Discovery of a Novel Metallo-β-Lactamase Inhibitor that Potentiates Meropenem Activity against Carbapenem-Resistant Enterobacteriaceae

Discovery of a Novel Metallo-ß-Lactamase Inhibitor, which can Potentiate Meropenem Activity against Carbapenem-Resistant Enterobacteriaceae

Martin Everett[1]*, Nicolas Sprynski[1], Alicia Coelho[1], Jérôme Castandet[1], Maëlle Bayet[1], Juliette Bougnon[1], Clarisse Lozano[1], David T. Davies[1], Simon Leiris[1], Magdalena Zalacain[1,2], Ian Morrissey[3], Sophie Magnet[3], Kirsty Holden[4], Peter Warn[4], Filomena De Luca[5], Jean-Denis Docquier[5], & Marc Lemonnier[1].

[1] Antabio SAS, 31670 Labège, France; [2]Zala Drug Discovery Consulting LLC, West Chester, PA 19380, USA; [3]IHMA Europe, 1870 Monthey/VS, Switzerland; [4]Evotec, Manchester, M15 6SE, UK; [5]Department of Medical Biotechnology, University of Siena, 53100, Italy.

Running Title: Novel Metallo-β-lactamase Inhibitor (max 54 characters with spaces)

* corresponding author

E-mail address: martin.everett@antabio.com,

Keywords: β-lactamase, metallo-β-lactamase, NDM-1, carbapenem, inhibitor

ABSTRACT (Limit 250 words)

Infections caused by carbapenem-resistant Enterobacteriaceae (CRE) are increasingly prevalent and have become a major worldwide threat to human health. Carbapenem resistance is driven primarily by the acquisition of β-lactamase enzymes which are able to degrade carbapenem antibiotics (hence termed carbapenemases) and can result in high levels of resistance and treatment failure. Clinically relevant carbapenemases include both serine-β-lactamases (SBLs, e.g. KPC-2 and OXA-48) and metallo-β-lactamases (MBLs), such as NDM-1. MBL-producing strains are endemic within the community in many Asian countries, have successfully spread worldwide, and account for many significant CRE outbreaks. Recently approved combinations of β-lactam antibiotics with β-lactamase inhibitors are only active against SBL-producing pathogens. Therefore, new drugs that specifically target MBLs and which restore carbapenem efficacy against MBL-producing CRE pathogens are urgently needed. Here, we report the discovery of a novel MBL inhibitor, ANT431, that can potentiate the activity of MEM against a broad range of MBL-producing CRE, and restore its efficacy against an *Escherichia coli* NDM-1 strain in a murine thigh infection model. This is a strong starting point for a chemistry lead optimization program that could deliver a first-in-class MBL inhibitor/carbapenem combination. This would complement the existing weaponry against CREs and address an important and growing unmet medical need.

INTRODUCTION

Resistance to β-lactams, the most widely used class of antibacterial drugs, emerged very soon after these antibiotics were introduced into clinical practice (1). In fact, even before penicillin was used clinically it had already been noted that some bacteria were non-susceptible due to the production of an enzyme that inactivated penicillin (2). Such β-lactamase enzymes, as they came to be known, are the most widespread mechanism of resistance to β-lactam antibiotics, hydrolyzing the β-lactam ring and rendering them ineffective. Strategies to fight β-lactamase-mediated resistance have included modification of β-lactams, as well as the development of combinations of β-lactams with β-lactamase inhibitors. In 1981, the first such combination, amoxicillin/clavulanate, was launched following the discovery of the natural product clavulanic acid, an inhibitor of serine β-lactamases (SBLs) (3, 4). However, new β-lactamases have continued to emerge which are insensitive to inhibition by clavulanic acid and other marketed inhibitors (5). Several new β-lactam/inhibitor combinations brought to the market more recently, (e.g. ceftazidime/avibactam (6); meropenem (MEM)/vaborbactam (7)) address resistance due to extended spectrum β-lactamases (ESBLs), and also the Class A KPC and certain Class D OXA carbapenemases that are largely responsible for recent increases in carbapenem-resistant Enterobacteriaceae (CRE) strains.

The most recent class of β-lactamases to have come to prominence are the Class B metallo-β-lactamases (MBLs), which include the NDM, VIM and IMP sub-classes and multiple variants thereof. This situation is extremely concerning as MBLs impart resistance to nearly all β-lactams (only monobactams, e.g. aztreonam, have some stability to MBLs (8)) and are not inhibited by SBL inhibitors such as avibactam or vaborbactam. Furthermore, MBL-producing organisms very often exhibit multidrug-resistance phenotypes due to the acquisition of plasmid-borne resistance genes, which are co-located on the same plasmids which carry the MBL genes (9). The most widespread MBL comes from the most recently identified NDM sub-class. NDM-1 was first reported in 2008 in a Swedish patient who had recently returned from India (10), and has now been identified in all

62 continents, with rapid dissemination being observed from reservoirs in Asia, the Middle East and the
63 Balkans (11). While national surveillance programs are not available for many countries, recent
64 reports, including several prevalence surveys and outbreaks, suggest an alarming worldwide increase
65 in incidences of NDM-1 as a percentage of carbapenem non-susceptible or resistant
66 Enterobacteriaceae isolates, e.g. Bulgaria 68% (12), Turkey 30% (13), Iraq 67% (14), China 32%
67 (15), S. Africa 48% (16), and Mexico 92% (17). Numerous variants of NDM-1, having single or
68 double amino-acids changes (18), have been reported from animal and human sources, the most
69 recent being NDM-17 from a chicken in China (19). NDM, VIM and IMP enzymes have been
70 identified in all major Gram-negative pathogens, including the WHO priority pathogens *Klebsiella*
71 *pneumoniae, Pseudomonas aeruginosa* and *Acinetobacter baumanii;* however, as yet, there are no
72 MBL inhibitors in clinical use, despite there being a clear unmet medical need (20).
73 Herein we describe the *in vitro* and *in vivo* properties of ANT431 (Fig. 1), a specific inhibitor of
74 MBLs, which is the result of a medicinal chemistry hit-to-lead program (manuscript in preparation)
75 starting from pyridine-2-carboxylic acid, a compound with weak MBL inhibition, originally reported
76 as an inhibitor of the CphA enzyme from *Aeromonas hydrophila* (21).
77
78
79 **MATERIALS AND METHODS**
80 **Compounds.** Meropenem trihydrate was purchased from Sigma (M2574). Imipenem monohydrate
81 was purchased from Apollo Scientific (OR2453). ANT431 was synthesized as a sodium salt by
82 GVK-Bio (Hyderabad, India) and CRL Discovery (Harlow, UK).
83
84 **Bacterial Strains.**

A panel of MBL expressing *Escherichia coli* strains in an isogenic background was generated by transformation of *E. coli* BL21(DE3) with the pET-9a plasmid containing the cloned NDM-1, VIM-1, VIM-2 or IMP-1 genes under control of the T7 RNA polymerase IPTG inducible system.

The 94 MBL-positive Enterobacteriaceae clinical isolates tested in the susceptibility study were randomly selected from a collection of globally-sourced isolates assembled between 2012 and 2014, and included *Citrobacter freundii* (5), *Enterobacter asburiae* (1), *Enterobacter cloacae* (21), *E. coli* (11), *K. pneumoniae* (50), *Morganella morgannii* (1), *Proteus mirabilis* (2), and *Serratia marcescens* (3). All isolates were genetically characterized to determine their β-lactamase complement. Strains containing KPC or OXA variants were not included in this study since ANT431 has no inhibitory activity against these enzymes

Antimicrobial Agents and Susceptibility Testing. MICs were determined by broth microdilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines (22), using cation-adjusted Mueller Hinton (CAMH) broth (Becton Dickinson). MEM MIC determinations of *E. coli* BL21 (DE3) transformed with pET plasmid derivatives were performed in the presence of 1mM IPTG to ensure a sufficient expression of the MBL gene.

Colonies were taken directly from a culture plate and prepared to a suspension equivalent to the 0.5 McFarland standard using normal saline. MIC plates were seeded within 15 minutes after adjustment of the inoculum suspension turbidity. Trays were incubated at 35 °C for 16 to 20 hr. Quality control (QC) testing was performed each day of testing as specified by CLSI using the following isolates: *E. coli* ATCC 25922 and *P. aeruginosa* ATCC27853.

MBLs Inhibition Assays. Inhibitory activities against purified MBLs (23) were determined by following hydrolysis of 150 μM imipenem (IPM) in 10 mM HEPES pH 7.5 buffer (25 °C) in the presence of 0.025 to 500 μM inhibitor using a Perkin Elmer Envision (UV absorbance: 290 nm).

Compound dilutions were performed in DMSO. K_i values for the inhibition of each enzyme were calculated from IC_{50} measurements using the standard Cheng-Prusoff equation, $K_i = IC_{50} / (1+([S]/K_m)$, where the K_m values for NDM-1, VIM-2 and IMP-1 were 70 μM, 9 μM and 25 μM respectively. The mechanism of inhibition and *Ki* for VIM-1 were determined using the Dixon plot analysis, using MEM as the substrate, due to its higher K_m for this enzyme (50 μm), compared to IPM (1.5 μm), thus allowing for more accurate measurements to be taken.

ACE Inhibition Assay. Selectivity against rabbit Angiotensin Converting Enzyme (ACE; Sigma A6778) metallo-enzyme was determined by following hydrolysis of 10 μM fluorescent substrate Abz-FRK (DNP)-P (Enzo Life Science, BML-P161-0001) in 100 mM Tris HCl pH 7, 50 mM NaCl, 10 μM $ZnCl_2$ buffer in the presence of 0.4 to 200 μM inhibitor, using a Perkin Elmer Envision (fluorescence: Ex: 320 nm, Em: 420 nm). Compound dilutions were performed in DMSO.

GLY2 Inhibition Assay. Selectivity against the human Glyoxalase II (GLY2; R&D Systems 5944-GO) metallo-enzyme was determined by measuring hydrolysis of 500 μM S-lactoylglutathione (SLG, Sigma L7140) using 200 μM 5,5′-dithio-bis-(2-nitrobenzoic acid) thiol detection reagent (DTNB, Sigma D8130) in 50 mM Tris HCl pH7.5, 250 mM NaCl buffer in the presence of 0.4 to 200 μM inhibitor, using a Perkin Elmer Envision (absorbance: 405 nm). Compound dilutions were performed in DMSO

DMPK and cytotoxicity studies. All DMPK and cytotoxicity studies were performed at GVK-Bio following standard procedures. Briefly, plasma protein binding (PPB) was determined in mice and human plasma by ultrafiltration. Binding to the hERG ion channel was assessed using a fluorescence polarization assay (Life Technologies, Cat#PV5365). Inhibition of CYP450 enzymes 1A2, 2C9, 2C19, 2D6 and 3A4 was performed using pooled substrate mixtures in the presence of NADPH with

135 analysis by LC-MS/MS. HepG2 cytotoxicity was assessed using CellTitre Glo Luminescent reagent
136 (Promega, Cat# G7571) after incubation with compound for 72 hr in a 5% CO_2 incubator at 37 ºC.
137 Metabolic stability was determined in liver microsomes (30 min incubation) and plasma (1h
138 incubation) from both mice and humans. Low dose (1 mg/kg) PK studies were performed IV
139 (administration via tail vein) in male Swiss albino mice, using a solution of ANT431 prepared at 1
140 mg/mL in DMSO and then diluted to 0.1 mg/mL in 10% Solutol in PBS.
141
142 **Murine Thigh Infection Model**. Male CD-1 mice (16-18 g) (Charles River Laboratories, Margate,
143 Kent, UK) were rendered neutropenic by immunosuppression with cyclophosphamide by
144 intraperitoneal injection at 150 mg/kg 4 days before infection and 100 mg/kg 1 day before infection.
145 The immunosuppression regime leads to neutropenia starting 24 hr post administration of the first
146 injection continuing throughout the study. *E. coli* IR3 stocks were prepared by addition of glycerol
147 (10%) to logarithmically growing broth cultures in MHB medium and freezing. The frozen stocks
148 were thawed and diluted to give an inoculum of 1.5×10^6 CFU/thigh. Animals (five/group), under
149 inhaled anesthesia with isoflurane, received 0.05 mL of this suspension by intramuscular (IM)
150 administration into both thighs. The test articles were administered intravenously (IV) at 1, 3, 5, and
151 7 hr post-infection at 10 mL/kg. One group of animals was humanely euthanized using
152 pentobarbitone overdose 1-hour post-infection to provide a pre-treatment control group. All animals
153 in the additional groups were euthanized at the end of the study, 9 hr post-infection. Thigh samples
154 were homogenized in ice cold sterile phosphate buffered saline; the homogenates were quantitatively
155 cultured onto CLED agar in triplicate and incubated at 37^{o}C for 18 - 24 hr before colonies were
156 counted. The data from the culture burdens were analyzed using appropriate non-parametric
157 statistical models (Kruskal-Wallis using Conover-Inman to make all pairwise comparisons between
158 groups) with StatsDirect software v. 2.7.8., and compared to vehicle control. For all calculations, the
159 thighs from each animal were treated as two separate data points even though they are not

completely independent samples. All procedures were performed under UK Home Office Licence 40/3644, with local ethical committee clearance (The University of Manchester Standing Committee).

RESULTS

MBL Inhibition by ANT431.

Table 1 shows the inhibitory activities of compound ANT431 against purified NDM-1, VIM-1, VIM-2 and IMP-1 enzymes and potentiation of MEM activity against an *E. coli* laboratory strain expressing the same enzymes from a similar recombinant plasmid background. ANT431 was a potent inhibitor of NDM-1 and VIM-2 with *Ki* values of 290 nM and 195 nM, respectively. Furthermore, susceptibility testing of MEM against NDM-1 and VIM-2 expressing bacteria in the presence of 30 μg/mL ANT431 (97.6 μM) resulted in strong potentiation of MEM antibacterial activity with decreases in MICs of 128-fold and 64-fold, respectively (**Table 1**). This indicates that ANT431 is able to penetrate into the bacterial periplasm where the MBL enzymes are located and thus effect its inhibitory activity. In contrast, ANT431 was a comparatively weak inhibitor of VIM-1 and IMP-1 (*Ki* of 14.6 and 4.15 μM, respectively) and showed correspondingly little or no potentiation of MEM activity against the *E. coli* strain overexpressing these enzymes.

Kinetic analyses of enzyme inhibition demonstrated that ANT431 is a competitive inhibitor with respect to the MEM substrate of VIM-1 **(Figure 2),** NDM-1, VIM-2, and IMP-1 (data not shown), as indicated by Dixon plot analysis by by convergence of lines to an intersection above the X-axis.

Potentiation of MEM Activity Against Clinical Isolates

To investigate the spectrum of activity of ANT431 against medically important pathogens, the antibacterial activity of the MEM/ANT431 combination was profiled against a panel of 94 randomly selected NDM and VIM producing clinical isolates (many of which co-expressed other β-lactam

184 resistance determinants) (**Table 2**). The cumulative distributions of MEM MICs in the presence of 0,
185 10 and 30 μg/mL ANT431 are shown in **Figure 3**. The pronounced leftward shift of the curves
186 compared to the MEM control indicates the greatly improved activity of the ANT431 combinations
187 versus the majority of isolates. In fact, addition of 30 μg/ml of ANT431 resulted in a reduction of
188 the MEM MIC to susceptible levels (2 μg/mL EUCAST breakpoint) in 72% of the MBL-positive
189 isolates, increasing to 79% for the NDM-positive subset.
190 The species, source, and β-lactamase genotypes of the clinical isolates are shown in **Table 2**,
191 highlighting the wide geographical and genetic diversity of the isolate panel. In addition to MBLs,
192 the majority of isolates also expressed one or more SBLs (e.g. TEM, CTX-M-3, CMY-2). The strain
193 set included representatives of the major NDM variants commonly found in clinical isolates, namely
194 NDM-1, -4, -5, -6 and -7. The fact that ANT431 was able to potentiate the MEM MIC by at least
195 eight-fold in at least one strain from each NDM variant group, shows that this compound is active
196 against all these common NDM enzymes. Against VIM-positive isolates, the majority of which
197 carried VIM-1, ANT431 showed only a modest ability to potentiate MEM. This is not surprising
198 given the poor enzymatic inhibitory activity versus the purified VIM-1 enzyme and the lack of MEM
199 potentiation observed against the laboratory *E. coli* strain overexpressing VIM-1 (**Table 1**). Despite
200 this, MEM MICs were potentiated several-fold in many VIM-1-containing clinical isolates with
201 originally low levels of resistance to MEM, often bringing the MIC down to the susceptibility
202 breakpoint. As anticipated, no potentiation was observed against IMP-containing isolates.
203
204 **PK and Physicochemical Properties of ANT431.**
205 ANT431 is a highly water-soluble compound (the Na salt has a solubility of 30 mg/mL in PBS
206 buffer pH 7.4) which is important for IV delivery and possible co-formulation with MEM. The
207 ADME profile of ANT431 was promising, with good metabolic stability in both mice and human
208 liver microsomes and plasma, although moderate inhibition of the 2C9 and 3A4 isoforms of the

cytochrome P450 enzyme were observed (IC_{50} 9 μM and 45 μM, respectively) (**Table 3**). Furthermore, ANT431 showed no measurable inhibition of ACE (an important metallo-enzyme selectivity target involved in blood pressure regulation) or GLY2 (the closest human homologue of the MBL enzymes (24)) at the maximum concentration tested of 200 μM, indicating good selectivity towards bacterial MBLs compared to mammalian metallo-enzymes, and confirming the specific inhibitory mechanism of action of this compound, which does not behave as a general metallo-enzyme inactivator via metal removal from the active site. Consistent with this, ANT431 showed no cytotoxicity up to 100 μM (the highest concentration tested) against the HepG2 human cell line. Furthermore, the IV PK profile of ANT431 in mice indicated a much longer plasma half-life (T½) and greater total exposure (AUC) than MEM (**Figure 4**), suggesting that the PK of the inhibitor should not be a limiting factor in efficacy studies with this combination. Additionally, 20% of unchanged drug was recovered in the urine indicating clearance through the kidneys and illustrating the potential for treatment of urinary tract infections (UTIs)

ANT431 Restores MEM Efficacy in a Mouse Thigh Model of Infection

The *in vivo* efficacy of ANT431 was tested against the NDM-1-positive clinical isolate *E. coli* IR3 (MEM MIC = 32 μg/mL; MEM + ANT431 (at 8 μg/mL) MIC = 4 μg/mL) in a 9 hr murine thigh infection model. MEM is rapidly hydrolyzed by murine renal DHP-1 in mice; hence, this model, with its short dosing interval of 2 hours, has been specifically developed to compensate for the short half-life and so facilitate MEM efficacy experiments in mice. When dosed IV at 1, 3, 5, and 7 hours post-infection, the combination of ANT431 (at 30 or 300 mg/kg) with MEM (at either 50 or 250 mg/kg) resulted in a statistically significant reduction of bacterial counts in the infected thighs of at least 1 $\log_{10}$ with respect to the counts observed with the corresponding dose of MEM alone (**Figure 5**). The compound was well tolerated at 300 mg/kg (amounting to a total dose of 1.2 g/kg within an 8 hours period) with no observable indications of toxicity.

234

DISCUSSION

The global spread of MBL-expressing Enterobacteriaceae represents a major threat to the ongoing usefulness of carbapenem antibiotics to treat severe, often life-threatening, Gram-negative bacterial infections. A new drug which could render MBLs inactive, and hence maintain the effectiveness of carbapenems, would be a valuable adjunct to carbapenem therapies and would prolong the utility of this important class of antibiotics. The discovery of this chemical series, exemplified by ANT431, provides an opportunity to develop such a new combination therapy to treat MBL-CRE infections. This would address a significant unmet medical need since the current options to treat such infections are colistin, an old antibiotic with nephrotoxicity (25), and tigecycline, which is not recommended for bloodstream and UTIs due to its low levels in those body fluids, and has received an FDA warning regarding the increased mortality risk associated with its use (26). Although, new antibiotics are in development that should, in principle, cover MBL-producing CREs, including cefidericol (27), aztreonam-avibactam (28), LYS228 (29), the advantage of developing an MBLi is that it can be combined with a well-characterized and extensively used carbapenem, such as meropenem, in order to directly rescue its activity against MBL-CRE pathogens, and hence allow other new antibiotics to be reserved for situations where no other effective treatment is available.

Other MBL inhibitors have been reported (30-37), many of which display good *in vitro* activity but have not been shown to be efficacious in animal infection models. An exception to this is the natural product aspergillomarasmine A (37), a strong metal ion chelator whose further development is likely to be limited by toxicity (LD_{50} in mice is 159.8 mg/kg) (38). In contrast, ANT431 functions by specific inhibition of the MBL enzymes, as shown by substrate competition studies, and displays good selectivity over non-bacterial metallo-enzymes (ACE, GLY2). Additionally, ANT431 exhibits promising drug-like properties, namely excellent physicochemical properties (low molecular weight simple synthesis, high solubility and stability), lower than 100 μM toxicity against a human cell line (HepG2) and a promising DMPK profile. Furthermore, *in vivo* proof-of-concept has been

demonstrated against a clinical MBL-expressing isolate of *E. coli* in a mouse infection model, with ANT431 nullifying the effects of MBL expression and restoring the efficacy of MEM. This study also demonstrated tolerability of the compound at doses as high as 1.2g/kg in 8 hours.

The activity of ANT431, which at 30 μg/mL could reduce MEM MICs to EUCAST breakpoint susceptibility levels in over 70% of a large panel of highly resistant relevant clinical isolates, demonstrates the potential of such an inhibitor in the clinical setting. However, at the same time, there were nearly 30% of isolates where the MICs were not significantly potentiated. There are several factors which, individually or together, may influence the final MICs of the combination; these are, i) the level of expression of the MBL under the MIC testing conditions, ii) alterations in the structure or level of expression of outer membrane porins, limiting the penetrability of meropenem and/or ANT431, iii) expression of efflux pumps, enhancing the expulsion of meropenem and/or ANT431 from the periplasm. The specific combinations of factors at play will ultimately contribute to the final MIC of each strain and are the subject of ongoing investigations.

Although ANT431 is not in itself a development candidate, due to its limited MBL inhibition profile and modest potentiation of meropenem against certain clinical strains carrying key MBL enzymes, this prototype molecule represents an excellent starting point for chemical lead optimization. The goal of this program will be to improve intrinsic potency and broaden the spectrum of activity to include a higher proportion of MBL-positive isolates, while maintaining its promising drug-like characteristics, in order to deliver a first-in-class MBL inhibitor for the treatment of MBL-CRE infections. Given the rapid worldwide emergence of MBLs, NDM-1 in particular, and the lack of effective drugs targeting these resistance mechanisms, developing such a treatment is an urgent medical priority.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the support of the Wellcome Trust, through provision of a Seeding Drug Discovery Initiative award to Antabio SAS. Thanks also are due to David Pallin and colleagues (Charles River Laboratories, Harlow, UK) for their input into the medicinal chemistry program that led to the discovery of ANT431; Silvia Tanfoni (Department of Medical Biotechnology, University of Siena) for technical assistance with enzyme assays; Luisa Borgianni (Department of Medical Biotechnology, University of Siena) for technical assistance in enzyme production and purification.

TABLES

Table 1. Effect of ANT431 on inhibition of purified MBLs and potentiation of MEM activity against *E. coli* BL21 (DE3) transformed with pET plasmids containing NDM-1, VIM-1, VIM-2 or IMP-1.

Enzyme	NDM-1		VIM-1		VIM-2		IMP-1	
Compound	*Ki*[1]	MIC[2]	*Ki*	MIC	*Ki*	MIC	*Ki*	MIC
None	-	32	-	4	-	4	-	8
ANT431	0.29	0.25	14.6*	4	0.19	0.06	4.15	4

[1] K_i, enzyme inhibition constant (µM)
[2] MIC, minimum inhibitor concentration of MEM (µg/mL) determined alone or in presence of 30 µg/mL of compound
* *Ki* determined from Dixon-Plot analysis (Fig.2)

298 **Table 2. Susceptibility testing data of 94 MBL-positive *Enterobacteriaceae* to MEM alone and**
299 **in combination with ANT431 (at 10 and 30 µg/mL).** Strains highlighted in bold are colistin
300 resistant. Grey-shaded cells indicate MEM MIC ≤ 2 µg/mL

MBL type	Organism Name	Country	Characterized ß-lactamases	MER	MER with ANT431 10 µg/mL	MER with ANT431 30 µg/mL
NDM-positive	*C. freundii*	Serbia	TEM-OSBL(b); CMY; NDM-1;	16	0.25	0.12
		Serbia	TEM-OSBL(b); CTX-M-15; CMY; NDM-1;	64	4	1
	E. asburiae	**Kenya**	**TEM-OSBL(b); VEB-2; NDM-1;**	**128**	**8**	**2**
	E. cloacae	Germany	CTX-M-14; NDM-1;	64	8	4
		Philippines	**TEM-OSBL(b); CTX-M-15; ACT-TYPE; NDM-1;**	**32**	**0.12**	**0.12**
		Philippines	**MIR-TYPE; NDM-1;**	**32**	**0.25**	**0.12**
		Philippines	**NDM-7;**	**32**	**1**	**0.25**
		Romania	CTX-M-15; ACT-16; NDM-1;	16	0.12	0.25
		Romania	SHV-12(e); TEM-OSBL(b); CTX-M-3; ACT-TYPE; NDM-1;	128	4	2
		Serbia	TEM-OSBL(b); CTX-M-15; NDM-1;	8	0.25	0.12
		Serbia	TEM-OSBL(b); CTX-M-3; ACT-TYPE; NDM-1;	16	0.12	0.25
		Spain	TEM-OSBL(b); CTX-M-15; ACT-TYPE; NDM-1;	64	8	1
		UAE	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;	64	0.12	0.25
		Vietnam	SHV-12(e); TEM-OSBL(b); CTX-M-14; ACT-TYPE; NDM-1;	>128	128	64
		Vietnam	TEM-OSBL(b); CTX-M-15; CTX-M-14; ACT-TYPE; NDM-1;	16	8	1
		Vietnam	TEM-OSBL(b); ACT-TYPE; NDM-1;	128	64	32
	E. coli	Egypt	TEM-OSBL(b); CTX-M-15; NDM-1;	128	4	0.5
		Egypt	TEM-OSBL(b); CTX-M-27; NDM-5;	64	2	0.5
		India	CTX-M-15; CMY-2; NDM-1;	64	2	0.25
		India	CTX-M-15; CMY-2; NDM-4;	128	8	1
		India	TEM-OSBL(b); CTX-M-15; CMY; NDM-1;	64	2	0.5
		India	CTX-M-15; NDM-6;	>128	128	32
		UAE	TEM-OSBL(b); CTX-M-15; CMY-TYPE; NDM-4;	128	4	0.5
		Vietnam	TEM-OSBL(b); NDM-1;	32	0.25	≤ 0.06
		Vietnam	TEM-OSBL(b); CMY-TYPE; NDM-1;	32	0.5	0.12
		Vietnam	CTX-M-15; NDM-4;	128	4	0.5
		Vietnam	TEM-OSBL(b); CTX-M-15; CMY-TYPE; NDM-5;	64	4	2
	K. pneumoniae	Egypt	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;	128	4	0.5
		Egypt	SHV-OSBL(b); CTX-M-15; NDM-1;	128	8	1
		Egypt	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;	2	4	0.5
		Egypt	SHV-12(e); TEM-OSBL(b); CTX-M-15; CTX-M-14; NDM-1;	64	2	1
		Greece	**SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;**	**64**	**2**	**0.5**
		Guatemala	SHV-12(e); TEM-OSBL(b); CTX-M-15; NDM-6;	128	4	0.5

		India	CTX-M-15; CMY-2; NDM-1;	64	2	0.25
		India	SHV-2A(e); CTX-M-15; NDM-1;	64	2	0.5
		India	CTX-M-15; DHA; NDM-1;	32	0.25	0.12
		India	CTX-M-15; NDM-1;	16	2	0.5
		Jordan	SHV-OSBL(b); CTX-M-15; NDM-1;	64	1	0.25
		Kuwait	SHV-OSBL(b); CTX-M-15; CMY; NDM-1;	128	16	2
		Nigeria	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;	64	4	0.25
		Nigeria	SHV-OSBL(b); CTX-M-15; NDM-1;	64	1	0.25
		Philippines	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;	32	0.5	0.25
		Philippines	SHV-OSBL(b); NDM-1;	128	8	0.5
		Philippines	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-7;	64	1	0.25
		Philippines	**SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;**	**64**	**1**	**0.25**
		Philippines	SHV-OSBL(b); CTX-M-27; NDM-1;	64	4	1
		Philippines	SHV-12(e); TEM-OSBL(b); CTX-M-15; NDM-7;	128	4	0.5
		Romania	**SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;**	**64**	**4**	**2**
		Romania	SHV-OSBL(b); CTX-M-15; NDM-1;	64	4	0.5
		Russia	SHV-11(b); NDM-1;	128	16	8
		Saudi Arabia	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;	64	1	0.25
		Serbia	SHV-OSBL(b); TEM-OSBL(b); CMY; NDM-1;	4	≤0.06	0.12
		Serbia	SHV-OSBL(b); CTX-M-15; CMY; NDM-1;	32	0.25	0.12
		Serbia	**SHV-OSBL(b); CTX-M-15; NDM-1;**	**>128**	**64**	**32**
		Serbia	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; CMY-TYPE; NDM-1;	128	64	64
		Serbia	SHV-OSBL(b); CMY; NDM-1;	64	16	8
		Serbia	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;	64	8	4
		Thailand	TEM-OSBL(b); CTX-M-15; CTX-M-27; NDM-1;	128	8	1
		Thailand	SHV-OSBL(b); NDM-1;	128	64	32
		Turkey	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-1;	32	1	0.25
		Turkey	SHV-OSBL(b); CTX-M-15; NDM-1;	64	4	1
		UAE	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; NDM-5;	>128	128	64
		UAE	SHV-OSBL(b); CTX-M-15; NDM-1;	128	64	32
		UAE	**SHV-OSBL(b); NDM-1;**	**128**	**64**	**32**
		UAE	SHV-OSBL(b); TEM-OSBL(b); DHA-TYPE; NDM-1;	64	0.25	0.12
		UK	**SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; CMY-6; NDM-1;**	**64**	**0.5**	**0.5**
		Vietnam	SHV-OSBL(b); TEM-OSBL(b); CTX-M-27; DHA-TYPE; NDM-4;	>128	16	2
		Vietnam	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; CMY-TYPE; DHA-TYPE; NDM-1;	128	32	32
	M. morganii	**India**	**CTX-M-15; DHA; NDM-1;**	**4**	**0.25**	**0.12**
	S. marcescens	**Romania**	**TEM-OSBL(b); CTX-M-3; DHA; NDM-1;**	**64**	**1**	**0.25**
		Romania	**TEM-OSBL(b); CTX-M-15; NDM-1;**	**>128**	**>128**	**>128**
VIM-positive	*C. freundii*	United States	CMY-81; VIM-32;	32	16	4
		Italy	SHV-12(e); CMY; VIM-1;	2	0.5	0.25
		Italy	SHV-12(e); CMY; VIM-1;	4	1	0.5

	E. cloacae	Greece	TEM-OSBL(b); ACT-32; VIM-1;	4	1	0.25
		Greece	VIM-1;	32	32	16
		Greece	TEM-OSBL(b); VIM-1;	32	16	2
		Mexico	VIM-23;	4	2	1
		Greece	TEM-OSBL(b); VIM-1;	4	1	0.5
		Croatia	TEM-OSBL(b); CTX-M-15; ACT-TYPE; VIM-1;	32	32	32
	K. pneumoniae	Greece	SHV-OSBL(b); VIM-1;	>128	>128	128
		Greece	SHV-OSBL(b); VIM-1;	128	128	64
		Romania	TEM-OSBL(b); CTX-M-3; VIM-4;	8	0.25	0.25
		Hungary	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; VIM-4;	4	0.25	0.12
		Egypt	SHV-OSBL(b); TEM-OSBL(b); CTX-M-14; CMY-TYPE; VIM-1;	64	32	8
	P. mirabilis	**Greece**	**TEM-OSBL(b); CMY-16; VIM-1;**	**8**	**1**	**0.25**
	S. marcescens	**Turkey**	**VIM-5;**	**128**	**32**	**2**
IMP-positive	*E. cloacae*	Australia	TEM-OSBL(b); ACT-TYPE; IMP-4;	4	4	2
		Thailand	TEM-OSBL(b); CTX-M-15; ACT-TYPE; IMP-14;	16	8	16
	K. pneumoniae	Philippines	SHV-OSBL(b); CTX-M-15; IMP-26;	32	32	16
		Japan	SHV-OSBL(b); CTX-M-2; IMP-1;	64	64	64
		Japan	SHV-OSBL(b); CTX-M-2; IMP-6;	>128	>128	>128
		Philippines	SHV-OSBL(b); TEM-OSBL(b); CTX-M-15; IMP-26;	16	16	2
	P. mirabilis	**Philippines**	**DHA-1; IMP-26;**	**32**	**32**	**16**

301

302

303 **Table 3.** Physicochemical, ADME, selectivity, cytotoxicity and safety properties of compound
304 ANT431.
305

Property	ANT431
Molecular Weight, acid/sodium salt	285.3/307.3
LogD (pH 7.4)	-2.5
Solubility (sodium salt) in PBS pH 7.4	30 mg/mL
PPB % bound, mouse / human	82.5 % / 97.6 %
Microsomal stability, % remaining at 30 min, mouse / human	> 95 % (both)
Plasma stability, % remaining at 1 hr, mouse/human	100 % (both)
ACE inhibition (IC_{50})	> 200 μM
Glyoxalase II (IC_{50})	> 200 μM
HepG2 cytotoxicity IC_{50} at 24 hr	> 100 μM
CYP inhibition IC_{50} (1A2, 2C9, 2C19, 2D6, 3A4)	>200, 9, >200, >200, 45 μM
hERG inhibition IC_{50}	> 10 μM

306
307

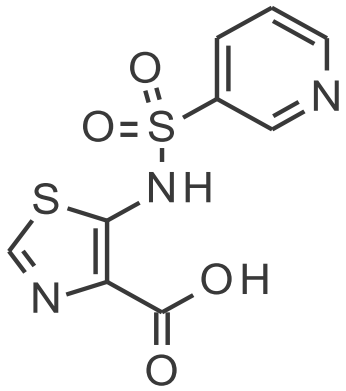
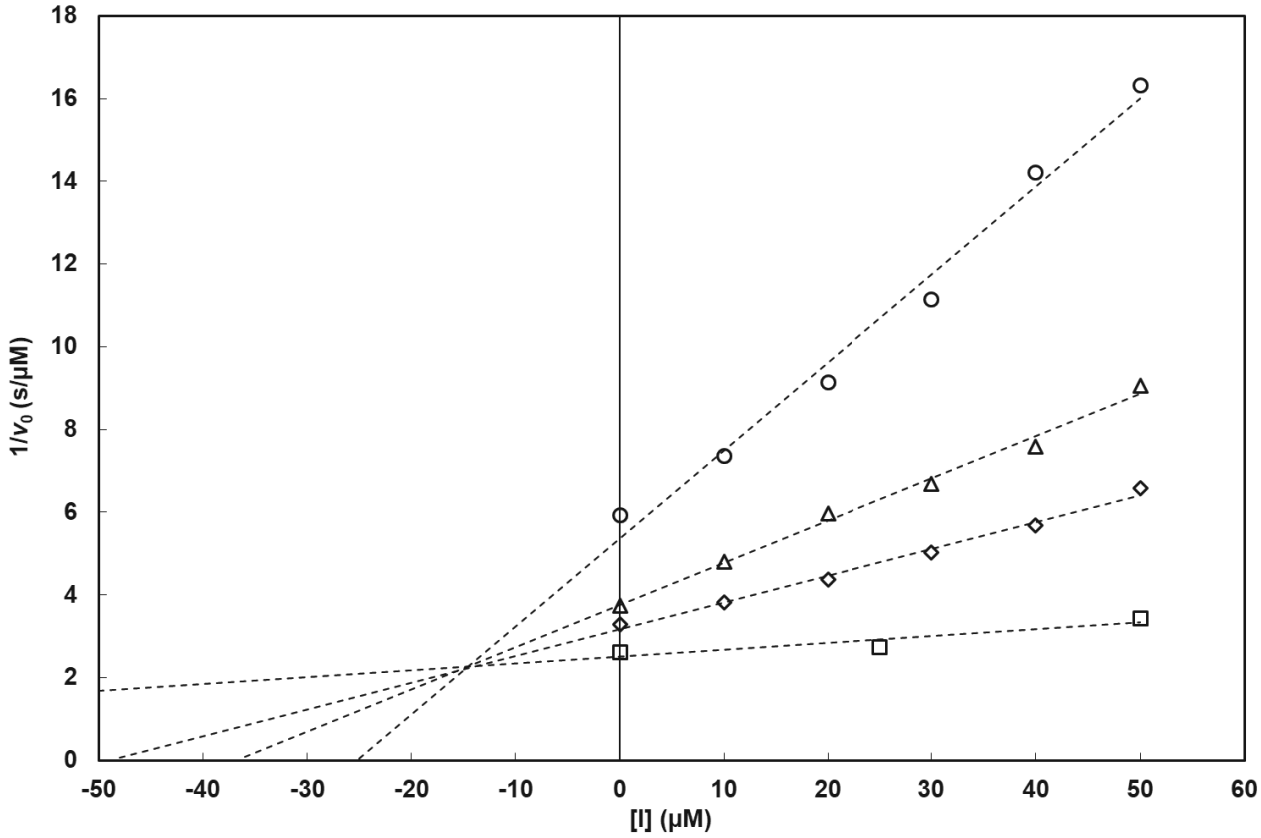


Figure 1. Chemical structure of ANT431



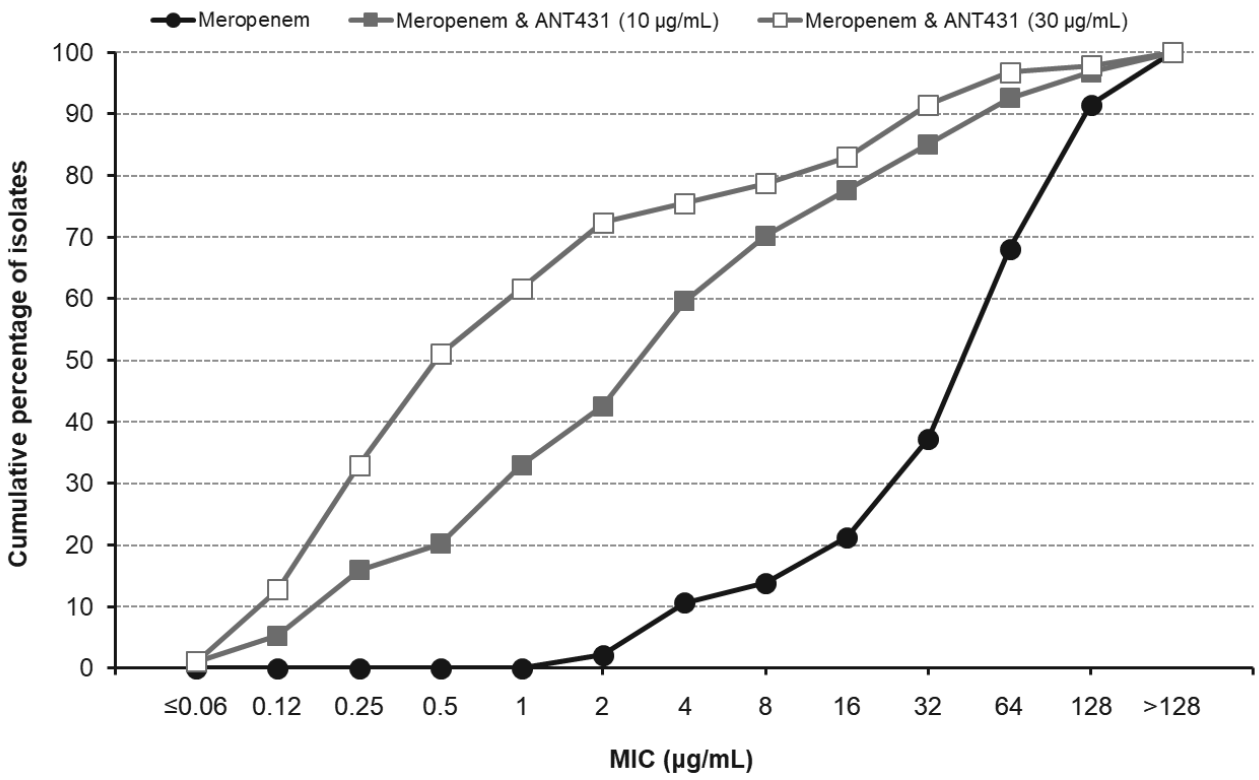
311

312

313 **Figure 2.** Dixon analysis of the inhibition of VIM-1 by ANT431. Initial rates of β-lactam hydrolysis
314 were measured spectrophotometrically using MEM (○, 40 μM; △, 90 μM; ◇, 130 μM; □, 800
315 μM) as the substrate in 50 mM HEPES buffer (pH 7.5), in the presence of 6.9 nM purified VIM-1.
316 Inhibitor concentrations ranged from 10 to 50 μM. Initial rates were measured in triplicates (SD,
317 ≤5%). V_{max} was unaffected by ANT431. These data fully support a competitive mode of inhibition of
318 the enzyme by ANT431, with a K_i value of 14.6 ± 0.6 μM. Similar conclusions (data not shown)
319 were obtained with the NDM-1, IMP-1 and VIM-2 MBLs.

320

321

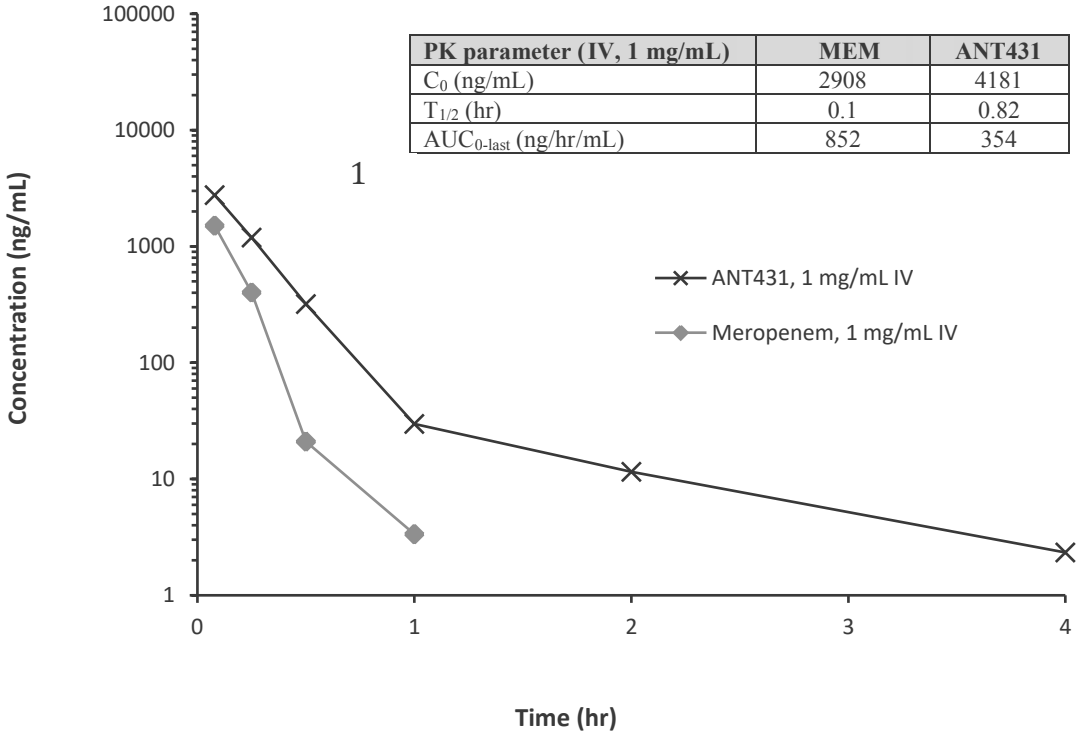


322

323 **Figure 3.** Cumulative MIC distribution of MEM alone and in combination with ANT431 against 94

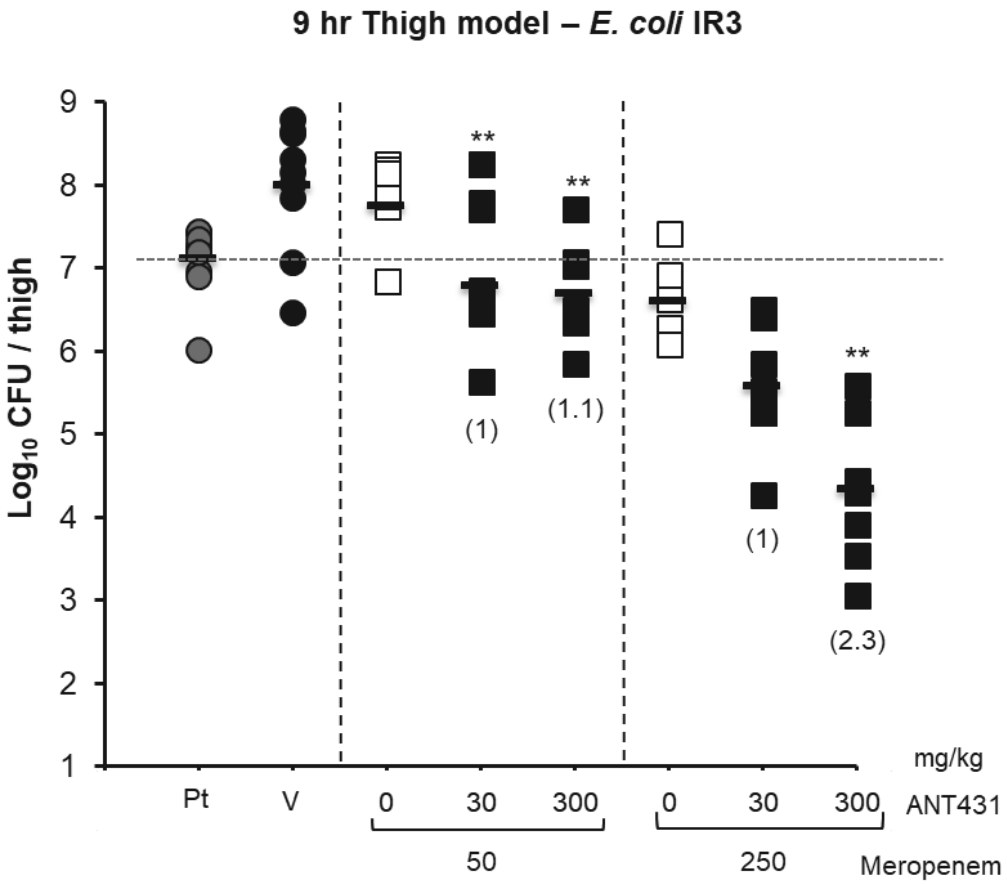
324 MBL-producing Enterobacteriaceae

325



PK parameter (IV, 1 mg/mL)	MEM	ANT431
C_0 (ng/mL)	2908	4181
$T_{1/2}$ (hr)	0.1	0.82
$AUC_{0\text{-last}}$ (ng/hr/mL)	852	354

Figure 4. Plasma pharmacokinetics of ANT431 and MEM in Swiss albino mice after 1 mg/kg intravenous administration. Table insert shows PK parameters (C_0, $T_{1/2}$, $AUC_{0\text{-last}}$).



MEM MIC (µg/mL)	Alone	+ANT431 (8 µg/mL)
E. coli IR3 (NDM-1)	32	4

Figure 5. Efficacy of MEM alone and in combination with ANT431 in murine thigh infection model, infected with *E. coli* IR3 (NDM-1). Bacterial counts (CFUs) were obtained from homogenized thighs of infected animals (n=5) treated IV at 1, 3, 5, and 7 h.p.i. Pt = pre-treatment group; V = vehicle only group. Numbers in brackets refer to log reduction in CFUs compared to respective MEM only group. ** Statistically significant difference (p = <0.005) compared to MEM only group. Table below figure shows MICs for MEM with and without ANT431 at 8 µg/mL.

References

1. Miller CP, Bohnhoff M. 1945. Studies on the action of penicillin; development of penicilli resistance by gonococcus. Proc Soc Exp Biol Med 60:354-6.
2. Abraham EP, Chain E. 1940. An Enzyme from Bacteria able to Destroy Penicillin. Nature 146:837.
3. Hunter PA, Coleman K, Fisher J, Taylor D. 1980. In vitro synergistic properties of clavulanic acid, with ampicillin, amoxycillin and ticarcillin. J Antimicrob Chemother 6:455-70.
4. De Koning GA, Tio D, Coster JF, Coutinho RA, Ansink-Schipper MC. 1981. The combination of clavulanic acid and amoxycillin (Augmentin) in the treatment of patients infected with penicillinase producing gonococci. J Antimicrob Chemother 8:81-2.
5. Bush K, Jacoby GA, Medeiros AA. 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 39:1211-33.
6. Temkin E, Torre-Cisneros J, Beovic B, Benito N, Giannella M, Gilarranz R, Jeremiah C, Loeches B, Machuca I, Jimenez-Martin MJ, Martinez JA, Mora-Rillo M, Navas E, Osthoff M, Pozo JC, Ramos Ramos JC, Rodriguez M, Sanchez-Garcia M, Viale P, Wolff M, Carmeli Y. 2017. Ceftazidime-Avibactam as Salvage Therapy for Infections Caused by Carbapenem-Resistant Organisms. Antimicrob Agents Chemother 61.
7. Hackel MA, Lomovskaya O, Dudley MN, Karlowsky JA, Sahm DF. 2017. Evaluation of the In Vitro Activity of Meropenem-Vaborbactam against Clinical Isolates of KPC-Positive Enterobacteriaceae. Antimicrob Agents Chemother doi:10.1128/aac.01904-17.
8. Queenan AM, Bush K. 2007. Carbapenemases: the versatile beta-lactamases. Clin Microbiol Rev 20:440-58, table of contents.
9. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N. 2010. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis 10:597-602.
10. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR. 2009. Characterization of a new metallo-beta-lactamase gene, bla(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 53:5046-54.
11. Li JJ, Munoz-Price LS, Spychala CN, DePascale D, Doi Y. 2016. New Delhi Metallo-beta-Lactamase-1-Producing Klebsiella pneumoniae, Florida, USA(1). Emerg Infect Dis 22:744-6.
12. Savov E, Politi L, Spanakis N, Trifonova A, Kioseva E, Tsakris A. 2017. NDM-1 Hazard in the Balkan States: Evidence of the First Outbreak of NDM-1-Producing Klebsiella pneumoniae in Bulgaria. Microb Drug Resist doi:10.1089/mdr.2017.0230.
13. Haciseyitoglu D, Dokutan A, Abulaila A, Erdem F, Cag Y, Ozer S, Aktas Z. 2017. The First Enterobacter cloacae Co-Producing NDM and OXA-48 Carbapenemases and Interhospital Spread of OXA-48 and NDM-Producing Klebsiella pneumoniae in Turkey. Clin Lab 63:1213-1222.

14. Hussein NH. 2017. Emergence of NDM-1 among carbapenem-resistant Klebsiella pneumoniae in Iraqi hospitals. Acta Microbiol Immunol Hung doi:10.1556/030.64.2017.026:1-17.
15. Dong F, Lu J, Wang Y, Shi J, Zhen JH, Chu P, Zhen Y, Han SJ, Guo YL, Song WQ. 2017. A Five-year Surveillance of Carbapenemase-producing Klebsiella pneumoniae in a Pediatric Hospital in China Reveals Increased Predominance of NDM-1. Biomed Environ Sci 30:562-569.
16. Singh-Moodley A, Perovic O. 2016. Antimicrobial susceptibility testing in predicting the presence of carbapenemase genes in Enterobacteriaceae in South Africa. BMC Infect Dis 16:536.
17. Bocanegra-Ibarias P, Garza-Gonzalez E, Morfin-Otero R, Barrios H, Villarreal-Trevino L, Rodriguez-Noriega E, Garza-Ramos U, Petersen-Morfin S, Silva-Sanchez J. 2017. Molecular and microbiological report of a hospital outbreak of NDM-1-carrying Enterobacteriaceae in Mexico. PLoS One 12:e0179651.
18. Khan AU, Maryam L, Zarrilli R. 2017. Structure, Genetics and Worldwide Spread of New Delhi Metallo-beta-lactamase (NDM): a threat to public health. BMC Microbiol 17:101.
19. Liu Z, Wang Y, Walsh TR, Liu D, Shen Z, Zhang R, Yin W, Yao H, Li J, Shen J. 2017. Plasmid-Mediated Novel blaNDM-17 Gene Encoding a Carbapenemase with Enhanced Activity in a Sequence Type 48 Escherichia coli Strain. Antimicrob Agents Chemother 61.
20. Dortet L, Poirel L, Nordmann P. 2014. Worldwide dissemination of the NDM-type carbapenemases in Gram-negative bacteria. Biomed Res Int 2014:249856.
21. Horsfall LE, Garau G, Lienard BM, Dideberg O, Schofield CJ, Frere JM, Galleni M. 2007. Competitive inhibitors of the CphA metallo-beta-lactamase from Aeromonas hydrophila. Antimicrob Agents Chemother 51:2136-42.
22. ClinicalandLaboratoryStandardsInstitute. 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 8th ed. Approved standard M07-A8. Clinical and Laboratory Standards Institute, Wayne, Pa, USA.
23. Docquier JD, Lamotte-Brasseur J, Galleni M, Amicosante G, Frere JM, Rossolini GM. 2003. On functional and structural heterogeneity of VIM-type metallo-beta-lactamases. J Antimicrob Chemother 51:257-66.
24. Pettinati I, Brem J, Lee SY, McHugh PJ, Schofield CJ. 2016. The Chemical Biology of Human Metallo-beta-Lactamase Fold Proteins. Trends Biochem Sci 41:338-55.
25. Ordooei Javan A, Shokouhi S, Sahraei Z. 2015. A review on colistin nephrotoxicity. Eur J Clin Pharmacol 71:801-10.
26. Dixit D, Madduri RP, Sharma R. 2014. The role of tigecycline in the treatment of infections in light of the new black box warning. Expert Rev Anti Infect Ther 12:397-400.
27. Choi JJ, McCarthy MW. 2018. Cefiderocol: a novel siderophore cephalosporin. Expert Opin Investig Drugs 27:193-197.
28. Marshall S, Hujer AM, Rojas LJ, Papp-Wallace KM, Humphries RM, Spellberg B, Hujer KM, Marshall EK, Rudin SD, Perez F, Wilson BM, Wasserman RB, Chikowski L, Paterson DL, Vila AJ, van Duin D, Kreiswirth BN, Chambers HF, Fowler VG, Jr., Jacobs MR, Pulse ME, Weiss WJ, Bonomo RA. 2017. Can Ceftazidime-Avibactam and Aztreonam Overcome beta-Lactam Resistance Conferred by Metallo-beta-Lactamases in Enterobacteriaceae? Antimicrob Agents Chemother 61.
29. Reck F, Bermingham A, Blais J, Capka V, Cariaga T, Casarez A, Colvin R, Dean CR, Fekete A, Gong W, Growcott E, Guo H, Jones AK, Li C, Li F, Lin X, Lindvall M, Lopez S, McKenney

D, Metzger L, Moser HE, Prathapam R, Rasper D, Rudewicz P, Sethuraman V, Shen X, Shaul J, Simmons RL, Tashiro K, Tang D, Tjandra M, Turner N, Uehara T, Vitt C, Whitebread S, Yifru A, Zang X, Zhu Q. 2018. Optimization of novel monobactams with activity against carbapenem-resistant Enterobacteriaceae - Identification of LYS228. Bioorg Med Chem Lett 28:748-755.

30. Arjomandi OK, Hussein WM, Vella P, Yusof Y, Sidjabat HE, Schenk G, McGeary RP. 2016. Design, synthesis, and in vitro and biological evaluation of potent amino acid-derived thiol inhibitors of the metallo-beta-lactamase IMP-1. Eur J Med Chem 114:318-27.
31. Klingler FM, Wichelhaus TA, Frank D, Cuesta-Bernal J, El-Delik J, Muller HF, Sjuts H, Gottig S, Koenigs A, Pos KM, Pogoryelov D, Proschak E. 2015. Approved Drugs Containing Thiols as Inhibitors of Metallo-beta-lactamases: Strategy To Combat Multidrug-Resistant Bacteria. J Med Chem 58:3626-30.
32. Yusof Y, Tan DT, Arjomandi OK, Schenk G, McGeary RP. 2016. Captopril analogues as metallo-beta-lactamase inhibitors. Bioorg Med Chem Lett 26:1589-93.
33. Brem J, van Berkel SS, Aik W, Rydzik AM, Avison MB, Pettinati I, Umland KD, Kawamura A, Spencer J, Claridge TD, McDonough MA, Schofield CJ. 2014. Rhodanine hydrolysis leads to potent thioenolate mediated metallo-beta-lactamase inhibition. Nat Chem 6:1084-90.
34. Liu XL, Yang KW, Zhang YJ, Ge Y, Xiang Y, Chang YN, Oelschlaeger P. 2016. Optimization of amino acid thioesters as inhibitors of metallo-beta-lactamase L1. Bioorg Med Chem Lett 26:4698-701.
35. Brem J, Cain R, Cahill S, McDonough MA, Clifton IJ, Jimenez-Castellanos JC, Avison MB, Spencer J, Fishwick CW, Schofield CJ. 2016. Structural basis of metallo-beta-lactamase, serine-beta-lactamase and penicillin-binding protein inhibition by cyclic boronates. Nat Commun 7:12406.
36. Yang SK, Kang JS, Oelschlaeger P, Yang KW. 2015. Azolylthioacetamide: A Highly Promising Scaffold for the Development of Metallo-beta-lactamase Inhibitors. ACS Med Chem Lett 6:455-60.
37. King AM, Reid-Yu SA, Wang W, King DT, De Pascale G, Strynadka NC, Walsh TR, Coombes BK, Wright GD. 2014. Aspergillomarasmine A overcomes metallo-beta-lactamase antibiotic resistance. Nature 510:503-6.
38. Matsuura A, Okumura H, Asakura R, Ashizawa N, Takahashi M, Kobayashi F, Ashikawa N, Arai K. 1993. Pharmacological profiles of aspergillomarasmines as endothelin converting enzyme inhibitors. Jpn J Pharmacol 63:187-93.