AAC Accepted Manuscript Posted Online 6 February 2017
Antimicrob. Agents Chemother. doi:10.1128/AAC.02243-16

Can ceftazidime/avibactam and aztreonam overcome β-lactam resistance conferred by metallo-β-lactamases in Enterobacteriaceae?

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27 **Running title:** Ceftazidime/avibactam combined with aztreonam

28 **Keywords:** ceftazidime, avibactam, aztreonam, disk diffusion, metallo-β-lactamases

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Abstract

Based upon knowledge of the hydrolytic profile of major β-lactamases found in Gram negative bacteria, we tested the effectiveness of the combination of ceftazidime/avibactam (CAZ/AVI) with aztreonam (ATM) against carbapenem-resistant enteric bacteria possessing metallo-β-lactamases (MBLs). Disk-diffusion and agar based antimicrobial susceptibility testing were initially performed to determine the *in vitro* efficacy of a unique combination of CAZ/AVI and ATM against 21 representative *Enterobacteriaceae* isolates with a complex molecular background that included bla_{IMP}, bla_{NDM}, bla_{OXA-48}, bla_{CTX-M}, bla_{AmpC}, and combinations thereof. Time-kill assays were conducted, and the *in vivo* efficacy of this combination was assessed in a murine neutropenic thigh infection model. By disk diffusion assay, all 21 isolates were resistant to CAZ/AVI alone, and 19/21 were resistant to ATM. The *in vitro* activity of CAZ/AVI in combination with ATM against diverse *Enterobacteriaceae* possessing MBLs was demonstrated in 17/21 isolates, where the zone of inhibition was ≥ 21 mm. All isolates demonstrated a reduction in CAZ/AVI agar dilution MICs with the addition of ATM. At 2 h, time-kill assays demonstrated a ≥ 4 $\log_{10}$ CFU decrease for all groups that had CAZ/AVI plus ATM (8 µg/ml) added, compared to the CAZ/AVI alone group. In the murine neutropenic thigh infection model, an almost 4 $\log_{10}$ reduction in CFUs was noted at 24 h for CAZ/AVI (32 mg/kg q8h) plus ATM (32 mg/kg q8h) vs. CAZ/AVI (32 mg/kg q8h) alone. The data presented herein, requires us to carefully consider this new therapeutic combination to treat infections caused by MBL-producing *Enterobacteriaceae*.

56 **Introduction**

57 As a consequence of the threat of rising antibiotic resistance, the Infectious Diseases
58 Society of America has challenged the pharmaceutical industry to develop novel antibiotics
59 ("The 10x20 initiative", http://www.idsociety.org/10x20/). Of particular importance are
60 carbapenem-resistant strains of bacteria, which are typically resistant to most or all commonly
61 used therapeutic options, and cause high morbidity and mortality (1). In response, the "antibiotic
62 pipeline" has delivered an important β-lactam β-lactamase inhibitor combination,
63 ceftazidime/avibactam, CAZ/AVI (2).

64 Avibactam (AVI) is a novel diazabicyclooctane (DBO) β-lactamase inhibitor with *in*
65 *vitro* activity against serine enzymes, such as the class A extended spectrum β-lactamases
66 (ESBLs) and *Klebsiella pneumoniae* producing carbapenemases (KPCs), as well as the class D
67 OXA-48 found in *Enterobacteriaceae*, and the class C cephalosporinases present in enteric
68 bacteria and *Pseudomonas aeruginosa*. AVI restores the activity of CAZ against a broad array of
69 resistance threats, making CAZ/AVI a welcome addition to the antibiotic armamentarium against
70 Gram-negative bacteria. However, CAZ/AVI is not active against strains bearing class B
71 metallo-β-lactamases (MBLs) such as NDM, VIM and IMP. The monobactam antibiotic
72 aztreonam (ATM) remains stable against MBLs, but is not a therapeutic option in many cases
73 because it is inactivated by ESBLs, KPCs, and other cephalosporinases frequently found in the
74 background of MBL-producing bacteria (2, 3).

75 Even before widespread clinical use of CAZ/AVI, case reports appeared that described
76 resistance to this novel inhibitor combination (4). As a result of these findings, we now know
77 that CAZ/AVI MICs may be elevated in the setting of unfavorable genetic backgrounds which

include resistance determinants that control entry and egress of antibiotics (5). Additionally, MBLs are a worldwide problem, with outbreaks reported in the United States in numbers that were previously unanticipated (6, 7).

Knowing that ATM is stable against hydrolysis by MBLs, we hypothesized that addition of ATM to the CAZ/AVI combination would result in enhanced activity by "protecting" ATM from the "attack" of ESBLs and other cephalosporinases. Therefore: 1) we tested the *in vitro* activity of the combination of CAZ/AVI with ATM against 21 carbapenem-resistant, MBL-producing Enterobacteriaceae isolates containing bla_{NDM} or bla_{IMP} genes, and a pan-susceptible control; 2) we evaluated the *in vivo* antibacterial efficacy of CAZ/AVI with ATM in a mouse thigh infection model using a MBL producing *K. pneumoniae* strain; 3) lastly, we report a case of infection caused by an *Enterobacter cloacae* containing a MBL treated with ATM in combination with CAZ/AVI. Our findings encourage further study of CAZ/AVI with ATM in experimental and clinical circumstances when therapeutic options against MBL producing strains are limited.

Materials and methods

Antimicrobial susceptibility testing

In vitro efficacy of the CAZ/AVI and ATM combination was performed on 21 previously characterized strains of diverse species of *Enterobacteriaceae* (8). Susceptibility testing was initially performed by disk diffusion. Disks containing CAZ (30 µg) and CAZ/AVI (30/20 µg) were obtained from Becton Dickinson (Sparks, MD) and Actavis (Schaumburg, IL), respectively. ATM (30 µg) disks were made by adding 10 µl of a 3000 µg/ml stock ATM

99 (Chem-Impex Int'l Inc -Wood Dale, IL) solution to blank disks. To test the activity of the
100 combination of CAZ/AVI with ATM, 30 ug of ATM were placed directly on the CAZ/AVI disk,
101 and allowed to dry for 30 minutes before use. Isolates were grown overnight in cation adjusted
102 Mueller-Hinton broth (CAMHB), diluted to a McFarland 0.5 standard and inoculated to form a
103 lawn on cation adjusted MH agar (MHA) plates. Disks containing CAZ, CAZ/AVI, and ATM
104 were placed onto the plates and then incubated overnight at 37° C. For CAZ and ATM, zones of
105 inhibition were interpreted according to the Clinical Laboratory Standards Institute (CLSI)
106 guidelines (9). For CAZ/AVI, zones of inhibition were interpreted according to FDA guidelines.
107 (Figure 1).

108 Agar dilution minimum inhibitory concentrations (MICs) for CAZ, ATM, CAZ/AVI,
109 CAZ/ATM and the CAZ/AVI-ATM combination were determined in triplicate and performed
110 according to CLSI protocol (9). Isolates were grown overnight in CAMHB and stamped on
111 MHA plates containing doubling dilutions of CAZ (Sigma Chemical Co. St Louis, MO), ATM
112 (Chem-Impex Int'l Inc -Wood Dale, IL) and CAZ/AVI (Avycaz™ 2g/0.5g per vial, Allergan)
113 using a Steers replicator that delivered 10^4 CFU/spot. The effect of adding a fixed concentration
114 of 8 or 16 µg/ml of ATM to CAZ or to CAZ/AVI was also assessed. Plates were incubated 18 -
115 20 h at 37° C and the antibiotic concentration (µg/ml) at which bacterial growth was no longer
116 observed defined the MIC. A minimum of 3 determinations were done for each susceptibility
117 with the most frequently observed result reported.

118 *Time-kill assays*

119 Time-kill assays were performed for isolate *K. pneumoniae* 1.41 as previously described
120 with some modifications (10). Briefly, freshly prepared colonies were resuspended in 5 ml
121 CAMHB and incubated overnight in a shaking incubator (37°C, 220 rpm). Cultures were then
122 diluted 1:100 and incubated in a shaking incubator until they reached 0.5 McFarland standard
123 (approximately 10^8 CFU/ml). A bacterial suspension was prepared in 50 ml conical tubes
124 containing 10 ml CAMHB so that the starting inoculum was approximately 10^6 CFU/ml.
125 CAZ/AVI (Avycaz™) was added to the prepared bacterial suspensions so that the final
126 concentration was 1×, 2×, 4× or 8× the agar MIC of the CAZ/AVI-ATM combination (1 µg/ml
127 CAZ:0.25 µg/ml AVI to 8 µg/ml CAZ:2 µg/ml AVI with ATM held constant at 8 µg/ml). A
128 control of CAZ/AVI alone was diluted in the prepared bacterial suspension at 8× the MIC, and a
129 growth control without antibiotics was also included. The starting inoculum was determined
130 from the growth control tube immediately after dilution and was recorded as the count at time
131 zero. After addition of antibiotics, the starting inoculum was 1×10^6 - 1×10^7 CFU/ml. Tubes
132 were incubated in a shaking incubator (37°C, 180 rpm), and viability counts were performed at 1,
133 2, 4, 6, and 24 h by removing 200 µl of the culture, diluting as appropriate, and plating 100 µl on
134 MHA. Plates were incubated at 37°C for 18 -20 h, colonies were counted on plates yielding 30-
135 300 colonies per plate, and the results were recorded as the number of CFU/ml. Three replicates
136 were conducted for each of the conditions reported in the time kill assay.

137 *Polymerase chain reaction (PCR) and sequencing*

138 PCR amplification of bla_{NDM} and other *bla* genes, in isolates not previously
139 characterized, was achieved using established primers and amplified with a MJ Research
140 Gradient Cycler Model PTC 225 using thermocycling conditions adjusted to the primer melting

141 temperatures (8). Positive controls included well characterized isolates (in the laboratory of
142 RAB). Amplicons were sequenced at a commercial sequencing facility (MCLAB, San Francisco,
143 CA). Sequence data were analyzed using Lasergene 7.2 software (DNAstar, Madison, WI), and
144 sequences were compared with BLAST online software (http://blast.ncbi.nlm.nih.gov), using the
145 megablast algorithm.

146 *Mouse thigh infection model*

147 For the thigh infection model, Female Hsd:ICR (CD-1) mice from Envigo were rendered
148 neutropenic by intraperitoneal (i.p.) injection of cyclophosphamide (Cytoxan, Baxter Healthcare,
149 Deerfield, IL) as previously described (11). Five mice per 2 control and 14 treatment groups
150 were infected by injecting a prepared inoculum of *K. pneumoniae* 1.41, input CFU = 5.01 $\log_{10}$
151 CFU/mouse into the right hind thigh muscle contained in a volume of 0.1 mL per animal. At two
152 hours post-infection, animals were administered subcutaneous doses (q8hr) of CAZ (Sigma,
153 Ceftazidime hydrate), CAZ + AVI (AvyCaz™), ATM (Azactam® for injection, USP) or
154 CAZ/AVI + ATM (1:1). All animals were euthanized 24 hours post-infection and thighs
155 aseptically removed into chilled PBS, homogenized (Kinematica® Polytron PT2100 tissue
156 homogenizer), 10-fold serially diluted, plated on Brain-heart infusion agar plates containing
157 0.5% activated charcoal and incubated for the determination of bacterial thigh titers (CFU).

158 *Statistical Analysis*

159 The 24 hour $\log_{10}$ CFU counts were compared across all treatment and control groups
160 using a One-way ANOVA. Post-hoc pairwise comparisons were performed across all treatment

and control groups and p-values were adjusted according to Tukey's method. All analysis was performed using GraphPad Prism 6.0 software.

Results and Discussion

As established by current and previous molecular testing (8), each of the isolates possessed an MBL with the exception of the *Escherichia coli* DH10B which served as a negative control. In Table 1, we show that isolates containing a MBL were resistant to CAZ (21/21 isolates), ATM (19/21 isolates), and CAZ/AVI (21/21 isolates) by disk diffusion (9). The combination of CAZ/AVI and ATM produced an inhibition zone ≥ 21 mm, suggesting susceptibility in 17 of the 21 MBL-producing strains that were tested (i.e., ≥ 21 mm is the S zone diameter for CAZ/AVI alone), with the measured zones being larger than with CAZ/AVI or ATM alone: 10-15 mm larger for 9 isolates, and 4-9 mm larger for 5 isolates (Table 1). In 3 cases where an increase in the inhibition zone was not found with the combination of CAZ/AVI and ATM, there was already a significant zone of susceptibility to ATM observed.

For the agar dilution MIC determinations we used a series of doubling dilutions of CAZ/AVI in the ratio that is found in the commercial preparation (Avycaz™) intended for patient use. This was a deliberate decision as we feel these dilutions reflect doses of CAZ/AVI that patients receive. The commercial preparation is formulated in a 4:1 ratio (CAZ 2g: AVI 0.5g /per vial). The values reported for CAZ/AVI in Table 1 reflect the concentration of the CAZ in the 4:1 ratio formulation. For MIC determinations where the efficacy of ATM addition was assessed, ATM was kept at a fixed concentration of either 8, 16, 32 or 64 µg/ml, and the

182 CAZ/AVI was added in doubling dilutions. These concentrations of ATM were chosen as they
183 represent the expected serum concentrations of the monobactam when delivered in 1 and 2 gram
184 doses throughout the dosing interval, particularly the 8 and 16 µg/ml doses. Single 30 minute
185 intravenous infusions of 1 and 2 gram doses of ATM produce peak serum levels immediately
186 after administration of 54 and 90 µg/ml, and 3 and 6 µg/ml at 8 h (end of the dosing interval),
187 respectively (Azactam package insert).

188 All 21 isolates were resistant to CAZ and CAZ/AVI alone by agar dilution MIC and disk
189 diffusion assay, and all demonstrated a reduction in CAZ/AVI MICs with the addition of ATM
190 (Table 1) in a dose dependent fashion. Notably, one *K. pneumoniae* and four *E. coli* isolates
191 demonstrated MIC values that remained at or above the resistance breakpoint for CAZ/AVI
192 alone (≥ 16/4 µg/ml) after the addition of ATM; genome sequencing is being performed on these
193 isolates to further explore the underlying mechanism of this resistance. However, addition of
194 ATM at higher concentrations of 32 and 64 µg/ml for all 5 isolates brought them into the
195 susceptible MIC range for CAZ/AVI alone (S ≤ 8/4 µg/ml), Table 1. Remarkably, results
196 presented herein accurately reflect the genetic backgrounds established in these isolates, and as
197 anticipated, the CAZ/AVI with ATM combination worked especially well in NDM producing
198 strains.

199 The time-kill curves detail the bactericidal activity of the combination of CAZ/AVI and
200 ATM against the MBL containing *K. pneumoniae* 1.41 isolate. ATM at a fixed concentration of
201 8 µg/ml was added to varying sub lethal concentrations of CAZ/AVI ranging from 8 µg/ml CAZ:
202 2 µg/ml AVI down to 1 µg/ml CAZ: 0.25 µg/ml AVI; corresponding to 4 different MIC
203 multiples of the CAZ/AVI plus ATM agar MIC of 1 µg/ml. Time-kill kinetics showed a time

204 dependent decrease in CFU/ml from 1 to 24 hours (Figure 2). A ≥ 4 $\log_{10}$ CFU decrease was
205 observed by 2 h for all combination concentrations and by 6 h, approximately 5 $\log_{10}$ reductions
206 were observed, when compared to the growth control to which antibiotics were not added. After
207 24 h there was minimal regrowth observed for all combinations, still keeping a 3 $\log_{10}$ decrease
208 as compared to t = 0 h, with the exception of the 8x MIC concentration that showed
209 approximately a 6 $\log_{10}$ decrease.

210 We next evaluated the *in vivo* antibacterial efficacy of CAZ/AVI in combination with
211 ATM in a mouse thigh infection model using the MBL producing *K. pneumoniae* 1.41 isolate.
212 The results are presented in Tables 2 and Figure 3. Both CAZ alone (256 mg/kg) and CAZ/AVI
213 alone (256/64 and 128/32 mg/kg) resulted in 3.1-, 4.7-, and 3.7-log CFU reductions respectively,
214 as compared to the untreated control group at 24 h. The lower dose of either CAZ or CAZ/AVI
215 were less effective. ATM alone at doses from 64 – 256 mg/kg q8h exhibited minimal efficacy
216 (1.16 – 1.99 log CFU reduction).

217 Our data in Table 2 show that in this model, the addition of ATM to the CAZ/AVI
218 treatment regimen results in significant enhancement in the reduction of infection as measured
219 by CFUs/thigh when compared to either agent alone and the untreated controls. Doses of 32/8
220 mg/kg q8h CAZ/AVI (4:1 ratio) plus 32 mg/kg q8h ATM reduced bacterial thigh titers by 3.95
221 log CFU below the 24 hr no antibiotic controls (p=0.0016), 3.79 log CFU below those of 32/8
222 mg/kg q8h CAZ/AVI alone (p=0.003, data not shown) and 2.08 log CFU below ATM 64 mg/kg
223 alone. Dosages of ≤ 4/1 mg/kg q8h CAZ/AVI (4:1 ratio) plus 4 mg/kg q8h ATM were unable to
224 reduce the CFU count.

225

226 *Clinical Commentary*

227 A 72-year-old woman without significant medical history fell and sustained a fractured
228 hip on an excursion. She was treated with a total hip arthroplasty performed in Eastern Europe
229 and returned home within 2 weeks with a painful, red surgical site. The arthroplasty was
230 removed in the United States and all intraoperative cultures grew carbapenem-resistant
231 *Enterobacter cloacae* susceptible to colistin, intermediate to tigecycline (FDA breakpoint), and
232 resistant to all other agents tested including fosfomycin. PCR and sequencing efforts revealed
233 that the *Enterobacter cloacae* possessed $bla_{\text{NDM-1}}$, Table 1. The culture also contained an ESBL-
234 producing *Klebsiella pneumoniae* and *Enterococcus faecalis* (ampicillin and vancomycin
235 susceptible). A hip spacer was not placed at this time, knowing further debridement would be
236 necessary.

237 By day 4 of colistimethate sodium (colistin), tigecycline, and meropenem treatment,
238 serum creatinine increased from 0.9 to 2.4 mg/dl. Colistimethate sodium was reduced to 1.5
239 mg/kg every 36 h and meropenem was adjusted for declining renal function. The patient
240 underwent repeat washout with cultures again yielding the carbapenem resistant *E. cloacae* and
241 ESBL-producing *K. pneumoniae*. CAZ/AVI 1.25 g thrice daily was added to the antibiotic
242 regimen; however, CAZ/AVI was discontinued after 4 days as the carbapenem resistant
243 *Enterobacter cloacae* demonstrated *in vitro* resistance by disk diffusion (ATM 30 µg disk: 6 mm
244 zone size, CAZ/AVI 50 µg disk: 18 mm zone size). Meropenem was soon discontinued due to
245 lack of evidence of efficacy. With normalizing serum creatinine, colistimethate sodium dose was
246 increased to 2mg/kg every 12 h.

247 Qualitative synergy in the *E. cloacae* isolate was demonstrated *in vitro* between
248 CAZ/AVI and ATM (inhibition zone of CAZ/AVI disk was increased from 18 mm to 28 mm
249 upon addition of ATM, Table 1. Therefore, the treatment regimen was modified so that the final
250 3 weeks of her 6-week post-operative antibiotic course were comprised of colistimethate sodium,
251 CAZ/AVI (2.5 g every 8 h), ATM (2 g every 8 h) and vancomycin. Six months after completion
252 of antibiotics, the patient underwent successful final reimplantation of a total hip arthroplasty,
253 with extensive inspection and washout. Six cultures were obtained from acetabular and femoral
254 spaces. All were negative for the 3 organisms originally isolated.

255 *Concluding remarks*

256 Based upon an understanding of the biochemical mechanism of action of these three
257 agents, we show that the combination of ATM and CAZ/AVI is able to help overcome
258 carbapenem and expanded-spectrum cephalosporin resistance in MBL producing strains of
259 enteric bacteria. AVI is a very potent β-lactamase inhibitor of class A and C enzymes, and since
260 most strains of *Enterobacter* spp. possess class C enzymes and possibly some class A ESBLs, we
261 reasoned that AVI would prevent the hydrolysis of CAZ and ATM (12). Although AVI cannot
262 restore susceptibility to MBL producing strains, ATM is not susceptible to hydrolysis by MBLs.
263 By inhibiting class A and C β-lactamases with AVI, and using ATM to "bypass" the class B
264 metallo-β-lactamase, susceptibility can be restored leading to a successful microbiological and
265 possible clinical outcome. Our clinical observation is supported by evidence from our *in vitro*
266 investigations.

267 An unexpected benefit from using CAZ and ATM may arise by the simultaneous
268 inhibition of multiple PBPs. CAZ has a high affinity for the penicillin-binding protein-3 (PBP-3)

and moderate affinity for the PBP-1a of certain Gram-negative organisms such as *E. coli* and *P. aeruginosa*. Binding to PBPs results in spheroplast formation followed by rapid lysis. Furthermore, AVI and developmental DBO inhibitors can also bind to certain PBPs (13-15). ATM is relatively PBP3 specific. The combination (CAZ/AVI and ATM) may completely affect the "divisome" of Gram-negative bacteria and have an independent impact on its own. Of note, the use of "double β-lactams" has some precedent in the literature. Overall, the existing data suggests that the potential benefit of this approach against Gram-negative pathogens should be explored carefully, especially given the recent availability of novel inhibitors and β-lactams (16).

There are important limitations that must be considered before uniform application of this promising combination: a) further animal testing with additional isolates and PK studies along with trials in humans are required before full endorsement by clinicians; and b) results from *Enterobacteriaceae* should not be extrapolated to non-fermenters without appropriate testing (studies in progress). At present, clinical experience using this combination against *Stenotrophomonas maltophilia* proved to be successful in a single case (17). Lastly, the possibility of cumulative toxicity from "double β-lactam" combinations must be considered when administering this regimen. However, one major advantage of using ATM is its safety profile. ATM is safe to use in patients with penicillin allergies, can be administered by prolonged or continuous infusion, and it is not associated with nephrotoxicity. The data presented here require us to carefully consider CAZ/AVI combined with ATM as a "new therapeutic opportunity" to treat infections caused by MBL producing strains, while recognizing the ongoing need for new antibiotics.

Acknowledgments

The authors wish to thank Ms. C. Lascols and the SMART study for sharing isolates with RAB. The animal studies were conducted under the IACUC approved protocol 2014/15-09-A04 (approved 10/31/14, expires 10/30/17).

Funding

This study was supported in part by funds and facilities provided by the Cleveland Department of Veterans Affairs, Veterans Affairs Merit Review Program award number 1I01BX001974, from the Biomedical Laboratory Research & Development Service of the VA Office of Research and Development and the Geriatric Research Education and Clinical Center VISN 10 to R. A. B.

This work was also supported by funds from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award numbers UM1AI104681, R01AI063517, R01AI072219, and R01AI100560 to R.A.B. The efficacy study in mice was supported by NIAID's Animal Models of Infectious Diseases program under contract HHSN272201500007C awarded to the University of North Texas.

Funding organizations were not involved in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Department of Veterans Affairs.

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365 Figure Legend

366 Figure 1. ATM placed directly on CAZ/AVI disk to evaluate synergy, isolate *E. cloacae* 6.31
367 was used in this assay.

368 Figure 2. Time kill curve for isolate *K. pneumoniae* 1.41. ATM concentrations were held
369 constant at 8 µg/ml for all combinations with two exceptions: 1) the growth control (no
370 antibiotics added), and 2) the CAZ/AVI NO ATM 8X MIC. Varying ceftazidime/avibactam
371 (CAZ/AVI) concentrations were added corresponding to 1x (1 µg/ml CAZ:0.25 µg/ml AVI), 2x
372 (2 µg/ml CAZ:0.5 µg/ml AVI), 4x (4 µg/ml CAZ:1 µg/ml AVI) and 8x (8 µg/ml CAZ:2 µg/ml
373 AVI) the MIC of the combination CAZ/AVI and ATM obtained by the agar dilution (1ug/ml).
374 Three replicates were conducted for each of the conditions reported in the time kill assay.

375 Figure 3. Individual and Mean Log_{10} CFU/thigh counts for various antibiotic/β-lactamase
376 inhibitor combinations in the neutropenic thigh infection model for *K. pneumoniae* 1.41 infected
377 mice: 5 log_{10} CFU/mouse, 5 mice per treatment group. LOQ: Limit of Quantitation; 2.35 log_{10}
378 CFU/thigh.

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383 Table 1. Evaluation of *in vitro* activity of CAZ/AVI plus ATM, by both disk diffusion and agar MIC testing. Genetic background of
384 isolates and inhibition zone diameters (in mm) of disks with CAZ, ATM, CAZ/AVI, and CAZ/AVI disks to which ATM was added is
385 presented. MIC values are in µg/ml.

		Disk Diffusion Assay Results				**Agar MIC Testing Results**							
Isolate	Known *bla* genes	CAZ 30 ≥21; 18-20; ≤17 S, I, R CLSI	ATM 30 ≥21; 18-20; ≤17 S, I, R CLSI	CAZ/AVI 50 ≥21; ≤20 S, R FDA	CAZ/AVI with ATM*	CAZ (µg/ml) ≤4; 8; ≥16 S, I, R CLSI	ATM (µg/ml) ≤4; 8; ≥16 S, I, R CLSI	CAZ (µg/ml) ATM at 16 µg/ml	CAZ/AVI (µg/ml) ≤8/4; ≥16/4 S, R FDA	CAZ/AVI (µg/ml) ATM at a constant 8 µg/ml	CAZ/AVI (µg/ml) ATM at a constant 16 µg/ml	CAZ/AVI (µg/ml) ATM at a constant 32 µg/ml	CAZ/AVI (µg/ml) ATM at a constant 64 µg/ml
Ec DH10B	none	33	35	35	40 (5)	0.125	0.25	<0.0625	0.25	<0.0625	<0.0625	---	---
Ecl 6.31	NDM-1, CTX-M-15, ACT/MIR, OXA-48	6	6	14	26 (12)	>512	>512	>512	64	0.5	0.5	---	---
Ecl 6.43	NDM-1, CTX-M-15, ACT/MIR	6	6	17	25 (8)	>512	>512	>512	128	0.5	0.25	---	---
Ecl joint inf	NDM-1, CTX-M, TEM-1, AmpC**	6	6	18	28 (10)	>512	256	>512	128	0.5	0.25	---	---
Ec 8.68	NDM-1, CTX-M-15, CMY-2, TEM	6	6	19	21 (2)	>512	>512	>512	64	16	16	8	1
Ec 8.69	NDM-1, CTX-M-15, CMY-2, SHV-5	6	6	17	24 (7)	>512	>512	>512	64	8	4	----	---
Ec 8.70	NDM-1, CTX-M-15, CMY-2, TEM, SHV-5	6	6	20	22 (2)	>512	512	>512	64	16	4	0.5	0.25
Ec 8.71	NDM-1, CTX-M-15, CMY-2, TEM	6	6	17	20 (3)	>512	>512	>512	64	32	8	1	0.25
Ec 8.72	NDM-1, CTX-M-15, CMY-2, TEM	6	6	18	23 (5)	>512	>512	>512	64	8	2	---	---

Ec 8.73	NDM-1, CTX-M-15, CMY-2, TEM	6	6	17	20 (3)	>512	>512	>512	32	8	2	---	---
Ec 8.74	NDM-1, TEM	6	6	20	27 (7)	>512	2	<0.0625	64	0.0625	<0.0625	---	---
Ec 6728	NDM-1	6	15	18	18 (0)	>512	32	>512	64	16	4	0.125	0.06
Kp 1.41	NDM-1, CTX-M-15, DHA, SHV, TEM	6	6	17	32 (15)	>512	128	>512	64	1	0.5	---	---
Kp 1.42	NDM-1, CTX-M-15, SHV-12	6	6	17	27 (10)	>512	>512	>512	64	1	0.5	---	---
Kp 1.44	NDM-1, CTX-M-15, CMY-2, DHA, SHV, TEM	6	6	15	25 (10)	>512	>512	>512	128	16	8	8	4
Kp 1.50	NDM-1, CTX-M-15, SHV	6	6	20	30 (10)	>512	>512	>512	128	0.25	0.25	---	---
Kp 1.63	NDM-1, CTX-M-15, CMY-2, SHV TEM	6	6	15	30 (15)	>512	512	>512	256	1	0.125	---	---
Kp 6913	IMP, SHV	6	28	17	28 (0)	256	0.5	0.0625	128	<0.0625	<0.0625	---	---
Kp 11-01-13	NDM-1, CTX-M-15, SHV	6	6	17	30 (13)	>512	512	>512	128	1	0.5	---	---
Pr 6384	NDM-1, SHV	6	37	6	34 (-3)	512	0.0625	<0.0625	256	<0.0625	<0.0625	---	---
Pr 1.27	NDM-1, CMY-2, DHA	6	14	10	20 (6)	>512	64	>512	>512	32	16	---	---
Mm 1.39	NDM-1, CTX-M-15, DHA	6	12	10	25 (13)	>512	256	>512	512	0.125	<0.0625	---	---

*Data presented in Table 1 is the zone diameter of the CAZ/AVI disk to which ATM was added. In parenthesis is the difference between this zone size and the zone of inhibition of either CAZ/AVI or ATM alone, whichever of the two was greater. Isolates in this

389 Table were available from the laboratories of the authors and characterized previously (8). **Presumed AmpC. CAZ/AVI 50 =
390 ceftazidime 30 µg/avibactam 20 µg; ATM 30 = aztreonam 30 µg; CAZ 30 = ceftazidime 30 µg. Interpretative criteria for CAZ and
391 ATM, MICs were according to the Clinical Laboratory Standards Institute (CLSI) guidelines (9). For CAZ/AVI, MICs were
392 interpreted according to FDA guidelines from the package insert. CAZ S ≤ 4, I = 8, R ≥ 16; ATM S ≤ 4, I = 8, R ≥ 16; CAZ/AVI S ≤
393 8/4, R ≥ 16/4 µg/ml. The values reported for CAZ/AVI reflect the concentration of the CAZ in the 4:1 ratio formulation. Green font
394 indicates susceptibility, and red font indicates resistance based upon the interpretive guidelines. Abbreviations: *Ec* is *Escherichia coli*;
395 *Ecl* is *Enterobacter cloacae*; *Kp* is *Klebsiella pneumoniae*; *Pr* is *Providencia rettgeri*; and *Mm* is *Morganella morganii*.

396 Table 2. Log_{10} CFU/thigh counts for various antibiotic/β-lactamase inhibitor combinations in the
397 neutropenic thigh infection model for *K. pneumoniae* 1.41 infected mice: 5 log_{10} CFU/mouse, 5
398 mice per treatment group. Bold font indicates a statistically significant value.

Test Article(s)	mg/kg SC q8h (5 mice per treatment)	Mean Log_{10} CFU/thigh ± SD	Change from control at 24 h no antibiotics (log_{10} CFU)	Tukey adjusted p-value (treatment vs 24-hour control)
24 h infection control	na	8.46 ± 1.15	na	na
CAZ	256	5.37 ± 1.17	**-3.08**	**0.0405**
	128	6.12 ± 1.34	-2.34	0.3152
	64	6.35 ± 1.57	-2.11	0.4909
ATM	256	7.30 ± 1.47	-1.16	0.9912
	128	6.46 ± 1.80	-1.99	0.5873
	64	6.59 ± 2.02	-1.86	0.6936
CAZ/AVI	256/64	3.77 ± 0.18	**-4.68**	**< 0.0001**
	128/32	4.75 ± 1.00	**-3.70**	**0.0042**
	64/16	6.23 ± 1.93	-2.22	0.4018
	32/8	8.30 ± 0.88	-0.16	> 0.9999
CAZ/AVI/ATM	32/8/32	4.51 ± 0.42	**-3.95**	**0.0016**
	16/4/16	6.88 ± 1.63	-1.58	0.8818
	8/2/08	6.57 ± 1.77	-1.88	0.6777
	4/1/04	8.47 ± 0.85	0.02	> 0.9999

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