam should be carefully evaluated depending on the predominant sequence types (STs). Lastly, with respect to MBLs and, as expected, cefepime/

enmetazobactam did not enhance the activity of cefepime in most of the strains, as previously described in literature (23) (Table S3 and Fig. S3).

The interpretations in this study have been obtained according the cefepime CLSI breakpoints (S ≤2 mg/L and R ≥16 mg/L); however, the EUCAST MIC breakpoints are slightly different (S ≤1 mg/L and R ≥ 8 mg/L). Following the EUCAST recommendations, cefepime/zidebactam and cefepime/taniborbactam continue to be the combinations with the highest activity against the whole collection, with 96.0% and 77.8% of strains showing a MIC ≤1 mg/L. EUCAST (MIC ≥8 mg/L) and CLSI (MIC ≥16 mg/L) cefepime resistance breakpoints recommendations do not differ by more than 6% for any combination studied.

Resistance to cefepime in the presence of BLIs and characterization of resistance mechanisms. Focusing particularly on the analysis of isolates showing MIC ≥16 mg/L (resistance breakpoint of cefepime) to the three new β-lactam/β-lactamase inhibitors, out of a total of 400 *Enterobacterales*, 4 isolates displayed MIC ≥16 mg/L to cefepime/taniborbactam (Table 3), 110 to cefepime/enmetazobactam (Tables S2 and S4), while none to cefepime/zidebactam.

(i) Clinical isolates with elevated MICs to cefepime/zidebactam. MICs ≤2 mg/L to cefepime/zidebactam were observed in 99% of strains included in this study, while only four (1%) strains displayed MIC of 4 to 8 mg/L (cefepime susceptible-dose dependent category), making cefepime/zidebactam the most active combination evaluated. The ability of zidebactam to moderately inhibit class A, C, and some class D together with its antimicrobial activity, through binding to PBP2, makes the cefepime/zidebactam combination extremely active against carbapenemase-producing *Enterobacterales*.

(ii) Clinical isolates with elevated MICs to cefepime/taniborbactam. In our study, four strains displayed cefepime/taniborbactam MICs ≥16 mg/l and all of them produced MBLs (NDM-5, NDM-7, VIM-1, and IMP-8) (Table 3). One of these strains was an IMP-8-producing *Enterobacter cloacae*, carbapenemase not inhibited by taniborbactam (13). Among NDM-producing strains, one strain presented a MIC of 16 mg/L for cefepime/taniborbactam, and another an elevated MIC of ≥128 mg/L. Although taniborbactam is able to inhibit NDM-like carbapenemases, these strains produced CTX-M-15 ESBL and OXA-1 β-lactamase, among others, and also exhibited relevant amino acid changes in PBP2 and/or PBP3, the main targets of cefepime (24). Both strains share a 4-amino acid insertion (YRIN) in PBP3, which directly affects the size of the Ω loop. This insertion has previously been associated with decreased susceptibility to several β-lactams including the ceftolozane/tazobactam combination (25, 26). Similarly, the V522I mutation in PBP2, found in the NDM-producing strain with the highest MIC to cefepime/taniborbactam also has been associated with increased resistance to β-lactams (27). Finally, a VIM-1-producing isolate of *K. pneumoniae* with a MIC to cefepime/taniborbactam of ≥128 mg/L, as well as several copies of OXA-1 and CTX-M-15, presented a nonfunctional OmpK35 due to a stop codon. Deficiencies in OmpK35 have been associated with increased resistance to ceftazidime/avibactam in the presence of β-lactamases such as KPC, which presents good hydrolytic activity against ceftazidime (28).

(iii) Clinical isolates with elevated MICs to cefepime/enmetazobactam. In this study, 110 isolates displayed MICs ≥16 mg/L to cefepime/enmetazobactam, including 40 OXA-48 producers, 25 KPC producers (22 KPC-3 and 3 KPC-2), and 49 MBL producers (Tables S2 and S4). Further analysis of the OXA-48-producing isolates revealed that 13% (40/304 isolates) displayed MICs ≥16 mg/L for cefepime/enmetazobactam, three of them also producing an MBL. A total of 37 OXA-48-producing strains not producing any other carbapenemase were either slightly or not inhibited by enmetazobactam, 35 out of 37 also produced an ESBLs, and interestingly 18 out of 37 exhibited important changes (frameshifts, insertions, deletions. . .) in OmpK35, OmpK36, PBPs, and/or AcrAB-TolC expression regulators (29). The remaining OXA-48-producing strains with high MICs to cefepime/enmetazobactam (19/37) showed no alterations in the β-lactam resistance genes analyzed in this study, highlighting only the presence of two or more ESBLs in most of these strains (Table S4).

With respect to the KPC-producing isolates, it should be highlighted that, in this study, enmetazobactam was only able to decrease the MIC of cefepime to ≤2 mg/L in

TABLE 3 Bacterial species, sequence type, and susceptibility to cefepime of clinical *Enterobacterales* showing high MIC values for cefepime/taniborbactam (MIC ≥16mg/L)[a]

Genome no.	Species	ST	MIC (mg/L)		Other β-lactamases					Transcription regulators AcrAB-TolC
			Cefepime	Cefepime/ taniborbactam	Carbapenemase	ESBL β-lactamases	Non- ESBL β-lactamases	Porins	PBPs	
AI2843	*E. coli*	410	≥128	16	NDM-5	CTX-M-15	EC-like, (2x)TEM-1, (2x)OXA-1, CMY-2		PBP3 (I532L, Y334_N337insYRIN)	
AI2858	*E. coli*	648	≥128	≥128	NDM-7	CTX-M-15	EC-like, CMY-6, OXA-1		PBP2 (V522I) PBP3 (I532L, Y334_N337insYRIN)	
AI2867	*K. pneumoniae*	39	≥128	≥128	VIM-1	(2x)CTX-M-15	(2x)OXA-1, SHV-11	OmpK35 NF (W316*)		
AI2992	*E. cloacae* complex	96	16	16	IMP-8		MIR-like, OXA-101			

[a](2x), the strain has two copies of the gene coding for the specified β-lactamase; NF, nonfunctional; asterisk (*), premature stop codon.

less than half the strains with the KPC carbapenemase (40.9%, Tables 2 and Table S2). This result is determined by the prevalence of ST512 (MICs ≥128 mg/L) and ST307 (MICs ≤1 mg/L), as exposed above. All ST512 isolates produced a nonfunctional OmpK35 and, interestingly, all the strains with high MICs showed a 2 amino acid insertion in OmpK36 (G134_D135insGD) in loop 3. This insertion has previously been involved in constriction of the porin channel and increased MICs to carbapenems, which is of greater significance when combined with deletion of OmpK35 (30). These alterations have been also associated with the increase of MICs to new combinations such as meropenem/vaborbactam (31, 32) or meropenem/QPX7728 (33). Other isolates displaying high MICs for cefepime/enmetazobactam were two strains of *Citrobacter freundii*, which also produced a nonfunctional OmpK35. This strongly suggests that secondary mechanisms such as porin modifications must be present in KPC-producing strains in order to show elevated MICs for this combination.

Finally, the inhibition spectrum of enmetazobactam does not cover MBLs (9), and therefore, most of the isolates producing MBLs in this study displayed MICs ≥16 mg/L for cefepime/enmetazobactam. However, two of the 56 MBLs producers cefepime-resistant isolates displayed a cefepime/enmetazobactam MIC of 4 mg/L (cefepime susceptible-dose dependent), producing VIM-1 and NDM-1 (Table S3 and Fig. S3).

Concluding remarks. Resistance to β-lactam antibiotics continues to increase and new β-lactamases with a broader spectrum and higher hydrolytic activity are constantly emerging. The current development and emergence of new classes of β-lactamase inhibitors such as the ones evaluated here is possibly one of the most promising aspects in the ongoing fight against bacterial resistance to antibiotics.

In our study, the combination that showed the highest activity against carbapenemase-producing *Enterobacterales* was cefepime/zidebactam, followed by cefepime/taniborbactam. Cefepime/enmetazobactam enhances the activity of cefepime alone in virtually all cases tested, but is less effective than the other two combinations, particularly against MBL carbapenemases. Cefepime/zidebactam and cefepime/taniborbactam, along with the β-lactam/β-lactamase inhibitor combinations that have already been approved, such as ceftazidime/avibactam and imipenem/relebactam, present results that lead us to think, with a certain degree of optimism, that in the near future we will have a wider therapeutic arsenal against the global expansion of MDR *Enterobacterales*.

MATERIALS AND METHODS

Bacterial isolates. Public hospitals in Spain participated in a nationwide survey of *Enterobacterales* with meropenem MICs above the screening cut-off recommended by EUCAST (>0.125 mg/L) (34) and carbapenemase production. Four-hundred *Enterobacterales* isolates in all were prospectively recovered during a 2-month period in 2018 (November to December) from 24 hospitals, and hence most regions of Spain (11). Bacterial strains were frozen in Luria Bertani broth (Sigma) with 15% glycerol and maintained at −80°C until analysis. The microbiological laboratory of the University Hospital Complex of A Coruña (CHUAC) served as reference laboratory.

Antimicrobial susceptibility testing. The *in vitro* antibacterial activity of cefepime (Sigma) alone and in combination with the BLIs zidebactam, taniborbactam, and enmetazobactam (provided by MedChemExpress) against 400 *Enterobacterales* isolates was determined in cation-adjusted Mueller-Hinton broth (Becton Dickinson and Company) using microdilution assays and following CLSI recommendations (35). To potentiate the antibacterial activity of the β-lactamase inhibitors, cefepime and zidebactam were tested in a 1:1 ratio, and taniborbactam and enmetazobactam at a fixed concentration of 4 and 8 mg/L, respectively. Serially diluted concentrations of cefepime and cefepime/BLIs ranging from 0.5 to 128 mg/L were performed. For the complete set of isolates evaluated in this study (n = 400), cefepime MIC values ≤2 and ≥16 mg/L were used as breakpoints to define the susceptible and resistant interpretive categories, respectively, following CLSI criteria. Thus, for the purposes of comparison, resistance to cefepime/BLIs was interpreted in this study as MIC values ≥16 mg/L (35). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 reference strains were used as controls; their MICs for these cefepime/BLI combinations have previously been described (35).

Whole-genome sequencing, hybrid assemblies, and resistance genomics. All clinical isolates were analyzed by whole-genome sequencing, as previously described (11). Total genomic DNA was obtained using a Genomic DNA Buffer Set with Genomic-Tip 20/G (Qiagen), following the manufacturer's instructions. The DNA yield was determined using the Qubit dsDNA HS assay kit (Thermo Fisher). Purified genomic DNA from all isolates was sequenced in parallel using both short- (Illumina MiSeq, Illumina) and long-read (MinION, Oxford Nanopore Technologies) approaches.

The resulting reads from each isolate were assembled using the Unicycler v0.4.6 (36) hybrid assembler and annotated using Prokka v1.13 (37). The antimicrobial resistance gene content of the isolates

was analyzed using Resfinder v3.2 software (38), ARMFinderPlus (39), and the Comprehensive Antibiotic Resistance Database (CARD) (38).

For the analysis of β-lactam resistance, the main genes involved in resistance were the *K. pneumoniae* porin genes, *ompK35* and *ompK36*, transcriptional activators of the *acrAB-tolC* efflux system (*marR*, *ramR*, and *acrR*), genes *mrdA* and *ftsI* (coding PBP2 and PBP3, respectively), and the possible presence of other β-lactamases. In other *Enterobacterales* species, homologous genes to the ones mentioned were evaluated. The sequences of resistance genes used as reference were obtained from strains selected according to the following criteria: the most conserved sequences between the strains in the multicenter study with low MICs to the cefepime/BLIs combinations were selected using BLASTP. Later, sequences were searched for 100% identity and coverage in both GenBank and Uniprot databases, in order to demonstrate they were conserved gene sequences in each *Enterobacterales* species.

Data availability. The BioProject accession number for strain genomes is PRJEB39112. Data will be available upon request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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