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Peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO) restores carbapenem susceptibility to NDM-1-positive pathogens *in vitro* and *in vivo*

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Objectives: The objective of this study was to test the efficacy of an inhibitor of the New Delhi metallo-β-lactamase (NDM-1). Inhibiting expression of this type of antibiotic-resistance gene has the potential to restore antibiotic susceptibility in all bacteria carrying the gene.

Methods: We have constructed a peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO) that selectively inhibits the expression of NDM-1 and examined its ability to restore susceptibility to meropenem *in vitro* and *in vivo*.

Results: *In vitro*, the PPMO reduced the MIC of meropenem for three different genera of pathogens that express NDM-1. In a murine model of lethal *E. coli* sepsis, the PPMO improved survival (92%) and reduced systemic bacterial burden when given concomitantly with meropenem.

Conclusions: These data show that a PPMO can restore antibiotic susceptibility *in vitro* and *in vivo* and that the combination of PPMO and meropenem may have therapeutic potential against certain class B carbapenem-resistant infections in multiple genera of Gram-negative pathogens.

Introduction

Antibiotic resistance is an escalating, worldwide problem that has gained urgency in the past decade. Many strains of bacterial pathogens have become resistant to multiple antibiotics, and some are now resistant to all standard antibiotics, making treatment challenging. From 2001 to 2012, acute-care hospitals reporting at least one healthcare-associated infection from a carbapenem-resistant Enterobacteriaceae (CRE) increased from 1.2% to 4.6%[1] and the mortality rate from a CRE infection is estimated to be between 40% and 50%.[2–9] This problem is confounded by a lack of development and approval of new classes of antibiotics in the last three decades.[10–12]

The New Delhi metallo-β-lactamase (NDM-1) is a plasmid-associated Ambler class B β-lactamase. It was first identified in 2008 in a strain of *Klebsiella pneumoniae* isolated from a patient in Sweden who had acquired the bacterium in India.[13] Subsequently, NDM-1-associated resistance has rapidly spread in a clonal fashion throughout the world at an alarming rate to many Gram-negative pathogens, including *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.[13–17] NDM-1 is particularly dangerous because it confers resistance to some of our most potent antibiotics, the carbapenems, and is accompanied by genes encoding resistance to most, if not all, classes of available antibiotics.[13,15,16] Although β-lactamase inhibitors have been approved for combination use in humans, these are primarily effective for the serine (class A) and *ampC* (class C) β-lactamases, and none is effective against metallo-β-lactamases, including NDM-1.[10,18,19]

A new strategy to combat antibiotic resistance is to design therapeutics that silence the expression of specific antibiotic-resistance genes. These therapeutics would then be used adjunctively with already-approved antibiotics. Targeting specific antibiotic-resistance genes or their mRNA transcripts has advantages. First, interference with human gene expression should be minimal, since there are no human homologues of antibiotic-resistance genes.[20] In addition, the therapeutic could potentially target multiple species of bacteria to which the antibiotic-

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resistance gene has spread, and recover the activity of existing small-molecule antibiotics in a manner analogous to β-lactamase inhibitors.

Phosphorodiamidate morpholino oligomers (PMOs) are synthetic nucleotide analogues that are thought to prevent translation of a specific gene by selectively binding mRNA in an antisense manner.[21–23] The structure differs from DNA by a six-member morpholino ring that replaces the five-member deoxyribose ring, and a charge-neutral phosphorodiamidate linker instead of the phosphodiester linker. The nucleobase is in the same 1′ position. Currently, we have designed and constructed a PMO targeted to the gene (bla_{NDM-1}) for the NDM-1 enzyme. This PMO was conjugated to an arginine-rich peptide, which improves penetration of the PMO into bacteria.[24,25] We show here a proof of the concept that this peptide-conjugated PMO (PPMO) targeted to bla_{NDM-1} can restore bacterial susceptibility to carbapenems and protect mice in a lethal model of sepsis when co-administered with meropenem. To our knowledge, this is the first time a gene-specific therapeutic targeted to NDM-1 has been shown to work *in vivo*.

Materials and methods

Reagents

All PPMOs were synthesized and purified at Sarepta Therapeutics (Corvallis, OR, USA) as described previously.[26] The PPMO nucleobase sequences are: NDM-1, 5′-TCCTTTTATTC; NDM-1′, 5′-GGCAATTCCAT; and scrambled base sequence control (Scr), 5′-TCTCAGATGGT. Each PPMO was conjugated to $(RXR)_4XB$, where R is arginine, X is 6-aminohexanoic acid and B is β-alanine. Meropenem was purchased from Hospira Inc. (Lake Forest, IL, USA), doripenem (Doribax®) from Shionogi and Co. Ltd (Osaka, Japan) and imipenem from LKT Laboratories, Inc. (St Paul, MN, USA).

Bacterial strains and growth conditions

E. coli CVB-1 was kindly provided by Dr Gian Maria Rossolini (University of Siena, Italy). *A. baumannii* BCT-B-026 and *E. coli* BCT-B-036 were kindly provided by Dr Patrice Nordmann (University of Fribourg, Switzerland). *E. coli* 1001728, 1101851, AIS070834 and AIS071077 were kindly provided by Dr J. Kamile Rasheed (CDC, Atlanta, GA, USA). *E. coli* NDM1-E was kindly provided by Dr Susan M. Poutanen (Mount Sinai Hospital, Toronto, ON, Canada). *K. pneumoniae* BAA-2146 and *E. coli* W3110 and 25922 were obtained from ATCC (Manassas, VA, USA). Liquid cultures were grown in either Mueller–Hinton II (MHII) (cation-adjusted) or LB broth. LB agar was used for growth on solid medium. To generate log-phase bacteria for *in vivo* studies, single colonies were cultured aerobically in LB broth overnight (18 h) at 37 °C with shaking (200 rpm). The bacteria were then diluted 4×10^{-2} and cultured for an additional 3 h. We determined cfu by plating serial dilutions on LB agar plates. Bacterial growth curves (culture volumes of 100 or 200 μL) were measured by spectrophotometer (OD_{595}) at 15 or 30 min intervals for 8–10 h in 96-well plates at 37 °C with orbital shaking.

Periplasm extraction

An overnight culture of NDM-1-expressing *E. coli* CVB-1 was diluted 1×10^{-2} in MHII and NDM-1 PPMO was added to final concentrations of 0, 2, 8 and 32 μM. A control culture included 32 μM of a PPMO with a scrambled base sequence (Scr). The cultures were grown aerobically at 37 °C until mid-log phase ($OD_{595} = 0.3$ of 100 μL in a 96-well microtitre plate). The cultures were cooled on ice and then 1.0 mL was centrifuged at 4×10^3 **g**, 4 °C, for 10 min. The supernatant was discarded and the pellet was resuspended in 200 μL of ice-cold 0.5 M sucrose. The suspension was centrifuged as before, and the supernatant was removed and discarded. The pellet was resuspended in 100 μL of ice-cold 0.1 M Tris-acetate/0.5 M sucrose/5 mM EDTA (pH 8.2). One hundred microlitres of ice-cold H_2O was added and gently mixed. The mixture was incubated for 10 min on ice and then 2 μL of 1 M $MgSO_4$/1 mM $ZnSO_4$ was added and gently mixed. The mixture was centrifuged at 1.5×10^4 **g**, 4 °C, for 10 min. The supernatant was removed and filtered through a 0.2 μm sterile filtration cartridge. The total protein concentration was measured by the method of Bradford.[27]

Carbapenemase enzymatic activity (disc diffusion assay)

Each periplasm extract (0.5 μg of protein) was mixed on ice with 2 mg/L meropenem in a final volume of 24 μL, and then incubated at 37 °C for 1 h. The mixtures were then immediately cooled on ice. A 20 μL aliquot of each mixture was transferred to a sterile 6 mm paper disc (Becton, Dickinson, and Company, Sparks, MD, USA), and then placed on a Petri dish with a freshly seeded top agar lawn (10 μL of an overnight culture of *E. coli* W3110 in 3 mL of LB broth top agar). The Petri dish was incubated aerobically at 37 °C for 18 h. The area of no growth surrounding the disc was measured. A control disc with 20 μL of 2 mg/L meropenem was placed on the surface of the agar and used to establish the maximal zone of inhibition (meropenem only). Carbapenemase activity was calculated by subtracting the area of each sample disc from the area of the meropenem-only control disc, and then dividing by the area of the meropenem-only control disc.

Blue-Carba test

The Blue-Carba test was performed as described previously.[28] CVB-1 was cultured in MHII with the indicated concentrations of PPMO or Scr for 8 h before being added (5 μL) to the 100 μL test solution [0.04% bromothymol blue (Sigma–Aldrich, St Louis, MO, USA), 0.1 mM $ZnSO_4$, (Mallinckrodt, St Louis, MO, USA) and 3 g/L imipenem at pH 7.0]. Bacteria were also added to a negative control solution (only bromothymol blue and $ZnSO_4$ at pH 7.0) to control for changes in the pH of the solution from the bacteria or PPMO. Results were quantified by measuring the absorbance at 620 nm.

MIC assays

The procedure for determining the MIC was based on the microdilution method of the CLSI.[29] Specifically, overnight cultures in MHII were diluted to 5×10^5 cfu/mL and used to fill wells of a 96-well microtitre plate (Costar 3370, Corning, NY, USA). Each row included a different, fixed concentration of the NDM-1 PPMO from 128 to 0 μM. A 2-fold dilution series of meropenem was made in each row from 64 to 0 mg/L. Plates were incubated at 35–37 °C with shaking for 18 h. The ODs of the cultures were measured in a spectrophotometer (OD_{595}). Bacterial viability was determined by plating in triplicate, dilutions of cultures on LB agar plates and counting cfu.

Ethics

All animal procedures were approved by the Oregon State University Institutional Animal Care and Use Committee (approval numbers 4355 and 4596) and comply with all local, state and federal laws.

Mouse sepsis model

Female BALB/c mice, aged 6–8 weeks (Jackson Labs, Sacramento, CA, USA) were randomly assigned to treatment groups, and then infected intraperitoneally with $\sim 3.0 \times 10^6$ cfu of log-phase *E. coli* CVB-1 in 5% mucin (type III, Sigma Chemical Co., St Louis, MO, USA) in PBS. Where indicated, NDM-1 PPMO or a non-specific, scrambled-base PPMO was included (100 μg/mouse, which is ∼5 mg/kg) in the bacterial inoculum. Meropenem was

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                                NDM-1                NDM-1′
NDM-1   TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-2   TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-3   TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-4   TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-5   TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-6   TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-7   TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-8   -----------------GCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-9   TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-10  -------------------------------------ATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-11  -------------------------------------ATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-12  TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-13  TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-14  TTTGCGGGGTTTTTAATGCTGAATAAAAGGAAAACTTGATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-15  -------------------------------------ATGGAATTGCCCAATATTATGCACCCGGTCG
NDM-16  -------------------------------------ATGGAATTGCCCAATATTATGCACCCGGTCG
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Figure 1. Alignment of partial sequences of all reported NDM alleles, including the 5′ non-coding region.[31] The start codon is underlined in bold font and the target regions of the two PPMOs are shaded. An asterisk indicates 100% identical bases in all 16 alleles. All bases in the 5′ non-coding region are 100% identical in all alleles for which sequence in this region is available. Additional accession numbers for 5′ non-coding regions of NDM-4, -6 and -8 are KP826707.1, KC887916.2 and JF798502, respectively. 5′ Non-coding sequence is not available for alleles 10, 11, 15 and 16.

administered (1 mg/mouse, which is ~50 mg/kg) subcutaneously to the groups indicated. Additional doses of PPMO and meropenem were given at 6, 12, 18 and 24 h. Mice were monitored up to 5 days post-infection. Body temperatures of mice were recorded using a tympanic infrared thermometer (Braun Thermoscan Pro 4000, Bethlehem, PA, USA). Blood was collected by venipuncture from the saphenous vein using Microvette CB 300 LH (Sarstedt, Germany) collection tubes. Blood was diluted 1×10^{-1} in PBS and serial dilutions were plated on LB agar plates for determination of cfu. Mice were euthanized when body temperatures fell below 30 °C. For delayed treatment experiments, mice were infected without concomitant treatment, and then treated subcutaneously with 1 mg of meropenem and intraperitoneally with 250 μg of PPMO (12.5 mg/kg) at 0.5 or 1 h after infection. Temperature and survival were monitored as before. For analysis of cytokines in the blood and bacterial burden in the spleens, mice were infected and treated as indicated, and then euthanized at 10 h post-infection. Spleens were collected, homogenized, diluted and plated on LB agar plates to determine cfu burden in the spleen.

Cytokine analysis

Blood was collected by cardiac puncture, and sera were analysed for cytokines using the Invitrogen Mouse Cytokine 10-Plex Panel (ThermoFisher, USA) and a Millipore Luminex 200 (EMD Millipore, Germany) with xPonent software.

Statistical analysis

In vitro data were analysed by the two-tailed Student's *t*-test. *In vivo* data were analysed by the two-tailed Mann–Whitney *U*-test for non-parametric experiments and by the Kaplan–Meier method for survival (log-rank, Mantel–Cox). All evaluations were conducted using GraphPad Prism v. 6.0 (La Jolla, CA, USA) and results were considered significantly different with $P < 0.05$.

Results and discussion

PPMO inhibits expression of NDM-1 protein

We constructed 11-nucleobase PPMOs that are complementary to the mRNA of NDM-1 in the region of the Shine-Dalgarno ribosome binding site (NDM-1) or the start codon (NDM-1′).[30] The region targeted by the NDM-1 PPMO is 100% conserved in 12 of the 16 sequenced non-coding regions of $bla_{\text{NDM-1}}$ alleles and NDM-1′ is 100% conserved in all alleles (Figure 1).[31]

The effect of the NDM-1 PPMO on expression of NDM-1 was tested by two different methods of measuring the enzymatic activity of NDM-1. The NDM-1 PPMO was added to growing cultures of *E. coli* CVB-1, an MDR pathogen that expresses the $bla_{\text{NDM-1}}$.[32] The carbapenemase enzymatic activity in periplasmic extracts was measured using a disc-diffusion assay. The results show a decrease in activity that was proportional to the amount of NDM-1 PPMO added to the growing cultures (Figure 2a and b). Notably, there was no loss of activity in cultures grown with a non-specific scrambled PPMO (Scr), which demonstrates specificity of the PPMO. As a control, the NDM-1 PPMO was added directly to a periplasmic extract from an untreated culture. The NDM-1 PPMO had no direct effect on the enzyme or its carbapenemase activity (data not shown). This is consistent with its role as an inhibitor of translation. The functional activity of the NDM-1 enzyme was also measured using a second method (Blue-Carba) that employs intact cells instead of periplasmic extracts. The Blue-Carba method uses a pH-sensitive dye that changes colour (from blue to yellow) when the β-lactam is hydrolysed by the carbapenemase. We found that adding the NDM-1 PPMO to a growing culture reduced carbapenemase activity in proportion to the concentration added (Figure 2c and d). The Scr PPMO had no effect. These results show that the NDM-1 PPMO inhibits $bla_{\text{NDM-1}}$ expression.

PPMO restores susceptibility of *E. Coli* CVB-1 to meropenem

The MIC of meropenem was measured with various concentrations of the NDM-1 PPMO. The MIC of meropenem was inversely proportional to the concentration of PPMO (Figure 3a). At 4 μM PPMO, the MIC of meropenem decreased 4-fold from 16 to 4 mg/L. Moreover, the initial viability was reduced by >3 logs in the culture treated with both meropenem and NDM-1 PPMO (Figure 3b). Importantly, the PPMO alone did not inhibit bacterial growth (Figure 3c), indicating that the PPMO alone has no antimicrobial activity. The NDM-1 PPMO was effective at lowering the MIC of meropenem for multiple NDM-1-positive strains of *E. coli* (Figure 3d). Similar results were found using a second PPMO (NDM-1′ PPMO) that is targeted downstream of the NDM-1 PPMO and entirely within the coding region of $bla_{\text{NDM-1}}$ (Figures 1 and 3d). Both PPMOs reduced the MIC of meropenem for all strains tested in a

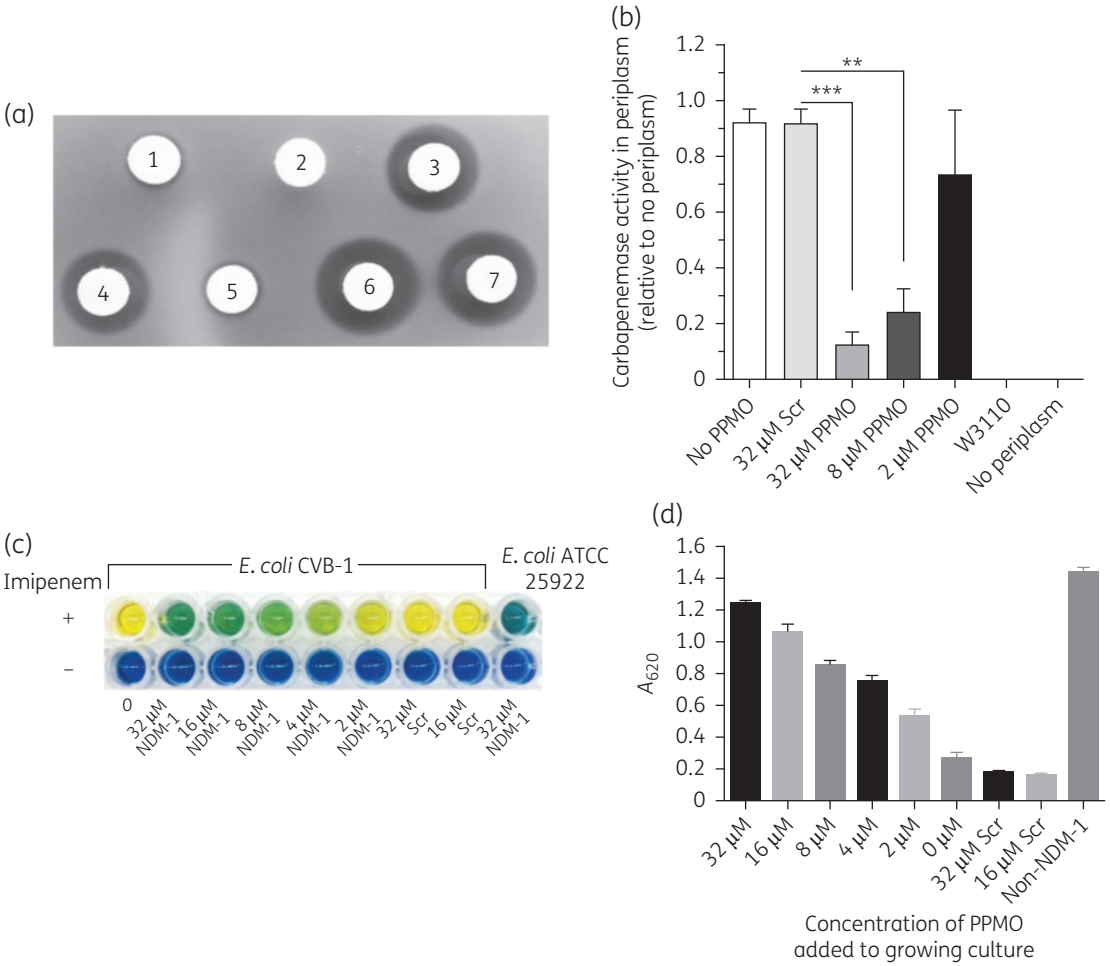
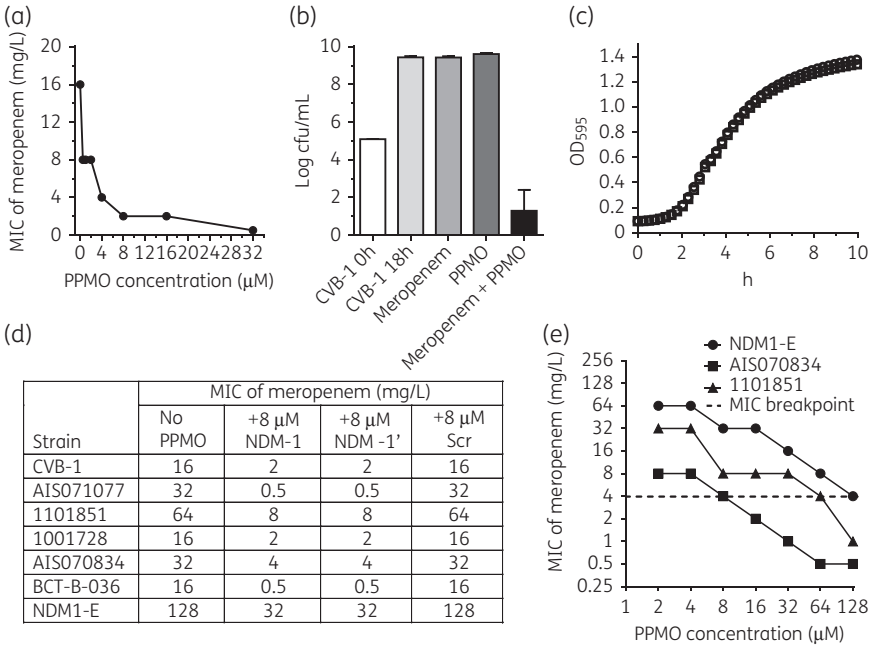


Figure 2. Carbapenemase activity in *E. coli* CVB-1 after treatment with NDM-1 PPMO. (a) An example of a microbiological assay for carbapenemase activity, showing zones of bacterial growth inhibition surrounding discs with meropenem on a lawn of the meropenem-susceptible *E. coli* W3110. A fixed amount of meropenem was mixed with periplasm extracts isolated from cultures treated as indicated below, incubated and then applied to the discs. The discs were then placed on a seeded lawn of *E. coli* W3110 and incubated overnight. The periplasm was extracted from cultures of NDM-1-expressing *E. coli* CVB-1 treated with: (1) no PPMO; (2) 32 μM Scr PPMO; (3) 32 μM NDM-1 PPMO; (4) 8 μM NDM-1 PPMO; or (5) 2 μM NDM-1 PPMO [(6) *E. coli* W3110 periplasm extract only; (7) control disc with meropenem only (no periplasm)]. (b) Quantitative analysis of carbapenemase activity from zones of inhibition shown in (a). (c) Representative results of the Blue-Carba test. Carbapenemase production of CVB-1 grown with NDM-1 PPMO (PPMO) or Scr PPMO (Scr), and a non-carbapenemase producer, ATCC 25922. (d) Absorbance of blue colour (620 nm) in the Blue-Carba test shown in (c). Data are represented as the mean ± SEM, $n = 3$. ***$P < 0.001$ and **$P < 0.01$ by two-tailed Student's *t*-test. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

dose-dependent fashion. However, the concentration of PPMO needed to achieve the breakpoint concentration of 4 mg/L for meropenem[33] varied (Figure 3e). It is unknown what causes this variability among strains, most of which are uncharacterized clinical isolates; indeed, only two of the strains that were tested (CVB-1 and 1001728) have published information on antibiotic resistance mechanisms.[32,34] The observed change in enhanced susceptibility does not seem to be sequence specific, because both NDM-1 PPMOs were equally effective among strains (Figure 3d), and the sequence of bla_{NDM-1} including its 5′ non-coding region was identical in each of the tested strains (data not shown). Additionally, the variability among strains does not correlate with the level of carbapenemase activity, as measured by the Blue-Carba method (data not shown). Perhaps these results are caused by differences in NDM-1 mRNA turnover rate, mechanisms controlling expression of bla_{NDM-1}, or simply redundant mechanism(s) of lactam resistance. The differing level of susceptibility among strains suggests that some pathogens may require higher doses of meropenem and/or PPMO to achieve beneficial clinical outcomes.

Since the bla_{NDM-1} is clonal and conserved across many bacterial genera, we next determined whether the PPMO would have a similar effect on additional Gram-negative pathogens. We found that the NDM-1 PPMO also reduced the MIC of meropenem for NDM-1-positive strains of *A. baumannii* (Figure 4a–c) and *K. pneumoniae* (Figure 4d–f) without affecting bacterial viability in the absence of the antibiotic. A scrambled-sequence PPMO had no effect on the MIC of meropenem for either pathogen (data not shown).

The NDM-1 PPMO also lowered the MIC of two other carbapenems, doripenem and imipenem, for CVB-1 (Figure 5a–d). This effect was specific to carbapenems because the NDM-1 PPMO had no effect on the MIC of other classes of β-lactamases, such as the cephalosporin ceftriaxone (data not shown).



Strain	MIC of meropenem (mg/L)			
	No PPMO	+8 μM NDM-1	+8 μM NDM -1′	+8 μM Scr
CVB-1	16	2	2	16
AIS071077	32	0.5	0.5	32
1101851	64	8	8	64
1001728	16	2	2	16
AIS070834	32	4	4	32
BCT-B-036	16	0.5	0.5	16
NDM1-E	128	32	32	128

Figure 3. Susceptibility of *E. coli* to meropenem in cultures treated with NDM-1 PPMO. (a) MIC of meropenem at various concentrations of NDM-1 PPMO using *E. coli* CVB-1 as indicator. (b) Bacterial viability in cultures of *E. coli* CVB-1 treated with meropenem (4 mg/L), NDM-1 PPMO (4 μM) or both. (c) Growth curves of untreated (squares) and 128 μM NDM-1 PPMO-treated (circles) cultures of *E. coli* CVB-1. (d) Comparison of NDM-1 and NDM-1′ PPMOs showing the MIC of meropenem using various NDM-1-positive *E. coli* strains. (e) MIC of meropenem at various concentrations of NDM-1 PPMO for three different strains of *E. coli*. The breakpoint (4 mg/L) between resistant and susceptible, as defined by the CLSI,[33] is shown by a broken line.

NDM-1 PPMO co-administered with meropenem confers protection in vivo

The efficacy of the NDM-1 PPMO was evaluated *in vivo* using a mouse sepsis model. Mice were infected and treated by intraperitoneal injection of a freshly prepared mixture of *E. coli* CVB-1 and 100 μg of NDM-1 PPMO. Meropenem was then immediately administered subcutaneously. Treatments were administered every 6 h post-infection for the first 24 h, and the mice were monitored for survival. The results show 92% survival of the mice treated with the NDM-1 PPMO and meropenem combination (Figure 6a). This is a significant increase in survival compared with mice treated with either agent separately, or with co-administration of a scrambled PPMO (Scr) and meropenem, all of which died by 18 h. Monitoring during survival demonstrated that after 6 h mice were healthier, as assessed by body temperature (Figure 6b) and bacterial burden in the bloodstream (Figure 6c). Additionally, at 10 h (two doses of PPMO and meropenem) similar and statistically significant reductions in bacteria in the blood and spleen were found (Figure 6d and e). Inflammation was also significantly reduced at 10 h as indicated by the levels of pro-inflammatory cytokines TNFα, IL-2 and IL-6 in the blood of mice treated with both NDM-1 PPMO and meropenem compared with the controls (Figure 6f). Importantly, the NDM-1 PPMO alone did not have any significant pro-inflammatory effect *in vivo*.

The response to treatment with NDM-1 PPMO was measured using various doses (33, 11 or 4 μg) of the NDM-1 PPMO. The results show that morbidity and mortality were inversely proportional to the dose of NDM-1 PPMO (Figure 7). Co-administration of 33 μg of NDM-1 PPMO and 1 mg of meropenem protected over 75% of the infected mice for the duration (5 days) of the experiment (Figure 7a), reduced the fall in body temperature (Figure 7b) and significantly decreased viable bacteria in the blood (Figure 7c). Lower doses of PPMO were less effective but still demonstrated significant improvement in survival, body temperature and bacteraemia as compared with the two negative controls. These data indicate that the PPMO increased bacterial susceptibility to antibiotic killing *in vivo* in a dose-dependent fashion.

The NDM-1 PPMO was tested therapeutically by administering it post-infection. Groups of mice were infected as described above and then treated with both meropenem (subcutaneously) and 250 μg of PPMO (intraperitoneally) at 0.5 h or 1 h after infection and every 6 h thereafter for 24 h. When treatment was delayed 0.5 h post-infection, 75% of the mice treated with both meropenem and PPMO survived (Figure 8). This is significantly ($P < 0.01$) more than mice in either of the control groups, which all died by 15 h post-infection. Treatment delayed 1 h was not statistically significant, but there was a trend towards an increase in mean time to death as compared with the control group that was treated with Scr PPMO with meropenem (14.86 ± 2.454 versus 11.29 ± 0.7469 h). A lower dose (100 μg) of the NDM-1 PPMO was ineffective when first administered at 0.5 h post-infection. These *in vivo* data demonstrate that the NDM-1 PPMO can be used therapeutically, analogously to a β-lactamase inhibitor, and has a protective effect when administered concurrently with meropenem. This therapeutic regimen compares favourably with other treatments targeted to NDM-1, particularly with those that have been tested in highly lethal models of infection.[35,36] Finally, the maximal doses used in our experiments (12.5 mg/kg) are in line with previous human trials with PMOs (≤16 mg/kg), although further studies

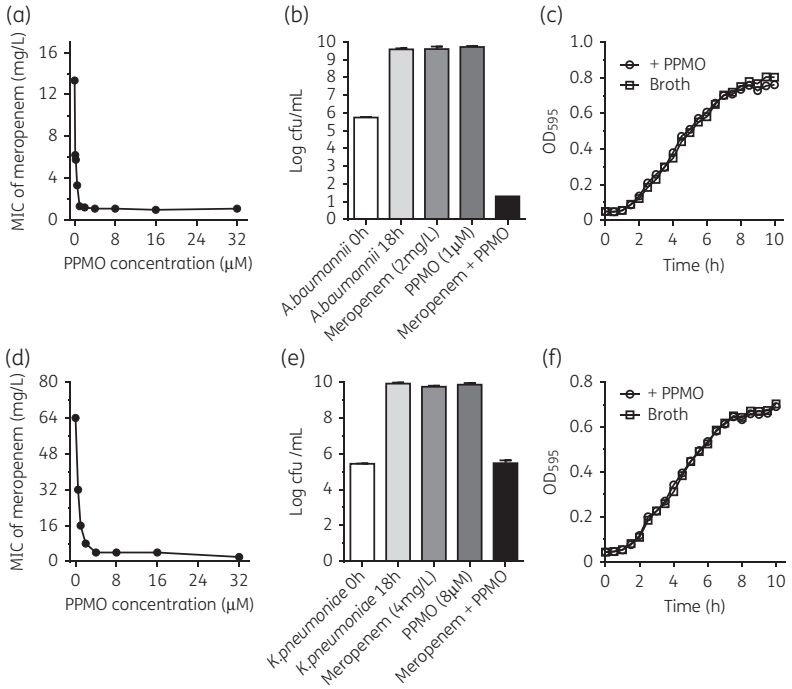


Figure 4. Susceptibility of other NDM-1-expressing Gram-negative pathogens to meropenem in cultures treated with NDM-1 PPMO. (a and d) MIC of meropenem as a function of NDM-1 PPMO added to the assay using either *A. baumannii* BCT-B-026 (a) or *K. pneumoniae* BAA-2146 (d) as indicator. (b and e) Bacterial viability in cultures of *A. baumannii* BCT-B-026 (b) or *K. pneumoniae* BAA-2146 (e) treated with meropenem, NDM-1 PPMO or both. (c and f) Growth curves of *A. baumannii* BCT-B-026 (c) or *K. pneumoniae* BAA-2146 (f) in the presence (circles) or absence (squares) of NDM-1 PPMO (16 μM). Bacterial density was measured at OD_{595} every 30 min. Data are represented as the mean ± SEM, $n = 5$ for *A. baumannii* and $n = 3$ for *K. pneumoniae*.

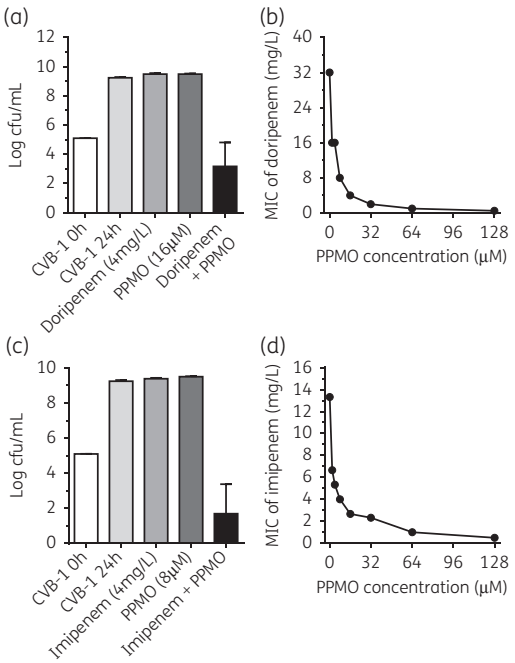


Figure 5. NDM-1 PPMO efficacy with other carbapenems. (a and c) Bacterial viability in cultures of CVB-1 treated with NDM-1 PPMO and doripenem or imipenem. Data are represented as the mean ± SEM, $n = 3$. (b and d) The MIC of doripenem or imipenem was measured in the presence of various concentrations of NDM-1 PPMO, using as indicator strain *E. coli* CVB-1.

will be needed to assess tolerability with the peptide-conjugated PMOs (PPMOs).[37–39]

Conclusions

Here, we have described a proof-of-concept, gene-specific therapeutic targeted to a highly conserved antibiotic-resistance gene. This PPMO was effective and well-tolerated *in vivo*, and has a unique mechanism of action. The strategy of suppressing the expression of a specific antibiotic-resistance gene is unique and significant for a number of reasons. First, instead of discarding standard antibiotics that are already approved for clinical use and searching for yet another new antibiotic, our approach restores utility to standard, marketed antibiotics that have lost usefulness. This speaks to antibiotic stewardship and our responsibility to use therapeutics wisely. Second, PPMOs are a nucleotide-based technology, which enables rapid sequence-specific design, synthesis and testing against bacterial gene targets.

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Some of these *in vitro* results were presented in an abstract/poster (F-1544) at the Fifty-fourth Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, USA, 2014.

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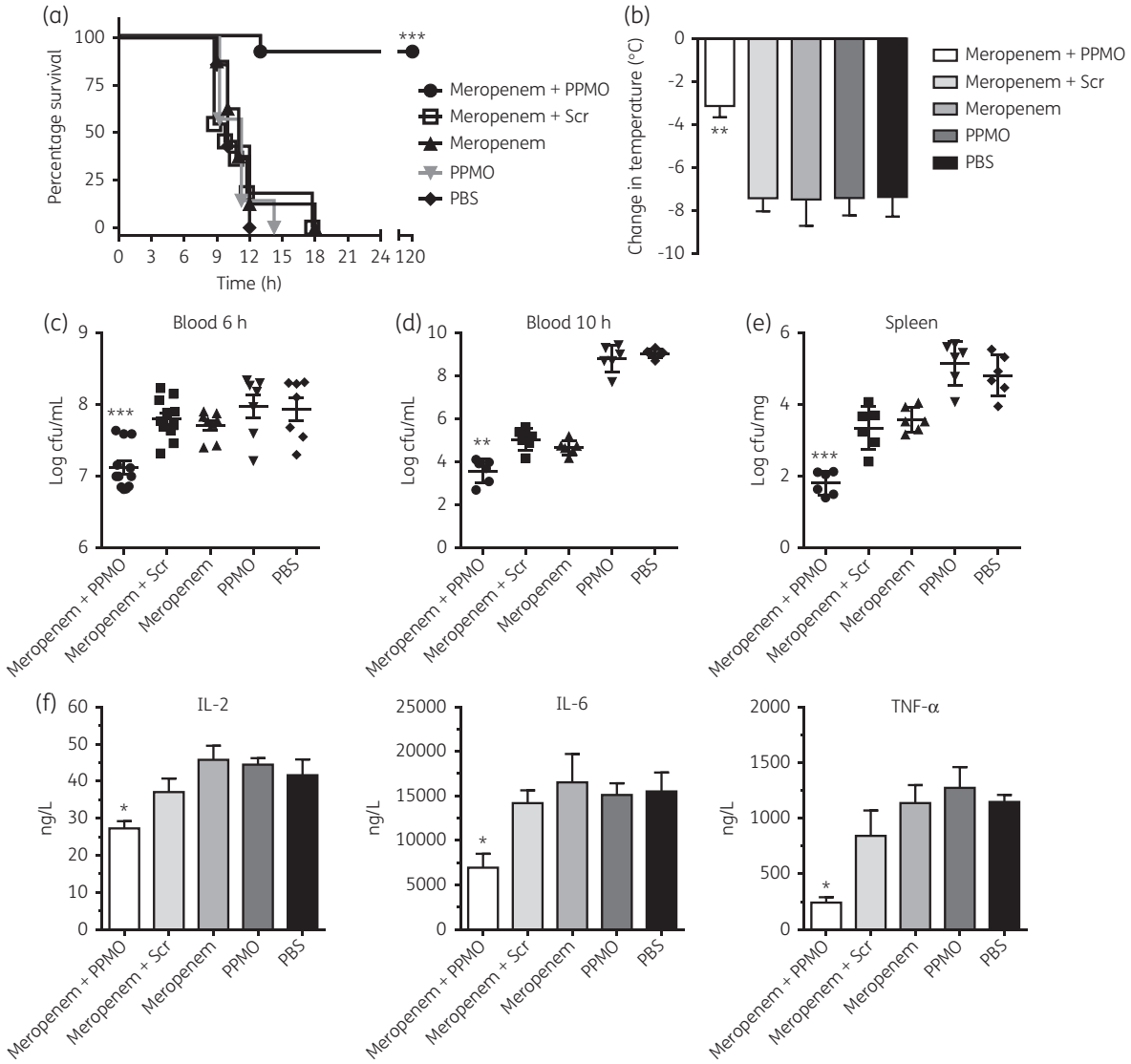

Figure 6. *In vivo* infectious challenge and treatment with meropenem and NDM-1 PPMO. Mice were infected with *E. coli* CVB-1 and treated at 0, 6, 12, 18 and 24 h post-infection with 1 mg of meropenem ($n = 8$) (given subcutaneously), 100 μg (5 mg/kg) of PPMO ($n = 7$) (given intraperitoneally), both treatments ($n = 12$), a scrambled PPMO (Scr) with meropenem ($n = 11$) or PBS ($n = 7$). (a) Survival and (b) body temperature (9 h post-infection) were recorded. (c) Bacteria in the blood were measured immediately prior to the 6 h treatment. In separate experiments, groups of mice ($n = 6$) were euthanized at 10 h post-infection (4 h after the second treatment) and bacteria in the (d) blood and (e) spleen were measured. (f) Cytokines were measured in sera sampled 10 h post-infection. For Kaplan–Meier survival curves, ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ by log-rank (Mantel–Cox) test. For other graphs, data are represented as mean ± SEM, ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ by two-tailed Mann–Whitney *U*-test.

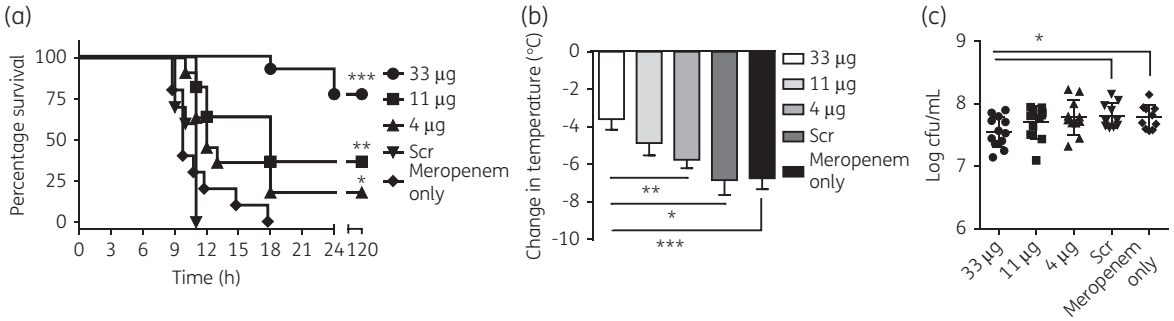

Figure 7. Dose–response relationship. Mice were infected and treated with meropenem and PPMO as described in the legend to Figure 6, except that the dose of NDM-1 PPMO varied as follows: 33 μg ($n = 13$), 11 μg ($n = 11$) or 4 μg ($n = 11$). Control groups included meropenem plus Scr PPMO (33 μg; $n = 10$) or meropenem only ($n = 10$). (a) Survival, (b) body temperature change at 9 h post-infection and (c) bacteria in the blood (6 h post-infection) were monitored. Asterisks indicate statistical significance as described in Figure 6.

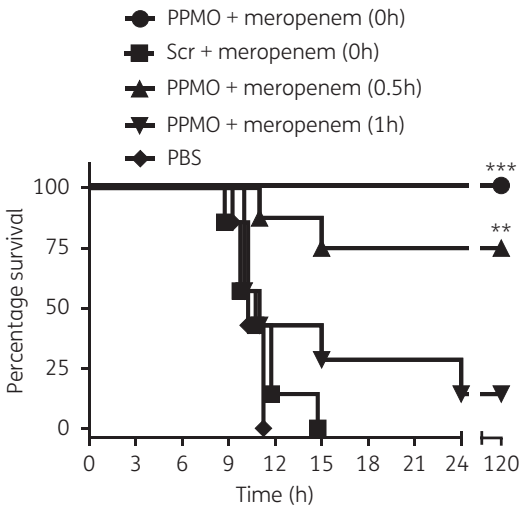

Figure 8. Delayed treatment. Survival was monitored in groups of mice treated at the time of infection (0 h, $n = 7$), 0.5 h post-infection (0.5 h, $n = 8$) or 1 h post-infection (1 h, $n = 7$), with meropenem (1 mg) and PPMO (250 μg). Mice treated with the same doses of meropenem and Scr PPMO at the time of infection ($n = 7$) and mice treated with PBS ($n = 7$) were used as negative controls. ***$P < 0.001$ and **$P < 0.01$ by log-rank (Mantel–Cox) test.

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Transparency declarations

B. L. G. is a consultant to Sarepta Therapeutics. B. L. G. and D. E. G. are inventors on numerous patents and patent applications involving PPMOs, receive research support and are the recipients of technology licensing fees from Sarepta Therapeutics. S. M. B., A. L. M. and M. W. are employees of Sarepta Therapeutics, which holds numerous patents on the methods of synthesis and use of PPMOs. All other authors: none to declare.

Author contributions

E. K. S., B. L. G. and L. L. performed all animal experiments. E. K. S., B. L. G., L. L., C. M. M., A. L. M., S. M. D. and C. R. S. performed *in vitro* experiments. S. M. B. synthesized the PPMOs. E. K. S., B. L. G. and D. E. G. designed experiments and analysed data. P. N. provided *A. baumannii* BCT-B-026. D. E. G. and M. W. assisted with manuscript preparation. E. K. S. and B. L. G. wrote the manuscript with contributions from all other authors.

References

1 Jacob JT, Klein E, Laxminarayan R *et al*. Vital signs: carbapenem-resistant Enterobacteriaceae. *MMWR Morb Mortal Wkly Rep* 2013; **62**: 165–70.

2 Patel G, Huprikar S, Factor SH *et al*. Outcomes of carbapenem-resistant *Klebsiella pneumoniae* infection and the impact of antimicrobial and adjunctive therapies. *Infect Control Hosp Epidemiol* 2008; **29**: 1099–106.

3 Schwaber MJ, Lev B, Israeli A *et al*. Containment of a country-wide outbreak of carbapenem-resistant *Klebsiella pneumoniae* in Israeli hospitals via a nationally implemented intervention. *Clin Infect Dis* 2011; **52**: 848–55.

4 Chitnis AS, Caruthers PS, Rao AK *et al*. Outbreak of carbapenem-resistant Enterobacteriaceae at a long-term acute care hospital: sustained reductions in transmission through active surveillance and targeted interventions. *Infect Control Hosp Epidemiol* 2012; **33**: 984–92.

5 Nguyen M, Eschenauer GA, Bryan M *et al*. Carbapenem-resistant *Klebsiella pneumoniae* bacteremia: factors correlated with clinical and microbiologic outcomes. *Diagn Microbiol Infect Dis* 2010; **67**: 180–4.

6 Borer A, Saidel-Odes L, Riesenberg K *et al*. Attributable mortality rate for carbapenem-resistant *Klebsiella pneumoniae* bacteremia. *Infect Control Hosp Epidemiol* 2009; **30**: 972–6.

7 Chang HJ, Hsu PC, Yang CC *et al*. Risk factors and outcomes of carbapenem-nonsusceptible *Escherichia coli* bacteremia: a matched case-control study. *J Microbiol Immunol Infect* 2011; **44**: 125–30.

8 Melzer M, Petersen I. Mortality following bacteraemic infection caused by extended spectrum β-lactamase (ESBL) producing *E. coli* compared to non-ESBL producing *E. coli*. *J Infect* 2007; **55**: 254–9.

9 Chang YY, Chuang YC, Siu LK *et al*. Clinical features of patients with carbapenem nonsusceptible *Klebsiella pneumoniae* and *Escherichia coli* in intensive care units: a nationwide multicenter study in Taiwan. *J Microbiol Immunol Infect* 2015; **48**: 219–25.

10 Drawz SM, Bonomo RA. Three decades of β-lactamase inhibitors. *Clin Microbiol Rev* 2010; **23**: 160–201.

11 Spellberg B, Bartlett J, Wunderink R *et al*. Novel approaches are needed to develop tomorrow's antibacterial therapies. *Am J Respir Crit Care Med* 2015; **191**: 135–40.

12 Outterson K, Rex JH, Jinks T *et al*. Accelerating global innovation to address antibacterial resistance: introducing CARB-X. *Nat Rev Drug Discov* 2016; **15**: 589–90.

13 Nordmann P, Poirel L, Walsh TR *et al*. The emerging NDM carbapenemases. *Trends Microbiol* 2011; **19**: 588–95.

14 Walsh TR. Emerging carbapenemases: a global perspective. *Int J Antimicrob Agents* 2010; **36** Suppl 3: S8–14.

15 Dortet L, Poirel L, Nordmann P. Worldwide dissemination of the NDM-type carbapenemases in Gram-negative bacteria. *Biomed Res Int* 2014; **2014**: 249856.

16 Dortet L, Nordmann P, Poirel L. Association of the emerging carbapenemase NDM-1 with a bleomycin resistance protein in Enterobacteriaceae and *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2012; **56**: 1693–7.

17 Yong D, Toleman MA, Giske CG *et al*. Characterization of a new metallo-β-lactamase gene, $bla_{\text{NDM-1}}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob Agents Chemother* 2009; **53**: 5046–54.

18 Queenan AM, Bush K. Carbapenemases: the versatile β-lactamases. *Clin Microbiol Rev* 2007; **20**: 440–58.

19 King DT, Worrall LJ, Gruninger R *et al*. New Delhi metallo-β-lactamase: structural insights into β-lactam recognition and inhibition. *J Am Chem Soc* 2012; **134**: 11362–5.

20 Woodford N, Wareham DW. Tackling antibiotic resistance: a dose of common antisense? *J Antimicrob Chemother* 2009; **63**: 225–9.

21 Geller BL. Antibacterial antisense. *Curr Opin Mol Ther* 2005; **7**: 109–13.

22 Summerton J, Stein D, Huang SB *et al*. Morpholino and phosphorothioate antisense oligomers compared in cell-free and in-cell systems. *Antisense Nucleic Acid Drug Dev* 1997; **7**: 63–70.

23 Stein D, Foster E, Huang SB *et al*. A specificity comparison of four antisense types: morpholino, 2'-O-methyl RNA, DNA, and phosphorothioate DNA. *Antisense Nucleic Acid Drug Dev* 1997; **7**: 151–7.

24 Mellbye BL, Puckett SE, Tilley LD *et al*. Variations in amino acid composition of antisense peptide-phosphorodiamidate morpholino oligomer affect potency against *Escherichia coli* in vitro and in vivo. *Antimicrob Agents Chemother* 2009; **53**: 525–30.

25 Greenberg DE, Marshall-Batty KR, Brinster LR *et al*. Antisense phosphorodiamidate morpholino oligomers targeted to an essential gene inhibit *Burkholderia cepacia* complex. *J Infect Dis* 2010; **201**: 1822–30.

26 Tilley LD, Hine OS, Kellogg JA *et al*. Gene-specific effects of antisense phosphorodiamidate morpholino oligomer-peptide conjugates on *Escherichia coli* and *Salmonella enterica* serovar typhimurium in pure culture and in tissue culture. *Antimicrob Agents Chemother* 2006; **50**: 2789–96.

27 Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–54.

28 Pires J, Novais A, Peixe L. Blue-Carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. *J Clin Microbiol* 2013; **51**: 4281–3.

29 Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically—Seventh Edition: Approved Standard M7-A7*. CLSI, Wayne, PA, USA, 2006.

30 Nasevicius A, Ekker SC. Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* 2000; **26**: 216–20.

31 Lahey Clinic. *β-Lactamase Classification and Amino Acid Sequences for TEM, SHV and OXA Extended-Spectrum and Inhibitor Resistant Enzymes*. http://www.lahey.org/studies/.

32 D'Andrea MM, Venturelli C, Giani T *et al*. Persistent carriage and infection by multidrug-resistant *Escherichia coli* ST405 producing NDM-1 carbapenemase: report on the first Italian cases. *J Clin Microbiol* 2011; **49**: 2755–8.

33 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Sixteenth Informational Supplement M100-S16*. CLSI, Wayne, PA, USA, 2006.

34 Rasheed JK, Kitchel B, Zhu W *et al*. New Delhi metallo-β-lactamase-producing Enterobacteriaceae, United States. *Emerging Infect Dis* 2013; **19**: 870–8.

35 King AM, Reid-Yu SA, Wang W *et al*. Aspergillomarasmine A overcomes metallo-β-lactamase antibiotic resistance. *Nature* 2014; **510**: 503–6.

36 Pan CY, Chen JC, Chen TL *et al*. Piscidin is highly active against carbapenem-resistant *Acinetobacter baumannii* and NDM-1-producing *Klebsiella pneumonia* in a systemic septicaemia infection mouse model. *Mar Drugs* 2015; **13**: 2287–305.

37 Mendell JR, Rodino-Klapac LR, Sahenk Z *et al*. Eteplirsen for the treatment of Duchenne muscular dystrophy. *Ann Neurol* 2013; **74**: 637–47.

38 Heald AE, Iversen PL, Saoud JB *et al*. Safety and pharmacokinetic profiles of phosphorodiamidate morpholino oligomers with activity against Ebola virus and Marburg virus: results of two single-ascending-dose studies. *Antimicrob Agents Chemother* 2014; **58**: 6639–47.

39 Heald AE, Charleston JS, Iversen PL *et al*. AVI-7288 for Marburg virus in nonhuman primates and humans. *N Engl J Med* 2015; **373**: 339–48.