J Antimicrob Chemother 2020; **75**: 559–565
doi:10.1093/jac/dkz468 Advance Access publication 13 November 2019

Journal of Antimicrobial Chemotherapy

In vitro selection of aztreonam/avibactam resistance in dual-carbapenemase-producing *Klebsiella pneumoniae*

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Received 12 August 2019; returned 30 September 2019; revised 3 October 2019; accepted 12 October 2019

Objectives: To examine the *in vitro* selection of aztreonam/avibactam resistance among MBL-producing *Klebsiella pneumoniae* and to understand the mechanism of increased resistance.

Methods: The MICs of aztreonam were determined with and without avibactam (4 mg/L) using a broth microdilution method. Single-step and multi-step mutant selection was conducted on five MBL-producing *K. pneumoniae* strains, including two dual carbapenemase producers. Genomic sequencing and gene cloning were performed to investigate the mechanism of increased resistance.

Results: We examined the MICs for 68 MBL-producing *K. pneumoniae* isolates, including 13 dual carbapenemase producers. Compared with aztreonam alone, the addition of avibactam (4 mg/L) reduced the MICs for all isolates by >128-fold, with MIC_{50} and MIC_{90} values of 0.25 and 1 mg/L, respectively. One NDM-1-, OXA-48-, CTX-M-15- and CMY-16-positive ST101 *K. pneumoniae* strain was selected to be resistant to aztreonam/avibactam, with a >16-fold increase in MIC (>128 mg/L). WGS revealed that the resistant mutants lost the bla_{NDM-1} gene, but acquired amino acid substitutions in CMY-16 (Tyr150Ser and Asn346His). Construction of bla_{CMY-16} mutants confirmed that the substitutions (Tyr150Ser and Asn346His) were primarily responsible for the decreased susceptibility to aztreonam/avibactam. In addition, transfer of bla_{CMY-16} mutant (Tyr150Ser and Asn346His) plasmid constructs into certain clinical carbapenemase-producing isolates demonstrated >64-fold increased MICs of aztreonam/avibactam and aztreonam/avibactam/ceftazidime.

Conclusions: Aztreonam in combination with avibactam showed potent *in vitro* activity against MBL-producing *K. pneumoniae*. However, our study suggested the likelihood of aztreonam/avibactam resistance among MBL- and AmpC-co-producing strains and clinical practice should beware of the possibility of the emerging resistance.

Introduction

The global spread of carbapenem-resistant Enterobacteriaceae, especially carbapenem-resistant *Klebsiella pneumoniae*, has emerged as a major public health concern. Three major classes of carbapenemases are largely associated with the global spread of carbapenem-resistant Enterobacteriaceae: KPC (Ambler class A), MBL (Ambler class B, e.g. NDM, VIM and IMP) and OXA-48-like (Ambler class D) carbapenemases. Class A and D enzymes have serine-based hydrolytic activity, while class B enzymes require the presence of metal, i.e. zinc, for their activity.[1]

The newly FDA-approved diazabicyclooctane β-lactamase inhibitors avibactam and relebactam and an acyclic boronic acid β-lactamase inhibitor, vaborbactam, are potent mechanism-based inactivators of KPC,[2,3] but do not inhibit MBLs, such as NDM.[4] As such, the MBLs remain to be a clinical challenge for antimicrobial treatment. The combination of aztreonam and avibactam has shown inhibitory effects on MBLs (NDM, IMP or VIM), including

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strains co-harbouring KPC or OXA-48-like carbapenemases,[5–9] and a clinical trial is ongoing using aztreonam/avibactam to treat serious infection due to MBL-producing Gram-negative bacteria (NCT03580044).

Currently, aztreonam/avibactam resistance is rarely observed among clinical isolates. Consequently, unravelling the molecular mechanism(s) underlying aztreonam/avibactam resistance, in order to guide appropriate clinical usage of this combination of antibiotics and to limit the emergence of resistance, is important. Here, we present *in vitro* susceptibility testing data for a collection of MBL-producing *K. pneumoniae* strains, including isolates co-producing KPC or OXA-48-like carbapenemases. Selected isolates were subjected to *in vitro* selection to study the molecular mechanism of aztreonam/avibactam resistance.

Materials and methods

Bacterial strains

Sixty-eight MBL-producing *K. pneumoniae* isolates were selected from the archived bacterial collection at the Center for Discovery and Innovation at Hackensack Meridian Health (HMH-CDI). The carbapenemase genotypes were previously characterized by PCR and Sanger sequencing. Among them, 55 strains were single carbapenemase producers, including 38 NDM-1, 5 VIM-1, 4 VIM-26, 3 NDM-7, 2 NDM-6, 1 VIM-4, 1 VIM-27 and 1 IMP-26. Thirteen were dual or triple carbapenemase producers, including two NDM-1- and OXA-48-, three VIM-1- and KPC-2-, two NDM-1- and OXA-181-, two NDM-5- and OXA-181-, two NDM-1- and KPC-2- and one NDM-5- and KPC-2-co-producing strains and one strain co-producing three carbapenemases (NDM-1, VIM-1 and OXA-244) (Table 1).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using a standard broth microdilution method following CLSI guidelines.[10] Avibactam was tested at a fixed concentration of 4 mg/L, in combination with 2-fold dilutions of aztreonam. When ceftazidime, aztreonam and avibactam were combined, aztreonam was fixed at 8 mg/L and avibactam was used at 4 mg/L, with 2-fold dilutions of ceftazidime.[11] MICs were interpreted using 2018 CLSI breakpoints[10] for all antimicrobial agents except for aztreonam/avibactam and ceftazidime/aztreonam/avibactam combinations. The testing was performed in duplicate on two different days. Quality control strains *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used in all testing.

In vitro selection

Single-step mutant selection was conducted as previously described.[12] In brief, approximately 10^9 cfu from overnight broth cultures were plated on LB agar with aztreonam/avibactam at 2× to 16× MICs. Multi-step selection was performed by inoculation of ~10^8 cfu in 2 mL of LB broth containing aztreonam/avibactam at 0.5× MIC and incubated for 24 h.[13] This procedure was repeated daily, each time doubling the aztreonam concentration up to a maximum of 128 mg/L. Resistant variants were selected by plating a bacterial suspension on agar plates of corresponding aztreonam/avibactam levels. The MIC values for the mutants and parent strains were determined by the broth microdilution method. The same single-step and multi-step selections were used to select ceftazidime/aztreonam/avibactam resistance, with aztreonam and avibactam concentrations fixed at 8 and 4 mg/L, respectively.

Genome sequencing

Six aztreonam/avibactam-resistant mutant colonies and the parental strain Kp202 were selected for next-generation sequencing using Illumina

Table 1. *In vitro* activities of aztreonam/avibactam against MBL-producing *K. pneumoniae*

Strains (*n*)	CAZ			IPM			ATM			CAZ/AVI			ATM/AVI			ATM/CAZ/AVI		
	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
All (68)	128 to >128	>128	>128	4 to >32	>32	>32	≤0.5 to >128	>128	>128	16 to >64	>64	>64	≤0.25–8	≤0.25	1	≤0.125	≤0.125	≤0.125
Single MBL (55)	128 to >128	>128	>128	4 to >32	32	>32	≤0.5 to >128	>128	>128	64 to >64	>64	>64	≤0.25–1	≤0.25	0.5	≤0.125	≤0.125	≤0.125
Dual/triple carbapenemases (13)	>128	>128	>128	8 to >32	>32	>32	≤0.5 to >128	>128	>128	16 to >64	>64	>64	≤0.25–8	0.5	8	≤0.125	≤0.125	≤0.125

CAZ, ceftazidime; IPM, imipenem; ATM, aztreonam; AVI, avibactam.

HiSeq. Illumina raw reads were *de novo* assembled using SPAdes v3.11.1.[14] MLST STs were determined using the *K. pneumoniae* MLST website (https://bigsdb.pasteur.fr/klebsiella), while the acquired resistance genes, including all β-lactamase-encoding genes, were determined by ResFinder 3.1.[15] Core SNPs were identified using the method described previously.[16] Outer membrane protein genes (*ompK35* and *ompK36*) were examined using BLAST. In addition, the parental strain Kp202 was also subject to Nanopore sequencing using the MinION platform. A hybrid assembly was conducted by Unicycler using the combination of Illumina HiSeq and Nanopore sequencing reads.[17]

Construction of recombinant strains

The promoter and full length of the bla_{CMY-16} gene and its mutant variants (Tyr150Ser and Asn346His) were amplified from the parental and mutant strains using primers CMY-F-EcoRI (5′-CCGGAATTCCAAAACCGCAAGATATGTAATCA-3′) and CMY-R-XbaI (5′-CTAGTCTAGATTATTGCAGCTTTTCAAGAATGCGCC-3′). The PCR products were then cloned into plasmid vector pET28a, followed by electroporation into *E. coli* DH10B. In addition, the recombinant plasmids, namely pET28a-CMY16-wt, pET28a-Tyr150Ser and pET28a-Asn346His, were electroporated into five selected carbapenemase-producing *K. pneumoniae* strains. The recombinant strains were selected with 1 mg/L aztreonam and 4 mg/L avibactam and confirmed by PCR and Sanger sequencing.

Expression analysis

Bacterial strains were grown to mid-log phase and total RNA was prepared using a QIAGEN RNeasy Mini Kit. A total of 5 ng RNA was used in an RT–PCR assay using a QIAGEN QuantiTect SYBR Green RT–PCR Kit (Germantown, MD, USA). The oligonucleotides used to detect the expression of the CMY allele in *K. pneumoniae* were CMY-F (5′-GATGCAGGAGCAGGCTATTC-3′) and CMY-R (5′-CCGATCCTAGCTCAAACAGC-3′) and the control oligonucleotides to detect expression of the control *gapA* gene were gapA-F (5′-TTTCTGAGCAGCACGGAA-3′) and gapA-R (5′-ATAGTCATATGT TCCTCCA-3′).

Accession numbers

The complete genome sequences of Kp202 were submitted to GenBank under the accession numbers CP041082 to CP041089.

Results

Activity of aztreonam/avibactam against MBL-producing K. pneumoniae

Among 68 MBL-producing isolates, all were resistant to imipenem (MIC_{50} >32 mg/L) and 95.6% (*n*=65) of them were resistant to aztreonam (MIC_{50} >128 mg/L). Compared with the result of aztreonam alone, the addition of avibactam resulted in a >512-fold reduction of aztreonam MIC_{50} (≤0.25 mg/L). Two dual-carbapenemase-producing *K. pneumoniae* strains had the highest aztreonam/avibactam MIC of 8 mg/L. All isolates were resistant to ceftazidime/avibactam, with an MIC_{50} of >64 mg/L, while the addition of aztreonam significantly lowered the MIC (MIC_{50} ≤0.125 mg/L) for MBL-producing isolates, including the two strains with aztreonam/avibactam MIC of 8 mg/L.

In vitro selection

We selected five MBL-producing strains, including three single MBL producers (IMP-4-producing ST1307, VIM-1-producing ST147 and NDM-1-producing ST11 strains) and the two dual carbapenemase producers (NDM-1- and OXA-48-co-producing ST101 and NDM-1- and OXA-232-co-producing ST14 strains) with aztreonam/avibactam MIC of 8 mg/L, for the *in vitro* selection experiment. The single-step selection failed to yield any colonies at 2× MIC or higher concentrations of aztreonam/avibactam. For the *in vitro* multi-step selection, the dual-carbapenemase-producing ST101 strain (namely Kp202) was successfully selected and was able to grow at concentrations up to 16× MIC (128 mg/L). However, the selection of the other four isolates was not successful and bacterial growth was not observed at 2× MIC. In addition, selection using aztreonam/ceftazidime/avibactam was not successful either.

The *in vitro*-selected Kp202 mutant culture was plated on LB agar with concentrations of aztreonam/avibactam used in the selection experiment (e.g. 2× MIC growth was plated on 16 mg/L aztreonam/avibactam agar plates). Two single colonies recovered from plates with 32 (namely Kp202_32A and Kp202_32B), 64 (Kp202_64A and Kp202_64B) and 128 mg/L (Kp202_128A and Kp202_128B) aztreonam/avibactam were randomly selected and subjected to next-generation sequencing and susceptibility testing. Susceptibility testing showed that the six mutants all demonstrated aztreonam/avibactam MICs ≥128 mg/L, while the MICs of imipenem and meropenem were decreased by ∼4-fold (Table 2). Interestingly, the aztreonam/ceftazidime/avibactam MIC also increased >8-fold for all six mutant colonies (from <0.125 to ≥16 mg/L) (Table 2).

Genomic sequencing of Kp202 and its mutants

The parental strain Kp202 had a circular chromosome of 5.46 Mbp and seven plasmids, belonging to IncA/C (pKp202_1: 179 254 bp), IncFIBkpn (pKp202_2: 158 438 bp), IncL/M (pKp202_3: 63 499 bp), IncFIA-IncR (pKp202_4: 61 718 bp) and three ColE-like groups (pKp202_5, pKp202_6 and pKp202_4: 5359, 4052 and 3541 bp, respectively). Acquired resistance gene analysis showed the parental Kp202 harboured eight different types of β-lactamase gene from the four Ambler classes, including bla_{NDM-1} (on pKp202_1), bla_{OXA-48} (pKp202_3), $bla_{CTX-M-15}$ (three copies: chromosome, pKp202_1 and pKp202_4), bla_{CMY-16} (pKp202_1), bla_{SHV-1} (chromosome), bla_{TEM-1} (two copies: pKp202_1 and pKp202_4), bla_{OXA-10} (pKp202_1) and bla_{SCO-1} (pKp202_2). In addition, Kp202 carried a mutant outer membrane protein OmpK35, with a premature stop codon at amino acid 63 due to a G deletion at nt 184, and a mutant OmpK36, with glycine and aspartic acid insertions at amino acid positions 134 and 135. We suspect the combination of diverse β-lactamases and the outer membrane protein defects may have contributed to the elevated aztreonam/avibactam MIC observed for Kp202.

A core SNP genomic analysis showed that the genomes from the six resistant colonies were nearly identical to the parental Kp202 (<2 SNPs). Further sequence inspection showed that the six mutants were all positive for bla_{OXA-48}, $bla_{CTX-M-15}$, bla_{SHV-1}, bla_{TEM-1} and bla_{OXA-10}, while Kp202_64A and Kp202_128A were negative for bla_{SCO-1}, due to the loss of the pKp202_2 plasmid in the two strains (data not shown). In addition, bla_{NDM-1} was lost in all six mutants. Examination of the bla_{NDM-1} neighbouring region in pKp202_1 showed that bla_{NDM-1} was located at a 9.3 kb region with the element *aph(3′)-VI*-IS_{Aba125}-bla_{NDM-1} flanked by two copies of *sul1*-ISCR1 (Figure 1a). A 6.45 kb fragment, encompassing *aph(3′)-VI*-IS_{Aba125}-bla_{NDM-1} and a copy of *sul1*-ISCR1, was deleted

Table 2. *In vitro* activity of different antibiotics against *K. pneumoniae* CMY-16 mutants

Strain	β-Lactamase(s)	MIC (mg/L)						
		AMP	ATM	CAZ	IPM	MEM	ATM/AVI	CAZ/ATM/AVI
K. pneumoniae strain Kp202 and the selected mutants								
Kp202	NDM-1, OXA-48, CTX-M-15, CMY-16, SHV-1, TEM-1, OXA-10, SCO-1	>1024	1024	>1024	16	16	8	≤0.125
Kp202_32A	OXA-48, CTX-M-15, CMY-16 (Tyr150Ser), SHV-1, TEM-1, OXA-10, SCO-1	>1024	1024	512	4	4	>256	16
Kp202_32B	OXA-48, CTX-M-15, CMY-16 (Tyr150Ser), SHV-1, TEM-1, OXA-10, SCO-1	>1024	1024	1024	4	4	128	32
Kp202_64A	OXA-48, CTX-M-15, CMY-16 (Asn346His), SHV-1, TEM-1, OXA-10	>1024	1024	512	4	4	>256	16
Kp202_64B	OXA-48, CTX-M-15, CMY-16 (Tyr150Ser), SHV-1, TEM-1, OXA-10, SCO-1	>1024	1024	1024	4	4	128	32
Kp202_128A	OXA-48, CTX-M-15, CMY-16 (Asn346His), SHV-1, TEM-1, OXA-10	>1024	1024	512	4	4	>256	16
Kp202_128B	OXA-48, CTX-M-15, CMY-16 (Tyr150Ser), SHV-1, TEM-1, OXA-10, SCO-1	>1024	1024	1024	4	4	128	32
E. coli DH10B CMY-16 constructs								
Ec202-WT	CMY-16 WT	512	256	>256	0.5	≤0.06	1	≤0.015
Ec202-R150	CMY-16 (Tyr150Ser)	64	256	8	0.25	≤0.06	64	1
Ec202-R346	CMY-16 (Asn346His)	256	128	64	0.125	≤0.06	16	≤0.015
DH10B	—	4	≤0.125	≤0.125	0.125	≤0.06	0.06	≤0.015

AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; AVI, avibactam; —, negative.

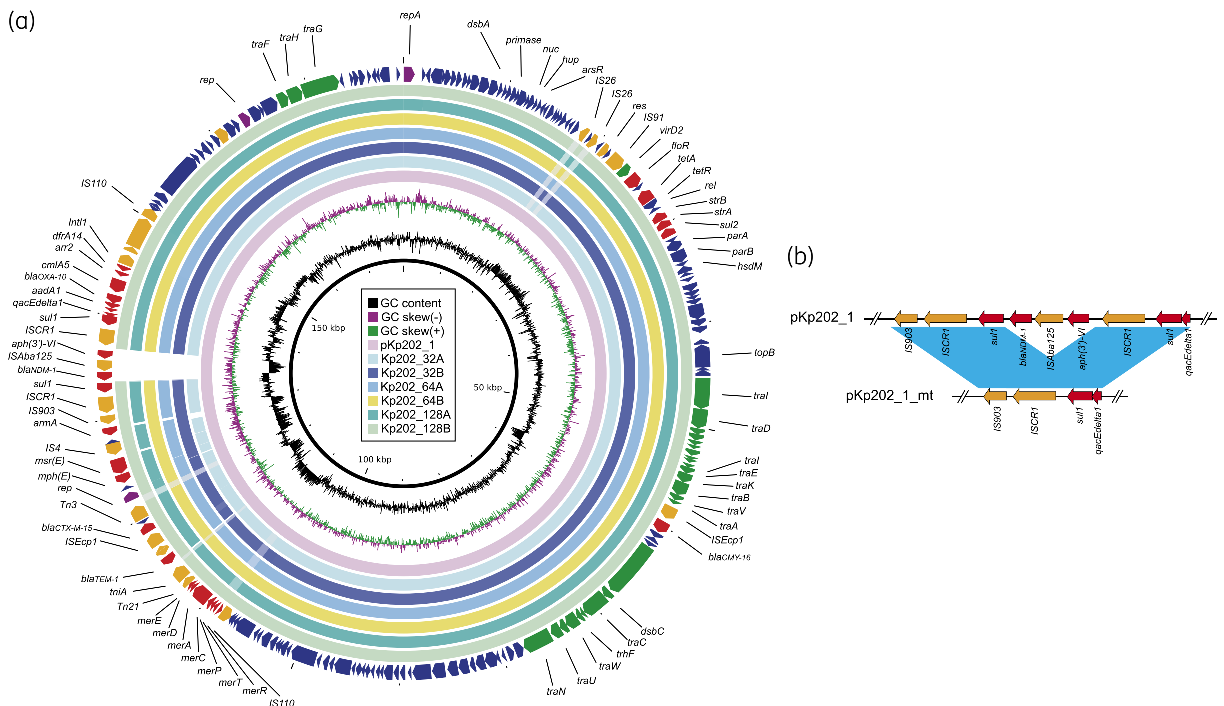

Figure 1. (a) Plasmid structure comparison between bla_{NDM-1}-harbouring pKp202_1 and the selected mutants. (b) The bla_{NDM-1} deletion in Kp202 selected mutants. Coloured arrows indicate ORFs, with purple, orange, green, red and blue arrows representing replication genes, mobile elements, plasmid transfer genes, the antimicrobial and heavy metal resistance gene, and plasmid backbone genes, respectively. Blue shading denotes regions of shared homology among different elements.

in all of the six mutants from the same site, likely through DR-mediated slippage (Figure 1b). The results indicated the elevated aztreonam/avibactam MIC level for the Kp202 selected mutants was unrelated to *bla*$_{NDM}$. Moreover, our results also indicate that the *bla*$_{NDM-1}$ in pKp202-1 was not stable under increased aztreonam challenge, as the gene was deleted in all selected mutants.

Interestingly, examination of the *bla*$_{CMY-16}$ sequence among the six mutants found that isolates Kp202_64A and Kp202_128A carried an asparagine-to-histidine substitution at Ambler amino acid position 346 (Asn346His), while the remaining four isolates harboured a tyrosine-to-serine substitution at position 150 (Tyr150Ser). We suspect that the two amino acid substitutions might have contributed to aztreonam/avibactam resistance among these mutant strains. Real-time quantitative RT–PCR did not show any significant differential expression of *bla*$_{CMY-16}$ between the parental Kp202 and the derived mutant strains (data not shown).

MICs for recombinant strains

The WT *bla*$_{CMY-16}$ and the two mutant *bla*$_{CMY-16}$ (Tyr150Ser and Asn346His) genes were cloned in plasmid vector pET28a, followed by electroporation into *E. coli* DH10B to generate constructs Ec202-WT, Ec202-R150 (Tyr150Ser) and Ec202-R346 (Asn346His), respectively. The MIC of aztreonam/avibactam increased from 1 to 64 mg/L for the CMY-16 Tyr150Ser construct (strain Ec202-R150) and from 1 to 16 mg/L for the CMY-16 Asn346His construct (strain Ec202-R346) (Table 2), indicating that the CMY-16 amino acid substitutions are responsible for the increased aztreonam/avibactam resistance. The MICs of aztreonam were not significantly affected and showed a <2-fold change in comparison with the WT CMY-16 construct (strain Ec202), suggesting that the increased aztreonam/avibactam resistance was mainly due to the reduced inhibition efficacy of avibactam against the CMY mutants. Along with the increase in aztreonam/avibactam resistance, the CMY-16 Tyr150Ser and Asn346His mutants also demonstrated lower ceftazidime MICs (>32- and >4-fold, respectively), suggesting the amino acid substitutions also reduce the ability of CMY-16 to degrade ceftazidime (Table 2).

To further evaluate the role of the CMY-16 mutations, we electroporated the CMY-16 mutant plasmid vectors into five clinical strains harbouring different carbapenemases and outer membrane porin defects. Among these transformants, the MICs of aztreonam/avibactam all increased by 16–128-fold (from ≤0.25 mg/L to 2–32 mg/L), which is consistent with the results from the *E. coli* DH10B constructs (Table 2). Interestingly, the MIC of aztreonam/ceftazidime/avibactam also increased by 256-fold for two dual-carbapenemase-producing mutant *K. pneumoniae* strains (NDM-5 and OXA-181, and NDM-1 and OXA-48) with OmpK defects (Table 3).

Discussion

As a novel non-β-lactam β-lactamase inhibitor, avibactam offers a broader β-lactamase inhibition profile than traditional β-lactamase inhibitors, such as clavulanic acid, tazobactam and sulbactam, which inactivate only specific class A enzymes. Avibactam

Table 3. *In vitro* activity of aztreonam/avibactam and ceftazidime/aztreonam/avibactam against recombinant *K. pneumoniae* strains (mg/L)

Strain	β-Lactamases	MLST ST	OmpK35/36	MIC (mg/L) ATM/AVI	MIC (mg/L) CAZ/ATM/AVI
Kp040	IMP-4, DHA-1	new	WT/WT	≤0.25	≤0.125
Kp040-CMY	IMP-4, DHA-1, CMY-16			≤0.25	≤0.125
Kp040-R150	IMP-4, DHA-1, CMY-16 (Tyr150Ser)			4	≤0.125
Kp040-R346	IMP-4, DHA-1, CMY-16 (Asn346His)			2	≤0.125
Kp189	NDM-7, CTX-M-15, SHV-11, TEM-1, OXA-1	ST16	WT/WT	≤0.25	≤0.125
Kp189-CMY	NDM-7, CTX-M-15, SHV-11, TEM-1, OXA-1, CMY-16			≤0.25	≤0.125
Kp189-R150	NDM-7, CTX-M-15, SHV-11, TEM-1, OXA-1, CMY-16 (Tyr150Ser)			4	≤0.125
Kp189-R346	NDM-7, CTX-M-15, SHV-11, TEM-1, OXA-1, CMY-16 (Asn346His)			4	≤0.125
Kp214	NDM-5, OXA-181, CTX-M-15, SHV-11, TEM-1	ST147	MT (IS*Ecp1* ins)/MT (135 DT ins)	≤0.25	≤0.125
Kp214-CMY	NDM-5, OXA-181, CTX-M-15, SHV-11, TEM-1, CMY-16			≤0.25	≤0.125
Kp214-R150	NDM-5, OXA-181, CTX-M-15, SHV-11, TEM-1, CMY-16 (Tyr150Ser)			128	32
Kp214-R346	NDM-5, OXA-181, CTX-M-15, SHV-11, TEM-1, CMY-16 (Asn346His)			64	32
Kp231	NDM-1, OXA-48, CTX-M-15, SHV-11, OXA-1	ST377	MT (IS*1* ins)/WT	≤0.25	≤0.125
Kp231-CMY	NDM-1, OXA-48, CTX-M-15, SHV-11, OXA-1, CMY-16			≤0.25	≤0.125
Kp231-R150	NDM-1, OXA-48, CTX-M-15, SHV-11, OXA-1, CMY-16 (Tyr150Ser)			128	32
Kp231-R346	NDM-1, OXA-48, CTX-M-15, SHV-11, OXA-1, CMY-16 (Asn346His)			32	32
Kp518	KPC-2, SHV-11	ST258	MT (stop)/WT	≤0.25	≤0.125
Kp518-CMY	KPC-2, SHV-11, CMY-16			≤0.25	≤0.125
Kp518-R150	KPC-2, SHV-11, CMY-16 (Tyr150Ser)			32	≤0.125
Kp518-R346	KPC-2, SHV-11, CMY-16 (Asn346His)			16	≤0.125

ATM, aztreonam; AVI, avibactam; CAZ, ceftazidime; MT, mutant type; ins, insertion; DT ins, aspartate and threonine insertion.

protects β-lactams from hydrolysis by class A (e.g. KPCs), class C (e.g. CMY) and some class D (e.g. OXA-48) enzymes. The combination of aztreonam/avibactam presents a novel approach to the treatment of infections caused by pathogens containing multiple β-lactamases, including isolates carrying MBLs. Our *in vitro* susceptibility testing also indicated that aztreonam/avibactam and ceftazidime/avibactam/aztreonam are potent against MBL-producing *K. pneumoniae*, including isolates co-harbouring more than one carbapenemase.

Our results demonstrated that selection of aztreonam/avibactam resistance is associated with mutations in bla_{CMY} (Tyr150Ser or Asn346His) during *in vitro* selection. In a previous study on the binding analysis of avibactam to *P. aeruginosa* AmpC β-lactamase, Lahiri *et al.*[18] identified eight key conserved amino acid residues (Ser64, Lys67, Gln120, Tyr150, Asn152, Lys315, Thr316 and Asn346) in chromosomal and plasmid AmpC β-lactamase enzymes that contribute to the binding interaction of avibactam with AmpC β-lactamase and other β-lactamases. The carboxamide group of avibactam interacted with the side chains of Asn152 and Gln120, and the sulphate moiety was positioned by Thr316, Lys315 and Asn346, whereas Tyr150 and Lys67 were positioned to participate in catalytic roles to enable formation of the covalent bond with Ser64.[18] The authors identified two amino acids substitutions (Asn346Tyr and Tyr150Ser) in *Citrobacter freundii* chromosomal AmpC and *E. coli* plasmid-encoded CMY-6 proteins through *in vitro* selection experiments.[18] The Tyr150Ser mutant significantly lowered the ability of avibactam against the hydrolysis of aztreonam, increasing the MIC value by 16-fold for the selected mutant strain, which is consistent with our results, except that we identified the same Tyr150Ser substitution in a different CMY-16 variant (Table 2). In addition, the authors suggested that the Asn346Tyr substitution, because of the increased size of the Tyr residue, could result in a steric clash with the sulphate group of avibactam, thus influencing the binding affinity of this inhibitor.[18] We suspect the Asn346His substitution described in this study may use a similar mechanism to reduce the binding to avibactam. Moreover, the Asn346His substitution also changed the charge from neutral to positive, which may interfere with the binding of avibactam.

Recent studies showed that ceftazidime/avibactam in combination with aztreonam demonstrated excellent *in vitro* synergistic activity against and bactericidal effect on MBL-producing Enterobacteriaceae and *P. aeruginosa* strains.[19,20] The success of treatment by ceftazidime/avibactam/aztreonam against MBL-producing strains has also been described in several clinical cases.[11,21,22] Our *in vitro* study also showed that the combination of ceftazidime/avibactam/aztreonam had great activity against MBL-producing *K. pneumoniae*, including strains co-producing more than one carbapenemase (Table 1). However, the Kp202 CMY-16 mutants showed more than 128-fold increases in ceftazidime/avibactam/aztreonam MIC (Table 2). In addition, the transformants of CMY-16 Asn346Tyr and Tyr150Ser of two clinical carbapenem-resistant *K. pneumoniae* also showed increased resistance to ceftazidime/avibactam/aztreonam, indicating that this triple combination may lose the activity in carbapenem-resistant *K. pneumoniae* strains with AmpC mutations and outer membrane porin defects. However, our results also suggested that the usage of the triple combination of ceftazidime/avibactam/aztreonam may lower the chance of bla_{CMY-16} mutations arising.

Taken together, our study showed that the aztreonam/avibactam combination is active against MBL-producing *K. pneumoniae*, including strains co-producing more than one carbapenemase. However, carbapenemase- and AmpC-co-producing strains showed selected resistance to aztreonam/avibactam as well as ceftazidime/avibactam/aztreonam. In order to prevent selection resistance in clinical practice, rational strategies for employing different combinational agents and dosing regimens that optimize pharmacokinetic/pharmacodynamic target attainment should be considered.

Funding

This work was in part supported by grants from the National Institutes of Health (R01AI090155 to B.N.K. and R01AI100560, R01AI063517 and R01AI072219 to R.A.B.). This work was in part supported by the Science and Technology Research Program of Chongqing Municipal Education Commission (KJ1702022) and the Medical Research Program of Chongqing Health and Family Planning Commission in 2016 (2016MSXM001).

Transparency declarations

None to declare.

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