


In vitro* activity of apramycin against multidrug-, carbapenem- and aminoglycoside-resistant Enterobacteriaceae and *Acinetobacter baumannii

Mario Juhas¹, Emma Widlake², Jeanette Teo³, Douglas L. Huseby⁴, Jonathan M. Tyrrell², Yury S. Polikanov⁵, Onur Ercan⁴, Anna Petersson⁴, Sha Cao⁴, Ali F. Aboklaish², Anna Rominski¹, David Crich⁶, Erik C. Böttger¹, Timothy R. Walsh², Diarmaid Hughes⁴  ⁴† and Sven N. Hobbie^{1*†}

¹Institute of Medical Microbiology, University of Zürich, Gloriastrasse 30, CH-8006 Zürich, Switzerland; ²Division of Infection and Immunity, School of Medicine, Cardiff University, Cardiff CF14 4XN, UK; ³Department of Laboratory Medicine, National University Hospital, 5 Lower Kent Ridge Road, 119074 Singapore, Singapore; ⁴Department of Medical Biochemistry and Microbiology, Uppsala University, Husargatan 3, 75123 Uppsala, Sweden; ⁵Department of Biological Sciences, College of Liberal Arts and Sciences, University of Illinois at Chicago, 900 South Ashland Avenue, MBRB 4170, Chicago, IL 60607, USA; ⁶Department of Chemistry, Wayne State University, 5101 Cass Ave, Detroit, MI 48202, USA

*Corresponding author. Tel: +41 44 634 2284; E-mail: sven.hobbie@uzh.ch

†These authors made an equal contribution to this work.

Received 13 August 2018; returned 2 October 2018; revised 17 November 2018; accepted 29 November 2018

Objectives: Widespread antimicrobial resistance often limits the availability of therapeutic options to only a few last-resort drugs that are themselves challenged by emerging resistance and adverse side effects. Apramycin, an aminoglycoside antibiotic, has a unique chemical structure that evades almost all resistance mechanisms including the RNA methyltransferases frequently encountered in carbapenemase-producing clinical isolates. This study evaluates the *in vitro* activity of apramycin against multidrug-, carbapenem- and aminoglycoside-resistant Enterobacteriaceae and *Acinetobacter baumannii*, and provides a rationale for its superior antibacterial activity in the presence of aminoglycoside resistance determinants.

Methods: A thorough antibacterial assessment of apramycin with 1232 clinical isolates from Europe, Asia, Africa and South America was performed by standard CLSI broth microdilution testing. WGS and susceptibility testing with an engineered panel of aminoglycoside resistance-conferring determinants were used to provide a mechanistic rationale for the breadth of apramycin activity.

Results: MIC distributions and MIC₉₀ values demonstrated broad antibacterial activity of apramycin against *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Morganella morganii*, *Citrobacter freundii*, *Providencia* spp., *Proteus mirabilis*, *Serratia marcescens* and *A. baumannii*. Genotypic analysis revealed the variety of aminoglycoside-modifying enzymes and rRNA methyltransferases that rendered a remarkable proportion of clinical isolates resistant to standard-of-care aminoglycosides, but not to apramycin. Screening a panel of engineered strains each with a single well-defined resistance mechanism further demonstrated a lack of cross-resistance to gentamicin, amikacin, tobramycin and plazomicin.

Conclusions: Its superior breadth of activity renders apramycin a promising drug candidate for the treatment of systemic Gram-negative infections that are resistant to treatment with other aminoglycoside antibiotics.

Introduction

Infectious diseases, particularly those caused by MDR pathogens, carbapenemase-producing Enterobacteriaceae (CPE) and carbapenemase-producing *Acinetobacter baumannii* (CPA), remain a major cause of morbidity and mortality worldwide.^{1–4}

Widespread antimicrobial resistance often limits the availability of therapeutic options to only a very few efficacious antibiotics.^{5,6} Last-resort drugs such as tigecycline and colistin are themselves increasingly challenged by emerging resistance⁷ and

compromised by significant adverse side effects.^{3,8} New therapeutic treatment options are therefore urgently needed.

Aminoglycoside antibiotics inhibit the essential process of protein biosynthesis^{9,10} by targeting the bacterial ribosome, one of the most effective targets in anti-infective history when looking back at seven decades of clinical track record.¹¹ Aminoglycoside antibiotics are recognized for rapid bactericidal activity in serious systemic infections.¹² Clinically relevant aminoglycoside antibiotics, however, are equally and increasingly challenged by emerging resistance¹³ due to aminoglycoside-modifying enzymes, and RNA methyltransferases frequently encountered in carbapenem-resistant isolates.¹⁴

Apramycin is a monosubstituted deoxystreptamine that differs in its chemical structure from clinically relevant aminoglycoside antibiotics that are all disubstituted (Figure 1). Its unique structure renders the apramycin molecule intrinsically resilient to almost all resistance determinants typically found in MDR and XDR Gram-negative bacteria.^{15–17} Apramycin has also been demonstrated to be of lower toxicity than other aminoglycosides of the disubstituted deoxystreptamine drug class.¹⁸

Here we report on a broad *in vitro* activity of apramycin against a variety of clinical isolates from patients in Europe, Asia, Africa and South America, including MDR, CPE and CPA. Furthermore, we elucidated the genotype of selected clinical isolates and tested engineered resistant strains to provide a mechanistic rationale for the superior spectrum of apramycin activity when compared with clinical benchmark drugs.

Materials and methods

Bacterial isolates

A total of 1132 Enterobacteriaceae and 100 *A. baumannii* clinical isolates were investigated in this study. The isolates were collected between 2014 and 2017 by clinical laboratories in Europe, Asia, Africa and South America, and donated without patient information.

Antimicrobial agents

Antimicrobial standards were obtained from the European Pharmacopoeia with microbiological potency values provided in each certificate of analysis. A microbiological potency value was not provided for apramycin sulphate purchased from Sigma-Aldrich. The lot-specific assay of active free base was therefore accurately determined by quantitative NMR as 554 µg of apramycin free base per mg of sulphate salt 'as is'. Stock solutions were prepared according to CLSI guideline M07.¹⁹

Aminoglycoside susceptibility testing

CLSI broth microdilution reference methodology M07¹⁹ was used to determine MICs of antimicrobial agents. The MIC values were assessed by visual inspection with the help of an MIC 2000 Illuminated viewer (Dynatech, Kloten, Switzerland). All susceptibility testing assays were quality controlled against *Escherichia coli* ATCC 25922. The MIC quality control range for apramycin was determined as 2–8 mg/L (modal MIC of 4 mg/L). For all other drugs, the quality control was run against CLSI performance standards for antimicrobial susceptibility testing (M100–S25). The most recent EUCAST interpretive guidelines (www.eucast.org/clinical_breakpoints) were used to determine EUCAST breakpoints for the categorical interpretations of susceptibility and resistance for gentamicin, tobramycin, kanamycin and amikacin. For the non-clinical aminoglycoside apramycin, the MIC distributions presented in this study were used to define the following tentative ECOFFS

(epidemiological cut-off values) as interpretative criteria of susceptibility and resistance: 4 mg/L for *K. pneumoniae* and *Enterobacter* spp., 8 mg/L for *E. coli* and 16 mg/L for *A. baumannii*.

Whole-genome sequencing

Clinical isolates of interest were analysed by MALDI-TOF and submitted to IIT Biotech GmbH, Bielefeld, Germany, for WGS and an ARG-ANNOT database search for antibiotic resistance genes. MALDI-TOF analysis, WGS and bioinformatics annotation of resistance genes were performed as described previously.^{20–22}

Construction of a strain panel expressing aminoglycoside resistance determinants

The amino acid sequence of all relevant aminoglycoside resistance genes was identified by reference genes listed in Table S1 (available as [Supplementary data](#) at JAC Online) and cloned downstream of an insulated constitutive promoter of defined promoter strength.²³ Each resistance cassette was then cloned into a low-copy plasmid with a pBR322 origin of replication, an ampicillin resistance cassette and a T7 terminator sequence downstream of the resistance gene. Chemically competent *E. coli* DH10B-derived TOP10 cells (Invitrogen) were transformed with the resulting expression plasmids, colony purified, quality-controlled by sequence analysis and antimicrobial susceptibility assessed by standard broth microdilution assays.¹⁹

Results

Broad antibacterial activity of apramycin

Apramycin demonstrated broad activity against all bacterial species tested, including MDR, CPE and CPA clinical isolates, collected between 2014 and 2017 in Europe, Asia, Africa and South America (Table 1).

Apramycin exhibited significantly better antimicrobial activity against *E. coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Providencia* spp., *Morganella morganii*, *Citrobacter freundii*, *Proteus mirabilis*, *Serratia marcescens* and clinical isolates of the *A. baumannii* complex than the clinical standard-of-care aminoglycosides gentamicin and amikacin (Table 1). An apramycin concentration of 8 mg/L inhibited 98% of all Enterobacteriaceae isolates tested. An apramycin concentration of 16 mg/L inhibited 97% of all *A. baumannii* isolates tested. At the level of individual Enterobacteriaceae species, an apramycin concentration of 4 mg/L inhibited 99% of *K. pneumoniae* and 93% of *Enterobacter* isolates. An apramycin concentration of 8 mg/L inhibited 99% of all *E. coli*, 98% of *C. freundii*, 96% of *Providencia* spp., 92% of *S. marcescens*, 97% of *M. morganii* and 100% of *P. mirabilis* clinical isolates (Table 1). In comparison, the MIC₉₀ of amikacin and gentamicin was >64 mg/L for most of the Enterobacteriaceae species tested (Table 1). The epidemiological distribution of MIC values highlighted the uncompromised *in vitro* activity of apramycin against clinical isolates of Enterobacteriaceae and *A. baumannii* (Figure 2 and Figures S1 and S2).

High activity of apramycin against CPE and *A. baumannii*

In addition to looking at all isolates collectively, we also analysed the activity of apramycin against the carbapenemase-producing subpopulation of isolates representing the most urgent unmet medical need.⁴ A total of 406 carbapenemase-producing *E. coli*,

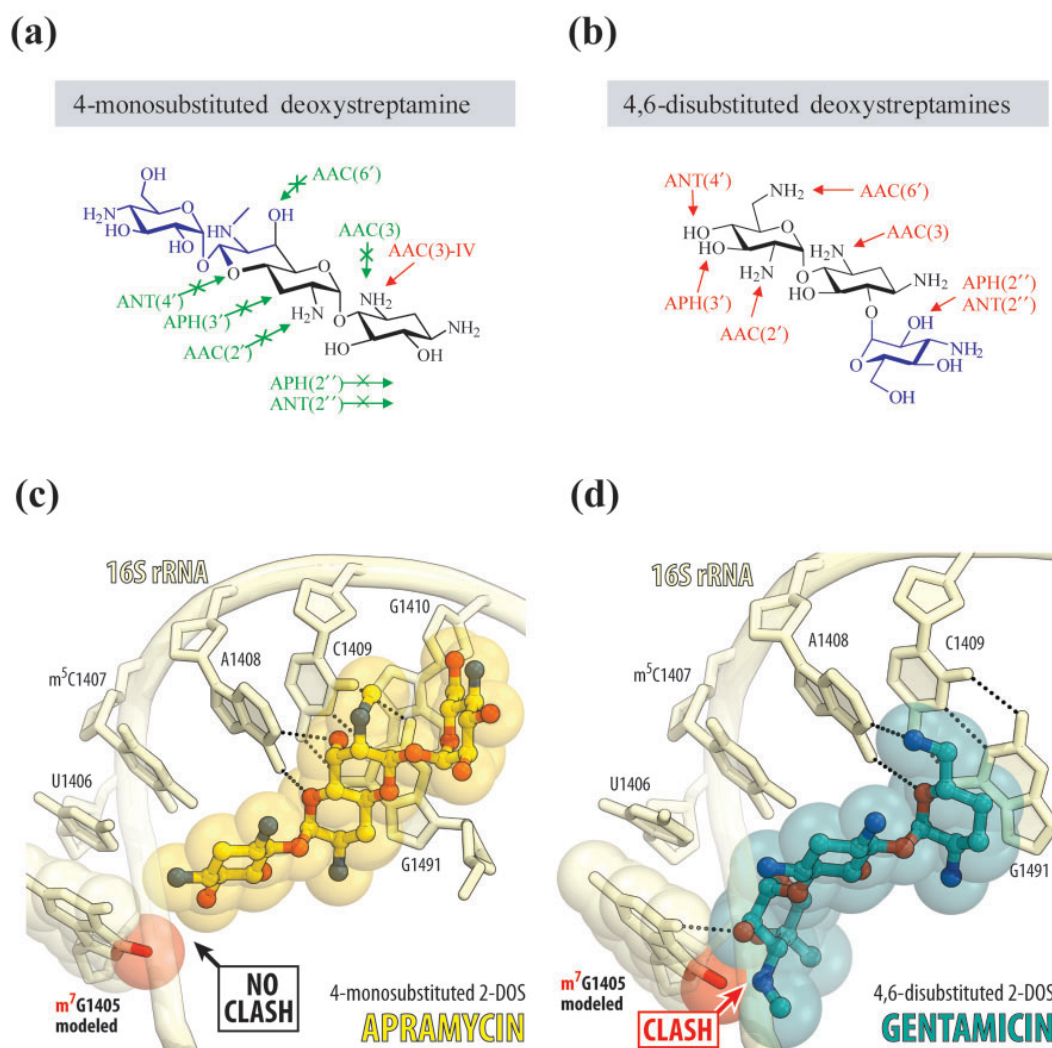


Figure 1. Structural rationale for the activity of apramycin in the presence of aminoglycoside resistance determinants. The chemical structures of monosubstituted (a) versus disubstituted (b) deoxystreptamine antibiotics indicating the reactive groups that are modified by acetyltransferases (AACs), phosphotransferases (APHs) and nucleotidyltransferases (ANTs). Molecular modelling of the RMTase-catalysed N7-methylation of G1405 (red sphere) onto the crystal structure of ribosome-bound apramycin (PDB entry 4AQY) reveals no clash of the methyl group with the 4-monosubstituted 2-DOS apramycin (c). Molecular modelling of the G1405 methylation onto the crystal structure of ribosome-bound gentamicin (PDB entry 4V53) reveals considerable clash with ring III of 4,6-disubstituted 2-DOS (d). Nucleotides of the 16S rRNA are shown in pale yellow, and apramycin and gentamicin are shown in yellow and teal, respectively. The *E. coli* nucleotide numbering is used throughout.

K. pneumoniae, *Enterobacter*, *C. freundii* and *Providencia* spp. isolates and 17 CPA were investigated in this study. Apramycin concentrations of 4 and 8 mg/L inhibited 93% and 97% of all CPE isolates, respectively. A concentration of 16 mg/L was required to inhibit 94% of CPA isolates (Table 1). In comparison, gentamicin and amikacin showed little activity against these isolates. In plotting the MIC distribution of apramycin for only CPE and CPA, the superiority of apramycin over clinical benchmark aminoglycosides became even more apparent (Figure 2). Against individual CPE species, apramycin inhibited 97% of *K. pneumoniae* and 94% of *C. freundii* clinical isolates at ≤ 4 mg/L, and 94% of *E. coli* and 93% of *Enterobacter* clinical isolates at ≤ 8 mg/L. In comparison, gentamicin and amikacin were found to

be inactive against a remarkable proportion of carbapenemase-producing *E. coli*, *K. pneumoniae*, *Enterobacter*, *C. freundii* and *Providencia* spp. clinical isolates (Figure 2). The higher activity of apramycin against individual CPE species became particularly apparent when plotting the MIC distribution (Figure S1).

Importantly, and in contrast to the clinically approved antibiotics gentamicin, kanamycin, tobramycin, amikacin, plazomicin and meropenem,^{24–27} apramycin was active against *E. coli* isolates that were characterized by WGS as encoding carbapenemases NDM-1, OXA-23 and IMP-1, which are the main causes of carbapenem resistance in CPE strains (Table 2).^{28,29} Apramycin was likewise active against *K. pneumoniae* strains encoding

Table 1. MIC₉₀ of apramycin in comparison with gentamicin and amikacin against clinical isolates of Enterobacteriaceae and *A. baumannii* isolated between 2014 and 2017

Species	No.	MIC ₉₀ (mg/L)		
		APR	GEN	AMK
Enterobacteriaceae (all)	1132	8	>64	>64
<i>Escherichia coli</i>	250	8	>64	>64
<i>Klebsiella pneumoniae</i>	372	4	>64	>64
<i>Enterobacter</i> spp.	179	4	>64	>64
<i>Morganella morganii</i>	37	8	>64	4
<i>Citrobacter freundii</i>	131	8	>64	>64
<i>Providencia</i> spp.	80	8	>64	>64
<i>Proteus mirabilis</i>	32	8	>64	>64
<i>Serratia marcescens</i>	51	8	>64	>64
CPE only (all)	406	4	>128	>128
<i>Escherichia coli</i>	74	8	>128	>128
<i>Klebsiella pneumoniae</i>	236	4	>128	>128
<i>Enterobacter</i> spp.	48	8	>128	>128
<i>Citrobacter freundii</i>	48	4	>128	>128
<i>A. baumannii</i>	100	16	>64	>64
CPA only	17	16	>256	>256
Geographic origin				
Europe	799	8	>64	>64
Asia	240	8	>256	>256
Africa	107	8	>256	>256
South America	86	4	>256	>256

APR, apramycin; AMK, amikacin; GEN, gentamicin; CPE, carbapenemase-producing Enterobacteriaceae; CPA, carbapenemase-producing *A. baumannii*.

carbapenemases KPC-2, OXA-181, OXA-232 and OXA-48 (Table 2), and against *E. coli* strains harbouring the plasmid-mediated colistin resistance gene, *mcr-1*.³⁰

Genotypic rationale for superior antibacterial activity

To assess the potency of apramycin against MDR bacteria, we compared the susceptibility and MIC distribution of MDR versus non-MDR clinical isolates of *E. coli*, *K. pneumoniae* and *E. cloacae* against a panel of aminoglycoside antibiotics. Clinical isolates in this analysis were classified as MDR if expressing resistance to at least three out of the following five drug classes: third-generation cephalosporins, quinolones, aminoglycosides, carbapenems and a β-lactam antibiotic with a β-lactamase inhibitor, namely piperacillin/tazobactam.³¹ The distribution of MICs for MDR clinical isolates was shifted to higher MIC values for the analysed benchmark aminoglycoside antibiotics, amikacin, gentamicin, kanamycin and tobramycin, when compared with non-MDR clinical isolates, but not for apramycin (Figure S3).

WGS of MDR clinical isolates of *E. coli*, *E. cloacae* and *K. pneumoniae* revealed the genotypes associated with the phenotypic resistance profiles of the analysed clinical isolates. The identified genotypes corresponded closely to the resistance phenotypes predicted by the aminoglycoside antibiogram (Figure 1). A broad variety of aminoglycoside resistance determinants and combinations

thereof were identified in the genomes of the sequenced MDR clinical isolates including several distinct subtypes of acetyltransferases (AACs), phosphotransferases (APHs), nucleotidyltransferases (ANTs) and 16S rRNA methyltransferases (RMTases) (Table 3).

The predominant resistance mechanisms identified were those facilitated by the acetyltransferases AAC(6') and AAC(3), which accounted for the majority of phenotypic non-susceptibility to gentamicin, tobramycin, amikacin and kanamycin (Table 3). The nucleotidyltransferase ANT(2''), phosphotransferase APH(3') and the RMTase-encoding genes *armA*, *rmtB* and *rmtC* were also found in clinical *E. coli*, *K. pneumoniae*, *Enterobacter* and *A. baumannii* isolates (Table 3). As predicted, the presence of these antibiotic resistance genes in the genomes of the analysed MDR clinical isolates did not negatively impact the antibacterial activity of apramycin. In conclusion, although many of the MDR clinical isolates analysed were resistant to benchmark aminoglycoside antibiotics, they retained a susceptible phenotype to apramycin when applying the ECOFF of 8 mg/L for *E. coli*, 4 mg/L for *K. pneumoniae* and *Enterobacter* spp., and 16 mg/L for *A. baumannii* (Table 3).

Apramycin evades almost all aminoglycoside resistance mechanisms

The high genotypic diversity and the presence of multiple antibiotic resistance determinants within individual isolates may in part blur a more mechanistic correlation between phenotypic resistance and individual resistance genes. Key resistance genes were therefore cloned into a DH10B-derived *E. coli* lab strain to allow promoter-controlled expression of individual resistance genes in an otherwise isogenic background. This enabled elucidation of the apramycin activity in the presence of individual and well-defined resistance genes including AACs, APHs, ANTs and RMTases. This analysis revealed high activity of apramycin against all known resistance mechanisms, with the exception of AAC(3)-IV (Table 4). In contrast, the clinical aminoglycosides gentamicin, amikacin, tobramycin and amikacin were each inactivated by a variety of resistance mechanisms as expected.³²⁻³⁴ Susceptibility to amikacin was reduced in the presence of AAC(6') and some subtypes of APH(3') and, similarly to plazomicin, was fully abolished in the presence of RMTases. When comparing the resistance profile of plazomicin with that of amikacin, it became apparent that susceptibility to plazomicin was retained in the presence of AAC(6') and APH(3'), but at the cost of lower susceptibility to plazomicin than amikacin in the presence of AAC(2') and APH(2''). The superiority of apramycin to other aminoglycosides was particularly remarkable for RMTases. Engineered *E. coli* strains expressing *armA*, *rmtB*, *rmtC* or *rmtF* genes were highly resistant (MIC >64 mg/L) to all other aminoglycoside antibiotics tested, including plazomicin, while remaining susceptible to apramycin (Table 4). The only resistance determinant investigated in this study that rendered the host *E. coli* cells non-susceptible to apramycin was the aminoglycoside 3-N-acetyltransferase subtype IV [AAC(3)-IV] (Table 4), a resistance mechanism that was previously known to confer apramycin resistance.³⁵

Discussion

The results presented in this study clearly demonstrate that apramycin has a broad antibacterial activity against a variety of clinical

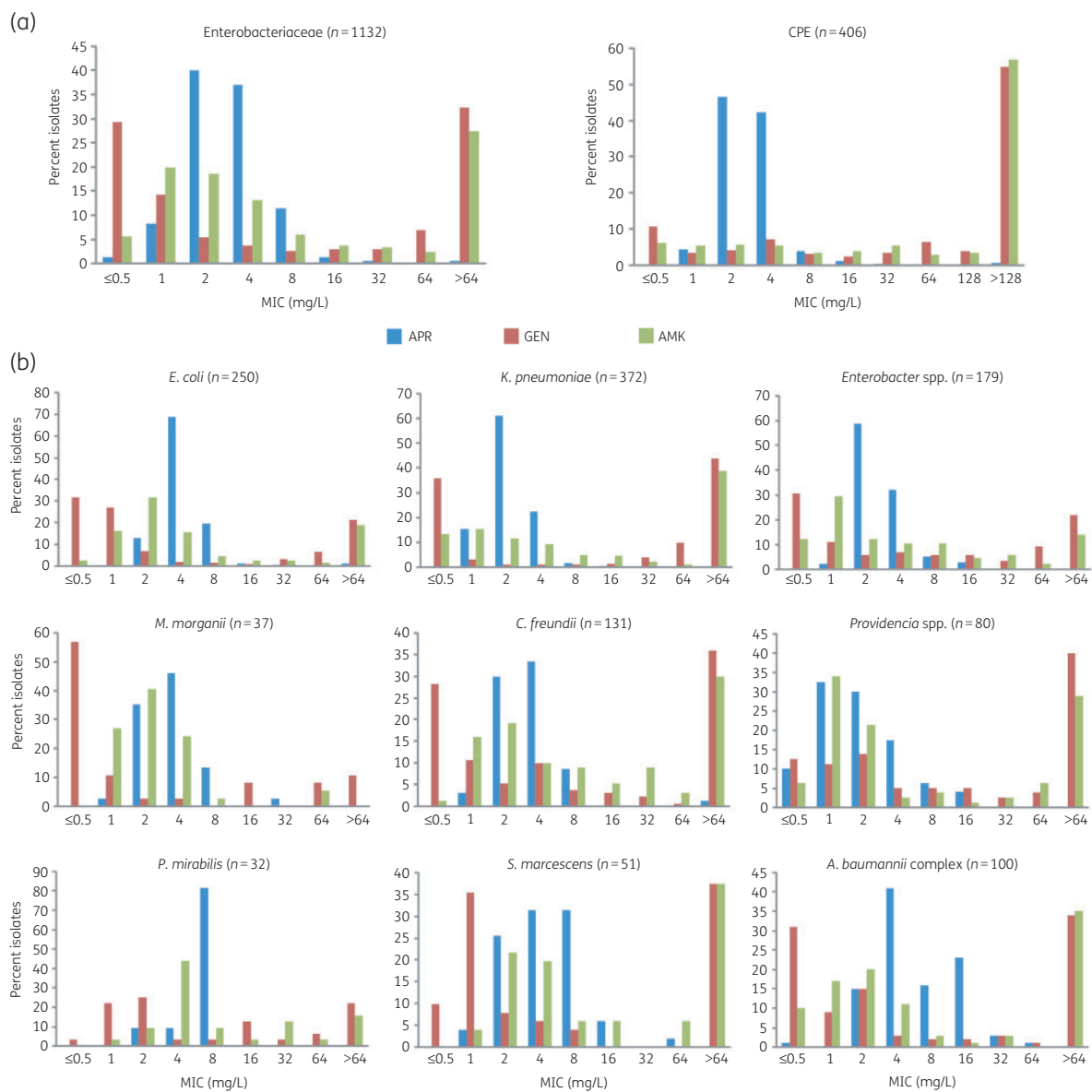


Figure 2. MIC distribution of apramycin in comparison with gentamicin and amikacin. (a) MIC distribution for Enterobacteriaceae clinical isolates of diverse geographic origin collected between 2014 and 2017 (left), and a subset of only carbapenemase-producing Enterobacteriaceae (CPE, right). (b) MIC distribution for Enterobacteriaceae and *A. baumannii* at the genus or species level. APR, apramycin; AMK, amikacin; GEN, gentamicin; CPE, carbapenemase-producing Enterobacteriaceae. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

isolates, including MDR, CPE and CPA clinical isolates of diverse geographic origin (Table 1). This is consistent with previous reports, which showed that the vast majority of the analysed CPE clinical isolates from the USA, the UK and China were more susceptible to apramycin than to other aminoglycosides.^{16,28,36} Our analysis provides strong evidence that the high susceptibility to apramycin is a widespread phenomenon and not geographically restricted.

Apramycin retains significant activity against a broad spectrum of MDR and XDR clinical isolates, including those with NDM-1,

IMP-1, OXA-23, OXA-48, OXA-181, OXA-232 and KPC-2 genotypes (Tables 1 and 2).^{28,29} Previously, it was shown that in comparison with amikacin, gentamicin and tobramycin, apramycin is highly active against MDR, XDR and pan-drug-resistant (PDR) clinical isolates that include *A. baumannii* and *P. aeruginosa*.¹⁵ Furthermore, our results also demonstrate uncompromised activity of apramycin against *E. coli* clinical isolates harbouring the plasmid-borne resistance gene *mcr-1*³⁰ (Table 2), which confers resistance to the last-resort drug colistin.

Table 2. Antibiotic susceptibility of selected genotypes of *E. coli* and *K. pneumoniae* clinical isolates isolated between 2014 and 2017

Organism	Characteristic	MIC (mg/L)						
		APR	GEN	KAN	TOB	AMK	PLZ	MEM
<i>E. coli</i>	NDM-1	8	>128	>128	>128	>128	>128	>128
	IMP-1	4	32	128	32	8	4	8
	MCR-1	2-4	2	>128	0.5	1	2	0.125
	OXA-23	8	64	>128	16	4	2	8
<i>K. pneumoniae</i>	KPC-2	2	128	>128	>128	>128	>128	128
	OXA-181	2	64	128	64	8	1-2	4
	OXA-232	2	>128	>128	>128	>128	>128	128
	OXA-48	2	128	>128	>128	>128	1	64

APR, apramycin; GEN, gentamicin; KAN, kanamycin; TOB, tobramycin; AMK, amikacin; PLZ, plazomicin; MEM, meropenem.

Perhaps the most striking observation is that the presence of various antibiotic resistance-conferring genes, such as those encoding aminoglycoside-modifying enzymes and RMTases, did not adversely affect the distribution of apramycin MICs in the analysed clinical isolate panels. Apramycin remained active against *E. coli* harbouring various individual aminoglycoside resistance determinants, including those which rendered the host *E. coli* cells resistant against all other antibiotics tested (Tables 3 and 4). The only mechanisms found to induce significant resistance to apramycin was that of the acetyltransferase AAC(3)-IV (Table 4), which has previously been identified as a pre-determinant of resistance to apramycin and other aminoglycosides.³⁷

Regio-specific acetylation of the 3-amino group is catalysed by a family of acetyltransferases that for the most part do not seem to recognize apramycin as a substrate. To date, AAC(3)-IV is the only subtype known to acetylate apramycin efficiently. Acetylation at C3 not only replaces an amino group with a less basic amide, but may also prevent its binding to the drug target site by means of a steric clash with the phosphate backbone of 16S rRNA (Figure S4).

Of 1232 clinical isolates tested in this study, 4 isolates (<0.5%) were found to be resistant to all antibiotics tested including apramycin. The fact that apramycin resistance was found to be rare in a relevant panel of clinical isolates suggests a very high (>99%) probability of coverage in empirical treatment of MDR pathogens. WGS of the four isolates resistant to apramycin revealed the presence of a variety of resistance genes including *aac(3)-IV* (Table S2), which was in agreement with the genotypic prediction inferred from the results with engineered *E. coli* strains (Table 4). The fact that resistance to apramycin was exclusively associated with an *aac(3)-IV*-positive genotype in all cases suggests feasibility of a PCR detection kit as a more rapid diagnostic than bacterial culture or WGS. Among over a thousand clinical isolates from across the globe included herein, no other apramycin resistance determinants were found.

A literature search for additional mechanisms of apramycin resistance suggested acetylation of the 1-amino group as another possible mechanism of apramycin resistance.^{38,39} However, little clinical or other evidence has since corroborated the hypothesis. Putative AAC(1) enzymes were found to acetylate aminoglycoside antibiotics inconsistently.⁴⁰ Structural modelling suggests that the drug-binding pocket can accommodate an acetamide at C1

Table 3. Phenotypes associated with the predominant aminoglycoside resistance determinants found in the analysed bacterial strains by WGS

Genotype	Resistance phenotype				
	APR	GEN	AMK	KAN	TOB
<i>Escherichia coli</i>					
AAC(3)-II	S	R	S	I	I
AAC(6')-I	S	S	I	R	R
AAC(6')-I ANT(2'')	S	R	R	R	R
AAC(6')-I AAC(3)-II	S	R	I	R	R
<i>rmtB</i> AAC(3)-II APH(3')	S	R	R	R	R
<i>Klebsiella pneumoniae</i>					
ANT(2'')	S	I	I	R	I
AAC(3)-II APH(3')	S	R	S	R	I
AAC(6')-I APH(3')	S	S	I	R	R
AAC(6')-I AAC(3)-II	S	R	I	R	R
AAC(6')-I AAC(3)-II APH(3')	S	R	I	R	R
<i>rmtB</i> AAC(6')-I AAC(3)-II	S	R	R	R	R
<i>Enterobacter cloacae</i>					
ANT(2'')	S	I	I	I	I
AAC(6')-I AAC(3)-II	S	R	S	R	R
AAC(6')-I ANT(2'')	S	I	I	R	R
AAC(6')-I AAC(3)-V ANT(2'')	S	R	I	R	R
AAC(6')-I AAC(3)-V APH(3')	S	R	R	R	R
<i>rmtC</i> AAC(6')-I AAC(3)-II	S	R	R	R	R
<i>Acinetobacter baumannii</i>					
<i>armA</i>	S	R	R	R	R
AAC(6')-I	S	I	R	R	R
APH(3')-I <i>armA</i>	S	R	R	R	R
AAC(6')-I APH(3')	S	I	R	R	R
AAC(3)-I <i>armA</i>	S	R	R	R	R
APH(3')-VI AAC(3)-I	S	R	R	R	S
APH(3')-VI AAC(3)-I ANT(2'')	S	R	R	R	R
AAC(6')-I AAC(3)-I APH(3')	S	R	R	R	R

APR, apramycin; GEN, gentamicin; AMK, amikacin; KAN, kanamycin; TOB, tobramycin.

In the case of apramycin, ECOFFs have been used as interpretative criteria.

Table 4. Apramycin activity in comparison with gentamicin, amikacin, tobramycin and plazomicin against engineered *E. coli* strains expressing individual aminoglycoside resistance mechanisms

Resistance mechanism	MIC (mg/L)				
	APR	GEN	AMK	TOB	PLZ
None	4	0.5	1–2	0.5	0.5
AAC(6′)-I	4	2	64	32–64	0.5
AAC(6′)-II	4	64	8	32–64	1
AAC(3)-I	8	>64	1–2	1	0.5–1
AAC(3)-II	8	>64	1	32	4
AAC(3)-III	4	>64	0.5–1	>64	0.5
AAC(3)-IV	>64	2	1–2	2	0.5
AAC(3)-VI	4	>64	1–2	4	1
AAC(2′)-I	2–4	4	1–2	8–16	8–16
APH(3′)-I	2	1–2	1–2	8	0.5
APH(3′)-II	4	0.5	8	0.5	0.5
APH(3′)-III	4	0.5	32	4–8	0.5
APH(3′)-VI	4	0.5	64	0.5	0.5–1
APH(2′′)-II	2–4	>64	2–4	64	8
APH(2′′)-IV	4	>64	1–2	32–64	8
ANT(4′)-II	2–4	0.5	1–2	0.5	0.5–1
ANT(2′′)-I	4	16–32	1	16–32	0.5
<i>armA</i>	2–4	>64	>64	>64	>64
<i>rmtB</i>	4	>64	>64	>64	>64
<i>rmtC</i>	2–4	>64	>64	>64	>64
<i>rmtF</i>	2–4	>64	>64	>64	>64

APR, apramycin; GEN, gentamicin; AMK, amikacin; TOB, tobramycin; PLZ, plazomicin.

without a prohibitive steric clash, unlike acetylation at C3 discussed above (Figure S4). This is in agreement with the fact that aminoglycosides such as amikacin, arbekacin or plazomicin, with the bulkier L-4-amino-2-hydroxybutyramide amide group at C1, are accepted by the drug-binding pocket without significant loss of activity when compared with the parent amines. Any loss of activity on acetamide formation at the 1-position would therefore be likely to arise owing to the loss of the basic amine (unlike amikacin, arbekacin and plazomicin in which the functionalized amide group itself carries a basic amine). The existence of such AAC(1) resistance mechanisms, however, has yet to be confirmed independently. Phylogenetic alignment of amino acid sequences revealed a very high sequence homology of putative AAC(1) sequences with AAC(3)-I proteins (Figure S5). Further studies are needed to re-analyse the functional conversion of aminoglycosides in the presence of AAC(1) proteins.

The high activity of apramycin against MDR, CPE and CPA clinical isolates is owing to the ability of apramycin both to evade aminoglycoside-modifying enzymes and to bind to a methylated drug target site.¹⁸ Drug target methylation at ribosomal site N7-G1405 by RMTases encoded by *armA* and *rmtC*, and related antibiotic resistance-conferring enzymes effectively distorts the binding pocket for clinically approved aminoglycoside antibiotics, including plazomicin (Figure 1).^{41,42} Consequently, although retaining antibiotic activity against many aminoglycoside-modifying enzymes, plazomicin is inactive against drug-resistant clinical

isolates encoding RMTases.⁴³ Methylation of N7-G1405 is frequently encountered in CPE clinical isolates^{17,36,44–47} and is therefore often associated with the resistance of CPE to all of the clinically relevant aminoglycoside antibiotics. In contrast, the binding and activity of apramycin are not prevented by methylation of N7-G1405.¹⁸ The crystal structure of apramycin bound to its target site (Figure 1) reveals sufficient space to accommodate methylation of N7-G1405, whereas otherwise it results in a steric clash with the glycosidic ring at C6 in standard-of-care aminoglycosides and plazomicin, all of which are 4,6-disubstituted deoxystreptamines.^{18,48} This ultimately leads to uncompromised activity of apramycin against a broad spectrum of pathogenic bacteria, including CRE and *A. baumannii*.

WGS of clinical *A. baumannii* isolates tested in this study revealed a relatively high incidence (44%) of *armA*-positive isolates. Because ArmA-mediated methylation inside the drug-binding pocket does not obstruct the binding of apramycin, the MIC distribution of apramycin was found to be superior to that of other aminoglycoside antibiotics for clinical isolates of the *A. baumannii* complex (Figure 2b).

Although some of the other clinically relevant aminoglycoside antibiotics, such as amikacin, tobramycin and gentamicin, have previously been successfully applied in the treatment of CPE infections either alone or in combination with other antimicrobial agents,^{49,50} increasing RMTase-mediated resistance represents a challenge for treatment of CPE and CPA bacterial infections, in particular in Southern Europe and in low- and middle-income countries in South America, Africa and Asia. The intrinsic resilience of apramycin to common mechanisms of aminoglycoside resistance including 16S rRNA-modifying enzymes translates into a superior spectrum of activity that comprises highly drug-resistant Gram-negative organisms and hence a possible therapeutic remedy for this issue. Collectively, our results are in agreement with previous reports suggesting the potential of apramycin.^{15,28,51}

In the present study, preliminary ECOFFs were used as interpretative criteria because the pharmacokinetics (PK), probability of target attainment and, therefore, clinical breakpoints for apramycin are currently unknown. Preliminary (and as yet unpublished) data on the apramycin PK in various animal species suggest a PK profile that resembles that of other aminoglycoside antibiotics. Further preclinical evaluation is required to study the *in vivo* efficacy and the PK/pharmacodynamics of apramycin, which will be important in assessing the clinical potential of apramycin in the treatment of MDR Gram-negative bacterial infections in humans.

Apramycin may also represent a promising lead scaffold for further derivatization, taking advantage of the various beneficial features of apramycin including its exquisite selectivity for the bacterial over the eukaryotic cytosolic and mitochondrial ribosomes.^{52–54} Together with its low inherent toxicity reported earlier,¹⁸ apramycin and apramycin-like monosubstituted deoxystreptamines may represent a promising new subclass of aminoglycoside antibiotics for further optimization and development.

Acknowledgements

The authors express their gratitude to Maisra El-Bouseary, Dr Diego Andrey, Dr Ana C. Gales, Dorota Żabicka, Marek Gniadkowski, Fernando Baquero, Rafael Canton, Niels Frimodt-Møller and Reinhard Zbinden for

kindly supplying clinical isolates. We acknowledge IIT Biotech GmbH Bielefeld and Dr Frank Imkamp for their expertise and services in whole-genome sequencing, and the staff at the Institute of Medical Microbiology at University of Zurich for their support in susceptibility testing contributing to Figure S3. We are grateful to Raymond Lin for critical comments on the manuscript.

Funding

Some of the research leading to these results was conducted as part of the ND4BB European Gram-Negative Antibacterial Engine (ENABLE) Consortium (www.nd4bb-enable.eu) and has received funding from the Innovative Medicines Initiative Joint Undertaking under grant agreement n°115583, resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007–2013) and The European Federation of Pharmaceutical Industries and Associations (EFPIA) companies in kind contribution. The ENABLE project is also financially supported by contributions from Academic and Small and medium-sized enterprise (SME) partners. The research leading to these results has been supported by the University of Zurich.

Transparency declarations

D. C., E. C. B. and S. N. H. are co-founders of Juvabis AG, a startup biotech company with an interest in aminoglycoside therapeutics. All other authors: none to declare.

Supplementary data

Figures S1–S5 and Tables S1 and S2 are available as [Supplementary data](#) at JAC Online.

References

- Potron A, Poirel L, Nordmann P. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. *Int J Antimicrob Agents* 2015; **45**: 568–85.
- Busani S, Serafini G, Mantovani E *et al.* Mortality in patients with septic shock by multidrug resistant bacteria. *J Intensive Care Med* 2017; doi: 10.1177/0885066616688165.
- Poirel L, Kieffer N, Liassine N *et al.* Plasmid-mediated carbapenem and colistin resistance in a clinical isolate of *Escherichia coli*. *Lancet Infect Dis* 2016; **16**: 281.
- WHO. *Prioritization of Pathogens to Guide Discovery, Research and Development of New Antibiotics for Drug-resistant Bacterial Infections*. Geneva: World Health Organization, 2017 (WHO/EMP/IAU/2017.12).
- Blair JM, Webber MA, Baylay AJ *et al.* Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 2015; **13**: 42–51.
- Fischbach M, Walsh C. Antibiotics for emerging pathogens. *Science* 2009; **325**: 1089–93.
- Liu YY, Wang Y, Walsh TR *et al.* Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 2016; **16**: 161–8.
- Osei Sekyere J, Govinden U, Bester LA *et al.* Colistin and tigecycline resistance in carbapenemase-producing Gram-negative bacteria: emerging resistance mechanisms and detection methods. *J Appl Microbiol* 2016; **121**: 601–17.
- Juhas M. *Pseudomonas aeruginosa* essentials: an update on investigation of essential genes. *Microbiology (Reading, Engl)* 2015; **161**: 2053–60.
- Lee SA, Gallagher LA, Thongdee M *et al.* General and condition-specific essential functions of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 2015; **112**: 5189–94.
- McCoy LS, Xie Y, Tor Y. Antibiotics that target protein synthesis. *Wiley Interdiscip Rev RNA* 2011; **2**: 209–32.
- Krause KM, Serio AW, Kane TR *et al.* Aminoglycosides: an overview. *Cold Spring Harb Perspect Med* 2016; **6**: a027029.
- Michiels JE, Van den Bergh B, Verstraeten N *et al.* In vitro emergence of high persistence upon periodic aminoglycoside challenge in the ESKAPE pathogens. *Antimicrob Agents Chemother* 2016; **60**: 4630–7.
- Karah N, Dwibedi CK, Sjöström K *et al.* Novel aminoglycoside resistance transposons and transposon-derived circular forms detected in carbapenem-resistant *Acinetobacter baumannii* clinical isolates. *Antimicrob Agents Chemother* 2016; **60**: 1801–18.
- Kang AD, Smith KP, Eliopoulos GM *et al.* In vitro apramycin activity against multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Diagn Microbiol Infect Dis* 2017; **88**: 188–91.
- Smith KP, Kirby JE. Evaluation of apramycin activity against carbapenem-resistant and -susceptible strains of Enterobacteriaceae. *Diagn Microbiol Infect Dis* 2016; **86**: 439–41.
- Wachino J, Arakawa Y. Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: an update. *Drug Resist Updat* 2012; **15**: 133–48.
- Matt T, Ng CL, Lang K *et al.* Dissociation of antibacterial activity and aminoglycoside ototoxicity in the 4-monosubstituted 2-deoxystreptamine apramycin. *Proc Natl Acad Sci USA* 2012; **109**: 10984–9.
- Clinical Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically—Tenth Edition: Approved Standard M07-A10*. CLSI, Wayne, PA, USA, 2015.
- Schulthess B, Bloemberg GV, Zbinden A *et al.* Evaluation of the Bruker MALDI biotyper for identification of fastidious Gram-negative rods. *J Clin Microbiol* 2016; **54**: 543–8.
- Stucki D, Ballif M, Egger M *et al.* Standard genotyping overestimates transmission of *Mycobacterium tuberculosis* among immigrants in a low-incidence country. *J Clin Microbiol* 2016; **54**: 1862–70.
- Gupta SK, Padmanabhan BR, Diene SM *et al.* ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrob Agents Chemother* 2014; **58**: 212–20.
- Davis JH, Rubin AJ, Sauer RT. Design, construction and characterization of a set of insulated bacterial promoters. *Nucleic Acids Res* 2011; **39**: 1131–41.
- López-Díaz MD, Culebras E, Rodríguez-Avil I *et al.* Plazomicin activity against 346 extended-spectrum-β-lactamase/AmpC-producing *Escherichia coli* urinary isolates in relation to aminoglycoside-modifying enzymes. *Antimicrob Agents Chemother* 2017; **61**: pii: e02454-16.
- Rodríguez-Avil I, Pena I, Picazo JJ *et al.* In vitro activity of the next-generation aminoglycoside plazomicin alone and in combination with colistin, meropenem, fosfomicin or tigecycline against carbapenemase-producing Enterobacteriaceae strains. *Int J Antimicrob Agents* 2015; **46**: 616–21.
- Denervaud-Tendon V, Poirel L, Connolly LE *et al.* Plazomicin activity against polymyxin-resistant Enterobacteriaceae, including MCR-1-producing isolates. *J Antimicrob Chemother* 2017; **72**: 2787–91.
- Zhang Y, Kashikar A, Bush K. In vitro activity of plazomicin against β-lactamase-producing carbapenem-resistant Enterobacteriaceae (CRE). *J Antimicrob Chemother* 2017; **72**: 2792–5.
- Hu Y, Liu L, Zhang X *et al.* In vitro activity of neomycin, streptomycin, paromomycin and apramycin against carbapenem-resistant Enterobacteriaceae clinical strains. *Front Microbiol* 2017; **8**: 2275.
- Logan LK, Weinstein RA. The epidemiology of carbapenem-resistant Enterobacteriaceae: the impact and evolution of a global menace. *J Infect Dis* 2017; **215**: S28–36.

- 30** MacNair CR, Stokes JM, Carfrae LA *et al.* Overcoming *mcr-1* mediated colistin resistance with colistin in combination with other antibiotics. *Nat Commun* 2018; **9**: 458.
- 31** Hombach M, Wolfensberger A, Kuster SP *et al.* Influence of clinical breakpoint changes from CLSI 2009 to EUCAST 2011 antimicrobial susceptibility testing guidelines on multidrug resistance rates of Gram-negative rods. *J Clin Microbiol* 2013; **51**: 2385.
- 32** Jakobsen L, Sandvang D, Jensen VF *et al.* Gentamicin susceptibility in *Escherichia coli* related to the genetic background: problems with breakpoints. *Clin Microbiol Infect* 2007; **13**: 830–2.
- 33** Rosvoll TC, Lindstad BL, Lunde TM *et al.* Increased high-level gentamicin resistance in invasive *Enterococcus faecium* is associated with *aac(6′)Ie-aph(2′′)Ia*-encoding transferable megaplasmids hosted by major hospital-adapted lineages. *FEMS Immunol Med Microbiol* 2012; **66**: 166–76.
- 34** Jaimee G, Halami PM. Conjugal transfer of *aac(6′)Ie-aph(2′′)Ia* gene from native species and mechanism of regulation and cross resistance in *Enterococcus faecalis* MCC3063 by real time-PCR. *Microb Pathog* 2017; **110**: 546–53.
- 35** Davies J, O'Connor S. Enzymatic modification of aminoglycoside antibiotics: 3-N-acetyltransferase with broad specificity that determines resistance to the novel aminoglycoside apramycin. *Antimicrob Agents Chemother* 1978; **14**: 69–72.
- 36** Livermore DM, Mushtaq S, Warner M *et al.* Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant Enterobacteriaceae isolates. *J Antimicrob Chemother* 2011; **66**: 48–53.
- 37** Magalhaes ML, Blanchard JS. The kinetic mechanism of AAC3-IV aminoglycoside acetyltransferase from *Escherichia coli*. *Biochemistry* 2005; **44**: 16275–83.
- 38** Lovering AM, White LO, Reeves DS. AAC(1): a new aminoglycoside-acetylating enzyme modifying the C1 aminogroup of apramycin. *J Antimicrob Chemother* 1987; **20**: 803–13.
- 39** Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. *Drug Resist Updat* 2010; **13**: 151–71.
- 40** Sunada A, Nakajima M, Ikeda Y *et al.* Enzymatic 1-N-acetylation of paromomycin by an actinomycete strain #8 with multiple aminoglycoside resistance and paromomycin sensitivity. *J Antibiot (Tokyo)* 1999; **52**: 809–14.
- 41** Schmitt E, Galimand M, Panvert M *et al.* Structural bases for 16 S rRNA methylation catalyzed by ArmA and RmtB methyltransferases. *J Mol Biol* 2009; **388**: 570–82.
- 42** Doi Y, Wachino JI, Arakawa Y. Aminoglycoside resistance: the emergence of acquired 16S ribosomal RNA methyltransferases. *Infect Dis Clin North Am* 2016; **30**: 523–37.
- 43** Cox G, Ejim L, Stogios PJ *et al.* Plazomicin retains antibiotic activity against most aminoglycoside modifying enzymes. *ACS Infect Dis* 2018; **4**: 980–7.
- 44** Poirel L, Bonnin RA, Nordmann P. Analysis of the resistome of a multidrug-resistant NDM-1-producing *Escherichia coli* strain by high-throughput genome sequencing. *Antimicrob Agents Chemother* 2011; **55**: 4224.
- 45** Ho PL, Lo WU, Yeung MK *et al.* Complete sequencing of pNDM-HK encoding NDM-1 carbapenemase from a multidrug-resistant *Escherichia coli* strain isolated in Hong Kong. *PLoS One* 2011; **6**: e17989.
- 46** Rahman M, Prasad KN, Pathak A *et al.* RmtC and RmtF 16S rRNA methyltransferase in NDM-1-producing *Pseudomonas aeruginosa*. *Emerg Infect Dis* 2015; **21**: 2059–62.
- 47** Poirel L, Goutines J, Aires-de-Sousa M *et al.* High rate of association of 16S rRNA methylases and carbapenemases in Enterobacteriaceae recovered from hospitalized children in Angola. *Antimicrob Agents Chemother* 2018; **62**: pii: e00021–18.
- 48** Polikanov YS, Melnikov SV, Söll D *et al.* Structural insights into the role of rRNA modifications in protein synthesis and ribosome assembly. *Nat Struct Mol Biol* 2015; **22**: 342–4.
- 49** Rafailidis PI, Falagas ME. Options for treating carbapenem-resistant Enterobacteriaceae. *Curr Opin Infect Dis* 2014; **27**: 479–83.
- 50** Shields RK, Clancy CJ, Press EG *et al.* Aminoglycosides for treatment of bacteremia due to carbapenem-resistant *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2016; **60**: 3187–92.
- 51** Truelson KA, Brennan-Krohn T, Smith KP *et al.* Evaluation of apramycin activity against methicillin-resistant, methicillin-sensitive, and vancomycin-intermediate *Staphylococcus aureus* clinical isolates. *Diagn Microbiol Infect Dis* 2018; **92**: 168–71.
- 52** Mandhapati AR, Yang G, Kato T *et al.* Structure-based design and synthesis of apramycin-paromomycin analogues: importance of the configuration at the 6′-position and differences between the 6′-amino and hydroxy series. *J Am Chem Soc* 2017; **139**: 14611–9.
- 53** Perez-Fernandez D, Shcherbakov D, Matt T *et al.* 4′-O-substitutions determine selectivity of aminoglycoside antibiotics. *Nat Commun* 2014; **5**: 3112.
- 54** Hobbie SN, Kalapala SK, Akshay S *et al.* Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria. *Nucleic Acids Res* 2007; **35**: 6086–93.