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LN-1-255, a penicillin sulfone able to inhibit the class D carbapenemase OXA-48

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31

32 **Abstract**

33 **Objectives.** Carbapenemases are the most important mechanism responsible for
34 carbapenem resistance in Enterobacteriaceae. Among carbapenemases, OXA-48
35 presents unique challenges as it is resistant to β-lactam inhibitors. Here, we test the
36 capacity of the compound LN-1-255, a 6-alkylidene-2`-substituted penicillanic acid
37 sulfone, to inhibit the activity of the carbapenemase OXA-48.

38 **Methods.** The OXA-48 gene was cloned into *Klebsiella pneumoniae* and
39 *Escherichia coli* in order to obtain MICs in presence of inhibitors (clavulanic acid,
40 tazobactam, and sulbactam) and LN-1-255. OXA-48 was purified and state-state
41 kinetics were performed with LN-1-255 and tazobactam. The covalent binding mode of
42 LN-1-255 with OXA-48 was studied by docking assays.

43 **Results.** Both OXA-48 producing clinical and transformant strains display
44 increased susceptibility to carbapenem antibiotics in the presence of 4 mg/L of LN-1-
45 255 (2-32-fold increased susceptibility) and 16 mg/L of LN-1-255 (4-64-fold increased
46 susceptibility). Kinetics assays demonstrated that LN-1-255 is able to inhibit OXA-48,
47 with an acylation efficiency (k_2/K) of 10±1 x 10^4 M^{-1} s^{-1} and a slow deacylation rate
48 (k_{off}) of 7±1 x 10^{-4} s^{-1}. Inhibitory concentration 50% (IC_{50}) was 3 nM for LN-1-255 and
49 1.5 µM for tazobactam. Lastly, turnover rate was 500-fold lower for LN-1-255 than for
50 tazobactam. Docking assays confirmed the anchored of LN-1-255 on the active binding
51 site of OXA-48.

52 **Conclusions.** In these studies, carbapenem antibiotics used in combination with
53 LN-1-255 are effective against the carbapenemase OXA-48, an important emerging
54 mechanism of antibiotic resistance. This provide stimulus for further investigations to
55 maximize the efficacy of penicillin sulfone inhibition of class D plasmid-carried
56 Enterobacteriaceae carbapenemases.

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58

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Introduction

Antimicrobial resistance rates on bacterial pathogens have steadily increased in recent years, and is now defined as a world health crisis by major international agencies.[1] Enterobacteriaceae are a bacterial family in which significantly increased rates of resistance have been observed. The main resistance mechanism - as for Gram-negative bacteria in general - is β-lactamase-mediated hydrolysis of antimicrobial compounds.[2] The prevalence and plasmid-mediated dissemination of β-lactamase genes are an important clinical challenge. Until the first decade of the XXI century, the more clinically relevant β-lactamases were the extended spectrum β-lactamases (ESBL) and the chromosomal and plasmid-mediated cephalosporinases (AmpC).[3] Although carbapenem antibiotics remained active against β-lactamase-expressing bacteria, in recent years there has been an increase in the number of β-lactamases able to hydrolyze carbapenems. Of these enzymes, denoted 'carbapenemases', a growing group of OXA enzymes - the class D β-lactamases - is especially relevant.[4,5] These carbapenemases are mostly observed in *Acinetobacter baumannii*,[5,6] but the frequency of OXA-48 expression is increasing in Enterobacteriaceae,[7] largely in *Klebsiella pneumoniae*, and in *Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii*, and *Providencia rettgeri.* [8-13]

An important epidemiological change is occurring nowadays, a rapid increase of the number of isolates of carbapenemase-producing Enterobacteriaceae, mainly *K. pneumoniae.* [14] Thus, numerous hospital outbreaks of OXA-48-carrying Enterobacteriaceae have been reported worldwide [8,10,15] and the rising numbers of positive isolates reported showing the OXA-48 the most frequently detected carbapenemase in France, Belgium and Malta.[12,16] The rapid increase of such carbapenem-resistant isolates is facilitated by two main routes of dissemination:

85 horizontal transfer of plasmids encoding OXA-48, and the vertical dissemination of
86 successful clones.[17,18] The *bla*$_{\text{OXA-48}}$-like genes are always encoded in transposons
87 Tn*1999*, plasmid-borne, broad-host-range self-conjugative, and with a high conjugation
88 rate IncL/M-type plasmid.[19] Along with KPC- and NDM-type carbapenemases, OXA-
89 48 represents a major obstacle in the preservation of carbapenem efficacy against
90 serious bacterial infections in Enterobacteriaceae.

91 Currently, few novel options for treatment of such multi-drug resistant bacterial
92 strains exist. One of the more promising avenues is the combination of β-lactam
93 antibiotics with β-lactamase inhibitors, but currently commercial inhibitors are not
94 effective against class D carbapenemases. Unlike class A enzymes, class D
95 carbapenemases are not inhibited by β-lactamase inhibitors such as clavulanic acid,
96 sulbactam or tazobactam.[2] Bacteria harboring and expressing these genes are resistant
97 to most antibiotics, with few exceptions (e.g. tigecycline and colistin) although it is also
98 increasing the resistance rates to these antimicrobials.[20-22] Considering that carbapenems
99 are the main drug of choice to treat multi-resistant hospital acquired infections, the
100 development of efficient inhibitors of OXA-48 enzymes is urgently needed in order to
101 maintain the efficacy of β-lactam antibiotics .[8,23] An example is the avibactam, which
102 has generated great interest among the medical community and has been very recently
103 approved by the FDA for treating complicated infections caused by antibiotic resistant-
104 pathogens.[24] It demonstrates activity against OXA-48, however not against the class D
105 carbapenemases of *Acinetobacter baumannii*, such as OXA-23 or OXA-24/40, main
106 responsible of carbapenem-resistance in this MDR pathogen.[25,26] Buynak *et al.* have
107 designed several β-lactamase inhibitors, [27] of which the compound LN-1-255 represents
108 a promising candidate in the quest for new OXA β-lactamase inhibitors. The design and
109 synthesis of this 6-alkylidene-2´-substituted penicillin sulfone was previously

reported,[28] and has previously demonstrated the efficacy of this compound to inhibit the class D carbapenemase OXA-24/40 of *A. baumannii* (implicating the hydrophobic barrier established through an arrangement of the Tyr112 and Met223 amino acids). This barrier is not present in the OXA-48 enzyme, similarly to OXA-10, another class D β-lactamase which lacks carbapenem hydrolytic activity.[29,30] The LN-1-255 compound is also able to inhibit class A β-lactamases, such as SHV-1 and SHV-2.[31] However the potential of LN-1-255 as an inhibitor of Enterobacteriaceae-derived class D carbapenemases has not yet been evaluated. Here, we analyze the capacity of LN-1-255 to inhibit such an OXA-48 carbapenemase, similarly to what happens with the OXA-24/40 carbapenemase. Also we study the synergy of this inhibitor with carbapenems against clinical isolates of *K. pneumoniae* and transformants of *E. coli* and *K. pneumoniae*.

Materials and methods

Bacterial strains, culture media and plasmids

For microbiological studies, the *bla*$_{\text{OXA-48}}$ gene was amplified from a clinical strain of *K. pneumoniae* derived from an outbreak in our hospital (A Coruña Hospital, Spain), which began in 2013 and continues until today, and has affected more than 160 patients. Epidemiological studies demonstrate that the outbreak resulted from the dissemination of a single clone of serotype ST-15. All carbapenem-resistant strains involved in the outbreak harbor the same plasmid, which carries the OXA-48 carbapenemase gene.[32] The strains utilized to transform the genetic constructions to study the MICs were obtained during previous studies, and have porin deficits: *E. coli* J53 Δ*ompC/F* lacks porins OmpC and OmpF, [33] and *K. pneumoniae* Δ*ompK35/36* lacks

134 porins OmpK35 and OmpK36.[34] These strains were chosen in order to increase the
135 genetic background of resistance upon transformation. All strains were cultured in Luria
136 Bertani (LB) medium at 37°C and stored at -80ºC until analysis in LB medium
137 containing 15% glycerol. When necessary the LB medium was supplemented with
138 ampicillin and/or kanamicyn (Sigma-Genosys Ltd, UK).

139

140 **Susceptibility testing antibiotics and inhibitors.**

141 OXA-48 was amplified from genomic DNA, using Expand High Fidelity PCR
142 System (Roche, Basel, Switzerland) and a primer pair containing recognition sites for
143 the restriction enzymes *BamH*I (5´-AAAGGATCCATGCGTGTATTAGCCTTAT-3')
144 and *EcoR*I (5´-AAAGAATTCCTAGGGAATAATTTTTTCCTGTTT-3'). The
145 amplified DNA fragment was then digested with *BamH*I and *EcoR*I (Fermentas, Glen
146 Burnie, Ma, USA) and ligated to a *BamH*I/*EcoR*I digested pBGS18 plasmid.[35] This
147 plasmid construct (pBGS18-OXA-48) was used to transform the strain *E. coli* DH5α for
148 subcloning and then transformed in *E. coli* J53 Δ*ompC/F,* and *K. pneumoniae*
149 Δ*ompK35/36.* Antibiotic susceptibility profiles were determined by microdilution
150 following CLSI criteria.[36] Susceptibility to imipenem, meropenem, and ertapenem
151 (Sigma-Genosys Ltd, UK), in combination with the inhibitor LN-1-255 at fixed
152 concentrations of 4 or 16 mg/L, was determined. For comparison, the inhibitor
153 tazobactam was used at the same concentrations, as well as sulbactam and clavulanic
154 acid (Sigma-Genosys Ltd, UK) at 4 mg/L. Etest of carbapenems (Biomerieux, Marcy
155 l'Etoile, France) in conjunction with inhibitors were performed on Mueller Hinton II
156 agar plates to confirm MICs. The MICs reported are the mean of three independent
157 replicates. LN-1-255 was prepared in the laboratories of the Southern Methodist

158 University, Dallas, USA, as described previously.[27] The chemical structures of LN-1-
159 255 and tazobactam are shown in Figure 1.
160 Synergy studies were also performed using the checkerboard method using both
161 K. pneumoniae strains. Synergy of imipenem (range of 0.12-256 mg/L) in absence and
162 presence of LN-1-255 at 16 mg/L was combined with important treatment option
163 against MDR pathogens, such as tigecycline, colistin and amikacin (range 0.12 -256
164 mg/L). The fractional inhibitory concentration index (FIC) was calculated and was
165 interpreted as follows: FIC_{index} of ≤0.5, synergy; FIC_{index} of >0.5 to 4, no interaction.[37]
166

167 **Purification of OXA-48**

168 For kinetic studies, *bla*$_{OXA-48}$ was directionally cloned into the p-GEX-6p-1
169 plasmid for expression and purification (GE Healthcare, Little Chalfont, United
170 Kingdom). As above, this gene was amplified from genomic DNA, using Expand High
171 Fidelity PCR System (Roche, Basel, Switzerland) with a primer pair containing
172 recognition sites for the restriction enzymes *BamH*I (5´-
173 AAAGGATCCAAGGAATGGCAAGAAAACAAA-3') and *EcoR*I (5´-
174 AAAGAATTCCTAGGGAATAATTTTTCCTGTTT-3'). The amplified DNA
175 fragment was then digested with *BamH*I and *EcoR*I and ligated to *BamH*I/*EcoR*I
176 digested p-GEX-6p-1. The recombinant plasmid was electropored in *E. coli* BL21
177 (DE3) - which has a proteases deficit - to generate the fusion protein GST/OXA-48.
178 This protein was purified to homogeneity using the GST Gene Fusion System
179 (Amersham Pharmacia Biotech, Europe GmbH) in conjunction with the manufacturer´s
180 instructions. The purification was confirmed using SDS-PAGE gels, as a band of
181 approximately 29 KD (>95% purity) and later identification with MALDI-TOF/TOF
182 spectrometer (Bruker Daltonics, Billerica, MA).

183

184 **Kinetic experiments.**

185 Kinetic constants of OXA-48 β-lactamase were determined by continuous assays
186 at room temperature, 25 ºC, using a Nicolet Evolution 300 spectrophotometer (Thermo
187 Fisher Scientific, Waltham, MA, USA). Each experiment was performed at least in
188 triplicate, in 50 mM sodium phosphate with 20 mM of sodium bicarbonate,[38] and using
189 0.2 and 1.0 cm pathlenght cuvettes. Measurements of hydrolysis were performed with
190 nitrocefin (NCF) (Oxoid, Hampshire, UK) at 590 nM, imipenem and ertapenem, both to
191 299 nM. Measurements of inhibition were performed in the presence of LN-1-255 and
192 tazobactam, using nitrocefin at 200 µM as the reporter substrate. The kinetics assays
193 were previously described [30,39,40] and are briefly explained below. V_{max}, k_{cat} and K_m were
194 determined by initial velocity kinetic analysis. The data were fitted to the Michaelis-
195 Menten equation (Equation 1) using nonlinear least-squares regression analysis.

196 $$v = V_{max} [S] / (K_m + [S]) \qquad (Eq. 1)$$

197 The inhibitor concentration resulting in 50% reduction of nitrocefin hydrolysis
198 after 10 min of pre-incubation of the enzyme and inhibitor at 25 °C was denoted as the
199 IC_{50}, as previously described.[30]

200 The apparent K_m for tazobactam and LN-1-255 was obtained as a competitive
201 inhibition constant ($K_{i\ app}$) in the presence of nitrocefin.[40] Inverse initial velocities ($1/v_o$)
202 were plotted against inhibitor concentration ([I]) to obtain linear plots. Initial velocity
203 (v_0) was determined by Equation 2.

204 $$v_0 = (V_{max} \times [S]) / [(K_{m\ NCF}) \times (1 + [I] / K_{m\ NCF}) + [S] \qquad (Eq. 2)$$

205 $K_{i\ app}$ was determined by dividing the value of the y-intercept of the linear plot by the
206 slope, and corrected for nitrocefin affinity using Equation 3.

207 $$K_{i\ app} (corrected) = K_{i\ app} (observed) / [1 + ([S] / K_{m\ NCF})] \qquad (Eq. 3)$$

208 The inhibitor complex inactivation rate (k_{inact}) in the presence of nitrocefin was
209 measured and K_I determined as previously described.[41] The k_{obs} values were determined
210 using non-linear least squares fit of the data, employing Origin 7.5® software and
211 Equation 4.

212 $$A = A_0 + v_f \text{ x } t + (v_0 - v_f) \text{ x } [1 - \exp(-k_{obs}t)] / k_{obs} \quad \text{(Eq. 4)}$$

213 Here, A is absorbance, v_0 (expressed in variation of absorbance per unit time) is initial
214 velocity, v_f is the final velocity, and t is time. Each k_{obs} was plotted against [I] and a fit
215 performed to determine k_{inact} and K_I using Equation 5. The K_I value was corrected using
216 equation 3.

217 $$k_{obs} = k_{inact} [I] / (K_I + [I]) \quad \text{(Eq. 5)}$$

218 The k_2/K (observed) was determined as the slope of the line obtained by plotting
219 [I] against $k_{obs.}$ This value was corrected for nitrocefin affinity as in equation 6.

220 $$k_2/K = k_2/K_{obs} \text{ x } (([S]/K_{m\ NCF}) + 1) \quad \text{(Eq. 6)}$$

221 The k_{off} for LN-1-255 was performed using a jump dilution method followed by
222 continuous assays, as previously described.[42] Briefly, 1 μM of enzyme was incubated
223 with 10 μM of LN-1-255 for 5 minutes at 37 ºC, the reaction solution was diluted
224 40,000-fold in assay buffer, and hydrolysis of 200 μM of nitrocefin was measured in
225 500 μL of reaction solution. Reaction solutions without OXA-48, or containing OXA-
226 48 without LN-1-255 were used as controls.

227 The K_d (dissociation constant) was determined by equation 7.

228 $$K_d = k_{off} / (k_2/K) \quad \text{(Eq. 7)}$$

229 The partition ratio (k_{cat} / k_{inact}) or turnover number (t_n) is defined as the ratio of
230 inhibitor enzyme ($I:E$) necessary for >90% inhibition of nitrocefin hydrolysis.[30] The t_n
231 values were determined after 5 hours of incubation with increasing concentrations of

232 inhibitor and 10 nM of enzyme, varying the molar ratios of *I:E*. Longer incubation
233 times could not be used due to enzyme instability and loss of activity. Incubations were
234 done in a final reaction volume of 500 µL and 200 µM nitrocefin added immediately
235 before measure, in order to determine the residual enzyme activity during 60 seconds.
236

237 **Docking studies**

238 The covalent binding mode of LN-1-255 with OXA-48 from *K. pneumoniae* was
239 studied using GOLD 5.2[43] and the enzyme geometries found in the crystal structure of
240 OXA-48/avibactam adduct (PDB 4S2K,[44] 2.1 Å). Water molecules and ligands were
241 removed from crystal structure. Ligand geometry was minimized using the AM1
242 Hamiltonian as implemented in the program Gaussian 09[45] and used as MOL2 files. The
243 ligand was docked in 25 independent genetic algorithm (GA) runs, and for each of these
244 a maximum number of 100000 GA operations were performed on a single population of
245 50 individuals. Covalent docking was applied between the catalytic Ser-81 (oxygen
246 atom side chain) and the carboxylate group (oxygen atom) of the indolizine derived
247 obtained after covalent modification of OXA-24/40 from *A. baumannii* with LN-1-
248 255.[26] Operator weights for crossover, mutation and migration in the entry box were
249 used as default parameters (95, 95, and 10, respectively), as well as the hydrogen
250 bonding (4.0 Å) and van der Waals (2.5 Å) parameters. The position of avibactam in the
251 crystal structure was used to define the active-site and the radius was set to 7 Å. The
252 "flip ring corners" flag was switched on, while all the other flags were off. The GOLD
253 scoring function was used to rank the ligands in order to fitness.
254

255 **Results**

256 **Antimicrobial susceptibility assays**

257 To determine the synergy of LN-1-255 with diverse carbapenems against OXA-
258 48, *in vitro* susceptibility to imipenem/LN-1-255, meropenem/LN-1-255, and
259 ertapenem/LN-1-255 were compared to susceptibility to these carbapenems in
260 combination with the classical inhibitors clavulanic acid, sulbactam and tazobactam.
261 (Results are shown in Table 1). Type-strains without any resistance mechanism were
262 not presented because they showed very low MIC values to carbapenems when OXA-
263 48 was cloned into them (data not shown), thus the used transformants were porins-
264 deficient. As discussed above, carbapenem MICs were investigated using 4 mg/L of all
265 inhibitors, and 16 mg/L of LN-1-255 and tazobactam. Clavulanic acid and sulbactam
266 could not be tested at 16 mg/L with *K. pneumoniae* strains or at 4 mg/L with *E. coli*
267 strains, as the MICs of these two inhibitors using *K. pneumoniae* strains were 32-256
268 mg/L, and using *E. coli* strains were 16 mg/L for sulbactam and 16-128 mg/L for
269 clavulanic acid. This intrinsic susceptibility to sulbactam and clavulanic acid prevents
270 clear observation of OXA-48 inhibition.

271 Neither sulbactam nor clavulanic acid at 4 mg/L were able at to inhibit OXA-48
272 activity at any dilution in the performed assays. At 4 mg/L, tazobactam was able to
273 inhibit OXA-48 activity in only one dilution, and otherwise did not reduce MICs
274 compared to carbapenems alone. At 4 mg/L, LN-1-255 was able to reduce carbapenem
275 MICs 2-32-fold. These differences were amplified when 16 mg/L of LN-1-255 or
276 tazobactam were used.

277 The clinical *K. pneumoniae* isolate displayed an imipenem MIC of 64 mg/L,
278 decreased 16-fold to 4 mg/L in the presence of LN-1-255, and 2-fold to 32 mg/L in the
279 presence of tazobactam. Similarly, transformed *K. pneumoniae* Δ*ompK35/36* displayed

280 an imipenem MIC of 128 mg/L, decreased 8-fold to 16 mg/L, in the presence of 16
281 mg/L of LN-1-255, but zero-fold in the presence of 16 mg/L of tazobactam.

282 More pronounced differences were observed in transformed *E. coli* Δ*ompC/F*,
283 with 8-, 32-, and 64-fold reductions in imipenem, meropenem, and ertapenem MICs in
284 the presence of LN-1-255, compared to a 2-fold MIC decrease in the presence of
285 tazobactam. The remaining data are shown in Table 1. Using transformed *E. coli* DH5α,
286 the effect of 4 mg/L LN-1-255 on carbapenem MIC was also more significant that the
287 effect of 4 mg/L tazobactam. However, at inhibitor concentrations of 16 mg/L, this
288 transformed strain was highly susceptible to the antimicrobial effect of these
289 combinations, precluding quantitative analysis of effect sizes (data not shown).

290 By checkerboard method the combination of imipenem/LN-1-255 or imipenem
291 alone were tested against tigecycline, colistin and amikacin. The presence of LN-1-255
292 decreased one dilution the MIC at tigecycline and amikacin in the *K. pneumoniae*
293 clinical strain, and also one dilution at amikacin in the Δ*ompK35/36* mutant. However
294 the FIC_{index} was > 0.5, no synergy was observed with these antimicrobials. (Data not
295 shown).

296

297 **Kinetics and inhibition studies**

298 Kinetic properties substrate hydrolysis by OXA-48 are shown in Table 2. The
299 protein concentration used on biochemical studies was 1.9 mg/mL. Similar K_m values
300 were observed for both carbapenems tested, imipenem and ertapenem, and for nitrocefin
301 (60-123 μM), but pronounced differences were observed in hydrolysis velocity (k_{cat}) for
302 these substrates. When converted to catalytic efficiency, this value was 10-fold higher
303 for imipenem than for ertapenem (0.025 and 0.002 $\mu M^{-1} s^{-1}$, respectively) while the

304 catalytic efficiency of nitrocefin (4.8 μM^{-1} s^{-1}) hydrolysis was 192-fold higher than that
305 of imipenem.
306 The OXA-48 inhibitory activities of LN-1-255 and tazobactam were determined
307 and are summarized in Table 3. Using nitrocefin as the indicator substrate, LN-1-255
308 showed inhibitory activity at much lower concentrations than tazobactam. Thus the K_{i}
309 $_{app}$ - analogous to the K_m for inhibitors - showed a higher affinity between the
310 carbapenemase and LN-1-255 than between the carbapenemase and tazobactam ($K_{i\ app}$ =
311 0.17 and 30 μM, respectively).
312 In order to determine k_{inact} and thus inactivation efficiencies (k_{inact} /K_I) of LN-1-
313 255 and tazobactam, progress curves were fitted to Equation 3 to obtain k_{obs} values
314 (Figure 2). These k_{obs} values were plotted against concentrations of both inhibitors, and
315 indicated a faster acylation at lower concentrations of LN-1-255 than of tazobactam.
316 However, in the case of LN-1-255, this determination could not be completed due to an
317 inability to achieve the maximum rate. Instead, k_2/K was determined as an
318 approximation of the inactivation efficiency (10 x10^4 M^{-1} s^{-1}). The k_{inact} /K_I of
319 tazobactam was 3000 M^{-1} s^{-1}, showing an OXA-48 inactivation efficiency of more than
320 33-fold lower than that of LN-1-255. Similarly, IC_{50} was much lower for LN-1-255
321 (0.003 μM) than for tazobactam (1.5 μM), about 500-fold lower, indicating a higher
322 relative efficacy of LN-1-255. Separate experiments (k_{off}) indicated that the off rate of
323 LN-1-255 was 7 x 10^{-4} s^{-1}, representing a slow return of carbapenemase activity, with a
324 residence time half-life ($t_{1/2}$) of 16.5 min (Figure 3). In the case of tazobactam, an
325 irreversible inhibitor, it was not possible to determine the off-rate. [46]
326 Lastly, in determining the partition ratio (t_n) of LN-1-255 and tazobactam, the
327 data showed a relative I:E significantly lower for LN-1-255 (t_n = 2) than for tazobactam

328 (t_n =1000), indicating a 90% inhibition of OXA-48 at much lower concentration

329 (approximately 500-fold) of LN-1-255 than of tazobactam (Figure 4).

330

331 **Docking studies**

332 As for previously reported OXA-48/avibactam adduct (PDB 4S2K,[38] 2.1 Å, Figures 5A

333 and 5C), our docking studies showed that the indolizine adduct obtained after covalent

334 modification of the catalytic Ser70 by LN-1-255 would also interact with residues Arg-

335 250, Lys-208, Tyr-211 and Thr-209 (Figures 5B and 5D). Specifically, the carboxylate

336 group of the modified ligand would be anchored in the active site by electrostatic

337 interactions with the guanidinium group of Arg-250 and the ε-amino group of Lys-208

338 and hydrogen-bonding interaction with the hydroxyl group of Thr-209. In addition, the

339 sulfinate group would establish an electrostatic interaction with the guanidinium group

340 of Arg-250 as well as the carboxylate group of the ester linkage would interact by

341 hydrogen-bonding with the NH main chain amide of Tyr-211. In contrast, our molecular

342 docking studies predict that the catechol side chain would be more flexible. One of the

343 phenol groups of the catechol moiety would interact by hydrogen-bonding with the ε-

344 amino group of Lys-208. This catechol moiety would partially occupy the large pocket

345 close to the active site.

346

347 **Discussion**

348 Carbapenem-hydrolyzing class D β-lactamases can confer resistance to

349 carbapenems, mostly in *A. baumannii* or Enterobacteriaceae with OXA-24/40 and

350 OXA-48 as respective examples of such enzymes, limiting therapeutic options for

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351 infection with these pathogens.[20,21] The increasing number of clinical isolates and
352 outbreaks of strains carrying class D carbapenemases underscores the need for new β-
353 lactamase inhibitors that can restore carbapenem efficacy. The penicillin sulfone
354 inhibitor LN-1-255 was developed by Buynak *et al.* as a novel inhibitor of β-
355 lactamases.[28] Initially, LN-1-255 was shown to be effective in inhibiting class A β-
356 lactamases,[31] and, subsequently, class D enzymes.[29,30] Similarly to OXA-24/40,
357 carbapenem MICs in the presence of LN-1-255 were more significantly reduced when
358 16 mg/L rather than 4 mg/L of LN-1-255 were used.[30]

359 We have thus observed inhibition of OXA-48 carbapenemases, both in a clinical
360 isolate of *K. pneumoniae* and in porin-deficient transformed *K. pneumoniae* and *E. coli*
361 strains. Depending on which carbapenem was used, we obtained 2- to 64-fold
362 reductions in carbapenem MICs. However, in most cases, use of the classical inhibitors
363 (clavulanic acid, sulbactam and tazobactam) did not alter carbapenem MICs. These
364 results are consistent with those obtained in similar studies on OXA-24/40 inhibition,
365 where carbapenem MICs decreased 8- to 32-fold in the presence of LN-1-255, but only
366 up to 2-fold in the presence of tazobactam.[30] In this study the porin deficit in *K.*
367 *pneumoniae* decreased slightly the efficacy of LN-1-255 (compared with the clinical
368 strain) when combined with imipenem and ertapenem, however increased the efficacy
369 when combined with meropenem, decreasing one fold the MIC. Thus it seems that LN-
370 1-255 is able to pass through the outer membrane, despite the porin deficit. In fact, this
371 inhibitor was designed with a catecholic functionality that resembles a natural bacterial
372 siderophore, enabling it to utilize the iron uptake system to traverse the outer
373 membrane.[28]

374 The carbapenem hydrolysis kinetics of OXA-48 (K_m and k_{cat}) are largely
375 concordant with those previously published by Poirel *et al.* [47,48] LN-1-255 was highly

376 effective at inhibiting OXA-48 β-lactamase. LN-1-255 demonstrated a 33-fold higher
377 inhibition efficiency than tazobactam, with k_2/K = 10 x10^4 M^{-1} s^{-1} and k_{inact} /K_I = 3000
378 M^{-1} s^{-1}, respectively. The inhibition due to LN-1-255 is characterized by a high binding
379 affinity, with K_i = 0.17 μM and IC_{50} = 0.003 μM, which are respectively 180 and 500-
380 fold lower than those yielded using tazobactam. The off rate of LN-1-255 was
381 moderately slow, with k_{off} = 7 x 10^{-4} s^{-1}, yielding an enzyme reactivation half-life of
382 16.5 min. Lastly, a partition rate (t_n) of 2 for LN-1-255 compares very favorably to the
383 more than 1000 for tazobactam required for 90% inactivation of OXA-48. These data
384 likely indicate that LN-1-255 molecules are more slowly hydrolyzed by OXA-48 than
385 tazobactam molecules.

386 Regarding the *A. baumannii* OXA-24/40 carbapenemase, LN-1-255-mediated
387 inhibition which was previously tested by our group, proved to be similar to the binding
388 affinity of LN-1-255 for OXA-48, with $K_{i\ app}$ = 0.7 μM and 0.17 μM, respectively. LN-
389 1-255 inhibition efficacies were also similar between these two enzymes, with k_2/K = 10
390 x10^4 M^{-1} s^{-1} for OXA-48 and k_{inact} /K_I = 21 x10^4 M^{-1} s^{-1} for OXA-24/40 [30]. The crystal
391 structure of both carbapenemases has been previously determined, OXA-48 by
392 Disquiet *et al* [49] and OXA-24/40 by our group.[50] These two β-lactamases were the first
393 class D carbapenemase crystal structures published and may be considered as two
394 different carbapenemase models: although they share a common evolutionary origin,
395 they eventually acquired their carbapenemase activity through distinct evolutionary
396 pathways. Significant differences in the size and shape of their active sites are evident.
397 OXA-24/40 presents a "hydrophobic barrier" (consisting of Tyr-112 and Met-223)
398 covering the active site, which is implicated in correct orientation of small substrates,
399 such as the carbapenems. However, this is not a conserved structure in the OXA
400 carbapenemases, and OXA-48 lacks this tunnel-like structure. By contrast, OXA-48

displays more homology with OXA-10, which is not a carbapenemase but is highly active in hydrolysing oxacillin. This ability is shared by OXA-48 but not OXA-24/40.[30,49] Despite these important differences, the compound LN-1-255 was able to inhibit both enzymes (OXA-24/40 and OXA-48) in the nM range. Later, De Luca *et al.* [51] showed that the small β5-β6 loop of OXA-48, close to the active site, is implicated in OXA β-lactamase ability to hydrolyze carbapenems by facilitating movement of the deacylating water molecule towards the acylated catalytic Ser-70 residue. The relevance of this loop has been confirmed, and is present in OXA-23, OXA-24/40 and OXA-48 carbapenemases, meanwhile it present significant structural differences in OXA-10 β-lactamase.[49,51] This suggests that LN-1-255 is interacting with this loop to prevent carbapenem access to the active site of the carbapenemases. However, further structural and biochemical work is necessary to identify the precise inhibition mechanism.

When comparing our results with those obtained by Ehmann *et al.* studying avibactam and OXA-48, a new β-lactamase inhibitor, we observed a faster on rate with LN-1-255 ($10\ x10^{4}\ M^{-1}\ s^{-1}$, compared with $1.4\ x10^{3}\ M^{-1}\ s^{-1}$ of avibactam, 71-fold lower). However, avibactam displayed a slower k_{off} ($1.2\ x10^{-5}\ s^{-1}$) compared with the off rate of LN-1-255 ($7\ x\ 10^{-4}\ s^{-1}$), resulting in a 60-fold higher avibactam half-life.[52] Thus the time half-life of 16.5 minutes of LN-1-255 could significant a drawback if used in the treatment in infections. How these data kinetics data affect to the clinical impact of these inhibitors is an issue that should be studied in experimental models of infection in the near future.

Figure 5 represents avibactam and LN-1-255 at the active binding site of OXA-48, the docking of LN-1-255 on the active site supports the inhibitory role of this compound. The LN-1-255 is anchored to OXA-48, similarly to the structure of avibactam and OXA-48 published by King and Strynadka[44]. Furthermore, although

426 avibactam is able to inhibit OXA-48, it poorly inhibits OXA-24/40 of *A. baumannii*, in
427 contrasting to LN-1-255.[26] Structurally, it has been suggested that the inhibition
428 efficiency of LN-1-255 is mainly due to: (1) the presence of the catechol moiety that
429 facilitates entry through the outer membrane via the iron-uptake cell pathway, and (2)
430 the incorporation of a (2-pyridyl)methylene group at C6 in the sulbactam core that
431 results with the formation of an heterocyclic (indolizine) ester derivative that is resistant
432 to hydrolysis.[26] In spite of the high stability of these type of heterocyclic esters, LN-1-
433 255 proved to have a higher k_{off} rate (7×10^{-4} s^{-1}) against OXA-48 from *K. pneumoniae*
434 than avibactam (1.2×10^{-5} s^{-1}). It has been suggested that avibactam does not
435 decompose via a hydrolytic mechanism of the carbamoyl linkage with the catalytic
436 serine. Instead, it seems to occur via regeneration of the diazobicyclooctane moiety. We
437 consider that, in addition to that, there are some differences in the binding mode of both
438 modified ligands that could also contribute to the lower stability of the OXA-48/LN-1-
439 255 adduct than the corresponding OXA-48/avibactam. Comparison of the binding
440 mode of the modified avibactam shown in the crystal structure of OXA-48/avibactam
441 adduct (PDB 4S2K)[44] and the predicted binding mode of the corresponding OXA-
442 48/LN-1-255 adduct obtained by molecular docking studies revealed that both adducts
443 interact with mainly the same active site residues, specifically, Arg-250, Lys-208, Tyr-
444 211 and Thr-209. However, as illustrated in Figure 5, there are some differences in the
445 binding contacts of the groups that most contribute to anchor the ligand in the active
446 site, the sulfate and sulfinate/carboxylate groups that might explain their different
447 behaviour. While the sulfate group in the avibactam adduct is deeply anchored in the
448 active site through a salt bridge with Arg-250 and a hydrogen-bonding with Thr-209
449 side chain (Figure 5C), the sulfinate group in the modified LN-1-255 only interacts by
450 electrostatic interaction with Arg-250 (Figure 5D). We consider that the latter is

451 consequence of the lack of the hydrophobic barrier present in OXA-24/40, which
452 involves mainly residues Tyr-112 and Met-223.[30] In this case, the carboxylate group of
453 the modified LN-1-255 interacts by hydrogen-bonding with the phenol group of Tyr-
454 112. As a consequence, the sulfinate group can be located pointing inwards. As in the
455 crystal structure of OXA-48/avibactam adduct, the latter group would interact by a salt
456 bridge with the guanidinium group of Arg-261 and hydrogen-bonding with Ser-128 side
457 chain. These binding differences might account in the overall different stability of the
458 two adducts as experimentally observed (on and off rates).

459 Because of the structural and mechanistic differences among class D
460 carbapenemases,[49] this area of drug discovery requires further studies to identify
461 additional inhibitors as possible therapeutic options. The results here reflected provide
462 stimulus for further *in vitro* and *in vivo* investigations to maximize the efficacy of
463 penicillin sulfone inhibition of class D carbapenemases in Enterobacteriaceae.

464

465

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Transparency declarations.

None to declare.

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Table 1. Carbapenem MIC values (mg/L) with clinical and transformed bacterial strains used.

β-Lactams + inhibitors[a]	*K. pneumoniae* clinical strain		*K. pneumoniae* Δ*ompK35/36*		*K. pneumoniae* Δ*ompK35/36* (OXA-48)		*E. coli* J53 Δ*ompC/F*		*E. coli* J53 Δ*ompC/F* (OXA-48)	
	4 mg/L [b]	16 mg/L	4 mg/L	16 mg/L	4 mg/L	16 mg/L	4 mg/L	16 mg/L	4 mg/L	16 mg/L
Imipenem	64	64	0.5	0.5	128	128	0.25	0.25	1	1
Imipenem + TAZ	64	32	0.5	0.25	128	128	0.25	0.125	1	0.5
Imipenem + SUL	64	NA	0.5	NA	128	NA	NA	NA	NA	NA
Imipenem + CLA	64	NA	0.5	NA	128	NA	NA	NA	NA	NA
Imipenem + LN	16	4	0.5	0.25	32	16	0.25	0.25	0.5	0.125
Meropenem	16	16	0.25	0.25	64	64	0.03	0.03	2	2
Meropenem + TAZ	16	8	0.25	0.25	32	32	0.03	0.015	1	1
Meropenem + SUL	16	NA	0.25	NA	32	NA	NA	NA	NA	NA
Meropenem + CLA	16	NA	0.25	NA	32	NA	NA	NA	NA	NA
Meropenem + LN	8	4	0.25	0.25	16	8	0.03	0.03	0.25	0.06
Ertapenem	128	128	2	2	256	256	0.015	0.015	0.5	0.5
Ertapenem + TAZ	128	32	2	1	256	128	0.015	0.007	0.5	0.25
Ertapenem + SUL	128	NA	2	NA	256	NA	NA	NA	NA	NA
Ertapenem + CLA	128	NA	2	NA	256	NA	NA	NA	NA	NA
Ertapenem + LN	64	16	2	1	64	64	0.015	0.015	0.015	0.007

[a] TAZ, tazobactam; SUL, sulbactam; CLA, clavulanic acid and LN, LN-1-255.

[b] The four inhibitors were tested at 4 mg/L using *K. pneumoniae* clinical and transformed strains. Tazobactam and LN-1-255 were tested using transformed *E. coli* up to 4 mg/L; and tazobactam and LN-1-255 were tested up to 16 mg/L using *K. pneumoniae* clinical and transformed strains.

NA: Some combinations of carbapenems and inhibitors could not be performed due to intrinsic strain susceptibility. MICs using transformed *E. coli* were 16 mg/L for sulbactam and 16-128 mg/L for clavulanic acid, and using *K. pneumoniae* strains were 32-256 mg/L for both inhibitors.

Data represent the means of three independent experiments.

Table 2. Carbapenem and nitrocefin hydrolysis kinetics of OXA-48

	OXA-48		
Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat} / K_m ($μM^{-1}$ s^{-1})
Nitrocefin	65.7 ± 18.9	314.7 ± 18.9	4.79
Imipenem	60.3 ± 12.4	1.5 ± 0.1	0.025
Ertapenem	123.7 ± 36.2	0.3 ± 0.02	0.002

Data represent the means of three independent experiments.

Table 3. OXA-48 inhibition kinetics of LN-1-255 and tazobactam.

Substrate	IC_{50} (μM)	$K_{i\ app}$ (μM)	K_I (μM)	k_{inact} (s^{-1})	k_{inact}/K_I ($M^{-1}\ s^{-1}$)	k_2/K ($M^{-1}\ s^{-1}$)	k_{off} (s^{-1})	$t_{1/2,Off}$ (min)	K_d (nM)	t_n
LN-1-255	0.003 ± 0.0003	0.17 ± 0.01	NA	NA	NA	(10 ±1) x10^4	(7 ±1)x10^{-4}	16.5	7	2
Tazobactam	1.5 ± 0.5	30 ± 3	14 ± 2	0.038 ± 0.004	3000 ± 500	NA	NA	NA	NA	1000

Data represent the means of three independent experiments.

Figure 1. β-lactamase inhibitors used in this study.

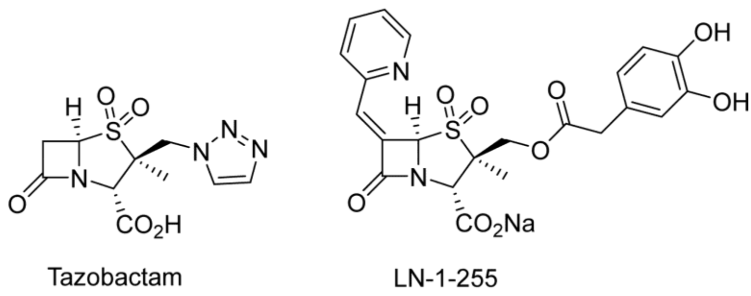

Figure 2. Progress curves of 200 μM nitrocefin hydrolysis with increasing inhibitor concentrations (0.2 μM to 3.3 μM LN-1-255 and 10 μM to 350 μM tazobactam) and a fixed concentration of OXA-48 (8.6 nM).

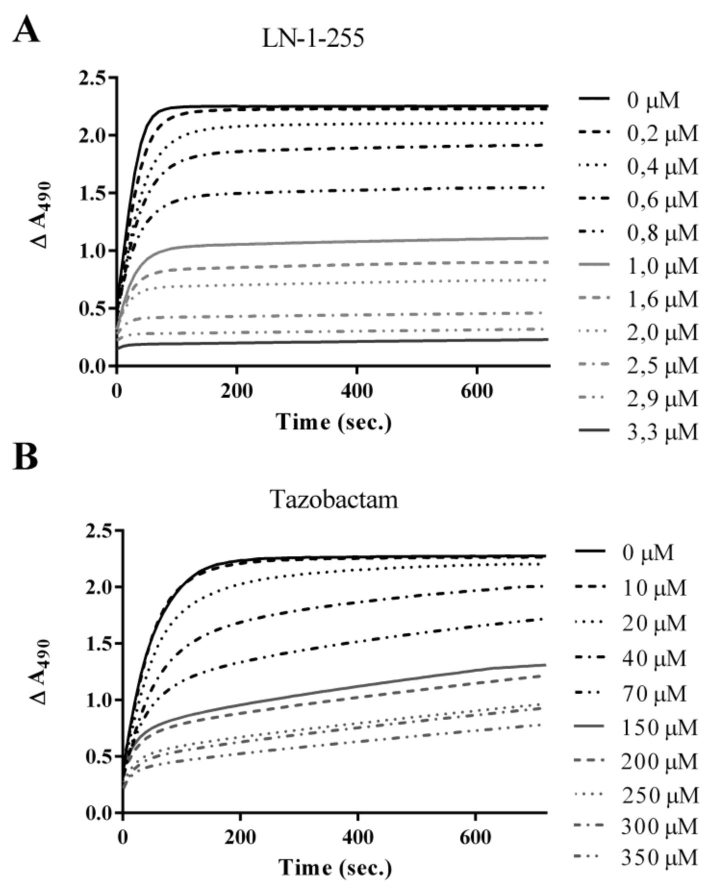

Figure 3. Progress curves for determination of off-rates (k_{off}) for OXA-48/LN-1-255 and controls (OXA-48 alone and LN-1-255 alone) with 200 μM of nitrocefin. OXA-48 was incubated with LN-1-255 to allow the formation of the acyl-enzyme adduct, then diluted to permit enzyme reactivation.

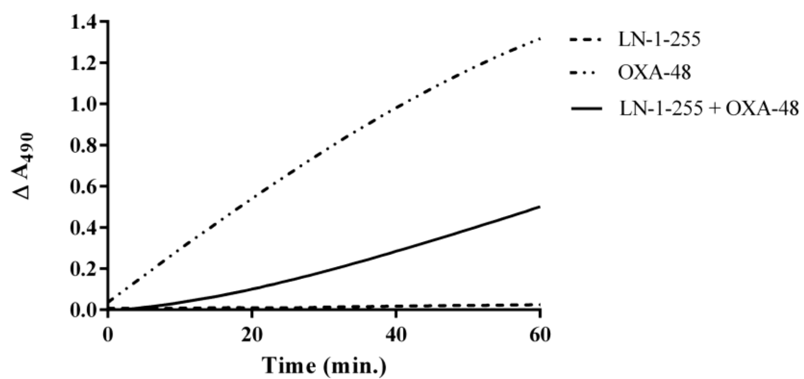

Figure 4. Progress curves for determination of turnover (t_n). OXA-48 at 10 nM was incubated with increasing concentrations of LN-1-255 (A) or tazobactam (B).

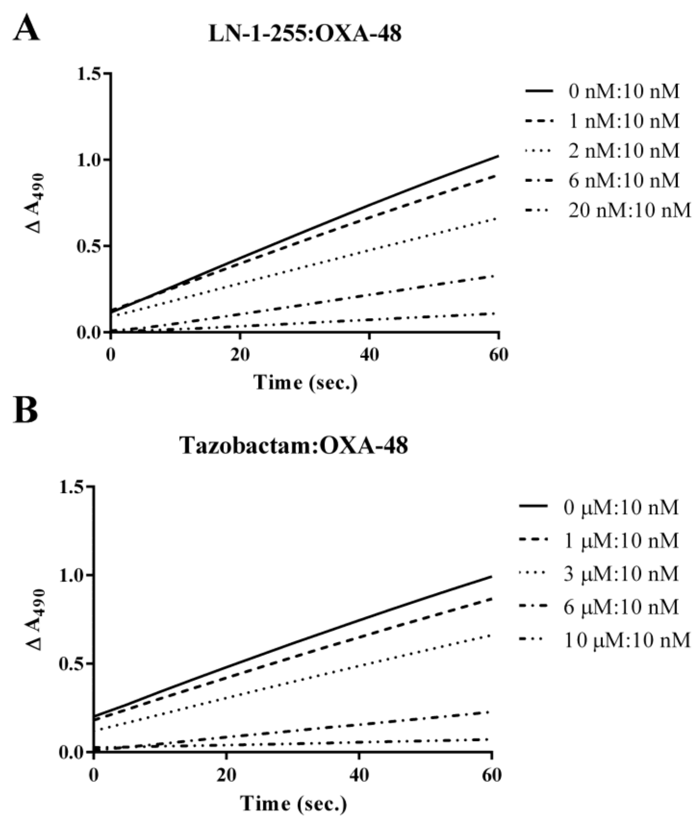

Figure 5. Carbapenemase OXA-48 from *K. pneumoniae* covalently modified by avibactam and LN-1-255. A,C) Crystal structure of OXA-48/avibactam adduct (PDB 4S2K,[44] 2.1 Å). B,D) Proposed irreversible modification of OXA-48 by LN-1-255 obtained by Molecular Docking studies. Hydrogen bonding and electrostatic interactions between the ligand and OXA-48 are shown as dashed blue lines. Relevant side chain residues are shown and labeled. Figure represented with PyMOL Molecular Graphics System.[53]