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How to cite

LE TERRIER, Christophe et al. Relative inhibitory activities of the broad-spectrum β-lactamase inhibitor taniborbactam against metallo-β-lactamases. In: Antimicrobial agents and chemotherapy, 2024, vol. 68, n° 2, p. e00991–23. doi: 10.1128/aac.00991-23

This publication URL: https://archive-ouverte.unige.ch/unige:181940

Publication DOI: 10.1128/aac.00991-23

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

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Relative inhibitory activities of the broad-spectrum β-lactamase inhibitor taniborbactam against metallo-β-lactamases

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ABSTRACT Taniborbactam (TAN) is a novel broad-spectrum β-lactamase inhibitor with significant activity against subclass B1 metallo-β-lactamases (MBLs). Here, we showed that TAN exhibited an overall excellent activity against B1 MBLs including most NDM- and VIM-like as well as SPM-1, GIM-1, and DIM-1 enzymes, but not against SIM-1. Noteworthy, VIM-1-like enzymes (particularly VIM-83) were less inhibited by TAN than VIM-2-like. Like NDM-9, NDM-30 (also differing from NDM-1 by a single amino acid substitution) was resistant to TAN.

KEYWORDS metallo-β-lactamase, NDM, IMP, VIM, taniborbactam

Metallo-β-lactamases (MBLs) increasingly encountered in Gram-negative bacteria are the most problematic carbapenemases (1). Indeed, MBLs exhibit several concerning features, including (i) an ability to confer resistance to all β-lactams with the exception of monobactams, (ii) a resistance to all clinically available β-lactamase inhibitors currently on the market, and (iii) a worldwide dissemination among Gram-negative bacteria (2). MBLs are class B β-lactamases which can be divided into three subclasses (B1, B2, and B3) (3). Subclass B1 includes the most acquired MBLs, mainly represented by NDM, IMP, and VIM enzymes, and also the less frequently identified SPM, SIM, DIM, and GIM enzymes (Fig. 1) (4). The subclass B2 enzymes are mainly naturally occurring and chromosomally encoded β-lactamases produced by diverse species such as CphA from *Aeromonas* spp., SFH from *Serratia fonticola*, or PFM from *Pseudomonas fluorescens* (5, 6). Most of the subclass B3 enzymes are also encoded by naturally occurring genes in some given species, such as L1 from *Stenotrophomonas maltophilia* or the GOB-like enzymes from *Elizabethkingia* spp. (7). AIM-1 is an exception from the B3 subgroup, being encoded on a mobile genetic element and identified as acquired in major human pathogens (8).

By contrast to most of the other recently developed β-lactamase inhibitors, belonging to the diazabicyclooctane family (avibactam, relebactam, zidebactam, nacubactam, and durlobactam) or boronate family (vaborbactam and ledaborbactam), that only inhibit serine-based β-lactamases, taniborbactam (TAN), alias VNRX-5133, possesses a significant and additional inhibitory action against MBLs (9–11). This novel boronate inhibitor is supposed to be commercialized in combination with cefepime (FEP) (https://clinicaltrials.gov/ct2/show/NCT03840148) and is currently under evaluation by the Food and Drug Administration. Indeed, TAN shows almost low K_i (inhibitory constant) values as well as avibactam (diazabicyclooctane) and vaborbactam (boronate) against the class A extended-spectrum β-lactamase CTX-M-15, the class A carbapenemase KPC-2, or the class D carbapenemases of the OXA-48 type, but its ability to inhibit the class B carbapenemases NDM-1 (K_i, 0.08 µM) and VIM-2 (K_i, 0.02 µM) is more effective than avibactam and vaborbactam (respective values being >30 µM for avibactam and vaborbactam in both cases) (10, 11). However, it has been recently demonstrated that the inhibitory property of TAN can be significantly affected by one single amino acid substitution in the

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The authors declare no conflict of interest.

See the funding table on p. 7.

Received 27 July 2023
Accepted 7 October 2023
Published 4 December 2023

FIG 1 Sequence alignment of subclass B1 metallo-β-lactamases. Nomenclature is according to the standard numbering scheme of class B β-lactamase (BBL) sequence numbering. (.), identical residue compared to NDM-1 sequence; (-), no corresponding residue compared to the NDM-1 sequence. Residues in bold are zinc ligands conserved in the subclass B1 MBL enzymes defining the BBL numbering. The Hα3 helix is overlined.

NDM-1 or the VIM-2 sequences, leading to variants such as NDM-9 or VIM-2 (Glu149Lys in BBL numbering), respectively, being considered as "resistant" to the inhibitory action of TAN (12, 13).

Following that observation, raising significant concern about the overall efficacy of TAN against MBLs, our aim here is to evaluate the relative inhibitory activity of TAN against a range of acquired MBLs belonging to sub-classes B1 and B3, in order to evaluate its effectiveness and spectrum of activity against acquired MBLs. The subclass B2 MBL PFM-1 was also added for comparison.

In order to compare the inhibitory activity of TAN against MBLs, the genes encoding different acquired β-lactamases classified as class B1 carbapenemases (*bla*$_{\text{VIM-1}}$, *bla*$_{\text{VIM-2}}$, *bla*$_{\text{VIM-4}}$, *bla*$_{\text{VIM-5}}$, *bla*$_{\text{VIM-6}}$, *bla*$_{\text{VIM-19}}$, *bla*$_{\text{VIM-53}}$, *bla*$_{\text{VIM-83}}$, *bla*$_{\text{NDM-1}}$, *bla*$_{\text{NDM-2}}$, *bla*$_{\text{NDM-4}}$, *bla*$_{\text{NDM-5}}$, *bla*$_{\text{NDM-7}}$, *bla*$_{\text{NDM-9}}$, *bla*$_{\text{NDM-14}}$, *bla*$_{\text{NDM-19}}$, *bla*$_{\text{NDM-30}}$, *bla*$_{\text{NDM-35}}$, *bla*$_{\text{NDM-47}}$, *bla*$_{\text{IMP-1}}$, *bla*$_{\text{IMP-2}}$, *bla*$_{\text{IMP-4}}$, *bla*$_{\text{IMP-13}}$, *bla*$_{\text{SIM-1}}$, *bla*$_{\text{DIM-1}}$, *bla*$_{\text{GIM-1}}$, *bla*$_{\text{SPM-1}}$), class B2 (*bla*$_{\text{PFM-1}}$), or class B3 (*bla*$_{\text{AIM-1}}$) were amplified by PCR, and corresponding amplicons were cloned into plasmid pUCP24 and expressed in *Escherichia coli* TOP10. Of note, while NDM-, IMP-, and VIM-type encoding genes are widely disseminated among Enterobacterales and *Pseudomonas aeruginosa*, those encoding DIM, GIM, SPM, and AIM are rarely identified and have so far been only identified in *Pseudomonas* sp.

To assess the inhibitory potency of TAN, susceptibility testing was performed by the broth microdilution (BMD) method, determining the minimal inhibitory concentrations (MICs) of different antibiotics or antibiotic plus β-lactamase inhibitor combinations, including FEP, ceftazidime (CAZ), imipenem (IPM), and their combinations with TAN (FEP-TAN, CAZ-TAN, and IPM-TAN). CAZ and FEP were purchased from Sigma-Aldrich (Saint-Louis, USA), while IPM from HuiChem (Shanghai, China). TAN (HY-109124) was purchased from MedChem Express (Luzern, Switzerland) and was used at a fixed concentration of 4 µg/mL (14). MICs were determined in duplicate using BMD in cation-adjusted Mueller-Hinton broth (Bio-Rad, Marnes-la-Coquette, France) according to the EUCAST guidelines (15). The results were interpreted according to the decrease in MIC value for the same antibiotic or combination of the corresponding recombinant strain expressing the MBL enzyme. The reference strains *E. coli* ATCC 25922 and *E. coli*

NCTC 13353, as well as the recombinant strains *E. coli* TOP10 (pNDM-1) and *E. coli* TOP10 (pNDM-9), were used as controls for all testing (13, 16). The 50% inhibitory concentrations (IC_{50}) of TAN were determined for all MBLs, using crude extracts of cultures of *E. coli* TOP10 recombinant strains, respectively, producing the different β-lactamases to be tested, as previously published (17). The nomenclature used in this article for numbering amino acid modifications is the standard numbering scheme of class B ß-lactamase (BBL numbering) (Fig. 1 and 2).

With the exception of the PFM-1 producer, all recombinant MBL-producing isolates exhibited resistance to CAZ, even though MICs were quite variable, with producers of SPM-1, VIM-like, IMP-like, and NDM-like enzymes showing higher resistance levels compared to those respectively producing DIM-1, GIM-1, SIM-1, and AIM-1 (Table 1). When supplementing CAZ with TAN, NDM-like producers still consistently showed very high MICs, likely resulting from very high MICs of CAZ alone. Hence, the inhibitory activity of TAN, despite being significant, was not enough to restore low MIC values of CAZ for those recombinant strains. By contrast, MICs of CAZ-TAN for VIM-2, VIM-4, VIM-5, VIM-19, and VIM-53 producers showed that supplementation with TAN resulted in at least a 128-fold reduction in the MIC of CAZ. MICs of CAZ-TAN were higher for the VIM-1, VIM-6, and VIM-83 producers, those latter enzymes being likely less inhibited by TAN. High MIC values of CAZ-TAN were also observed for producers of all tested IMP-like enzymes, in line with the lack of significant inhibitory activity of TAN against those enzymes. Surprisingly, no difference was observed between the MICs of CAZ alone and CAZ-TAN for the VIM-83-producing recombinant strain, likely suggesting a lack of significant inhibition of that MBL by TAN.

On the other hand, the determination of the MICs of FEP-TAN for all the recombinant strains revealed that a significant drop in the MIC of FEP could be observed for almost all strains (except the IMP-like producers, as expected). The discrepancy in terms of susceptibility restoration was likely related to less high MIC values of FEP alone compared to the ones of CAZ alone. The recombinant strains for which almost no difference was observed between the MICs of FEP compared to FEP-TAN were those producing NDM-9, NDM-30, VIM-2-E149K, VIM-83, and IMP-like enzymes.

Furthermore, the subclass B2 PFM-1-producing *E. coli* exhibited very low MIC values when testing CAZ and FEP, according to previous work (5). Therefore, TAN was evaluated at 4 mg/L in combination with imipenem only with this recombinant strain to be able to analyze the activity of TAN, and no decrease in MIC value was observed for this B2 MBL producer, as shown in Table 1. This result was in line with the IC_{50} measured (>100 µM) for this enzyme (Table 2). Hence, the activity of TAN appeared to be variable against B2 MBL, considering the IC_{50} reported in a previous work against another B2 MBL, CphA (11). Likewise, the high MIC values observed with the AIM-1-producing strain were in line with the lack of action of TAN against another subclass B3 MBLs, namely L1, as previously reported (11). Noticeably, a high MIC of CAZ-TAN suggesting a lack of inhibition of the corresponding enzyme by TAN was observed with the SIM-1-producing strain, revealing that several enzymes from subclass B1 can escape the action of TAN.

In accordance with the susceptibility data presented above, the determination of the IC_{50} values showed that TAN inhibited less efficiently VIM-1-like than VIM-2-like enzymes (with the exception of VIM-4 being conversely more sensitive), the difference being ca. 10-fold (Table 2). Strikingly, we showed that VIM-83, differing by a single amino acid substitution from VIM-1 (Glu149Lys) was "resistant" to TAN (IC_{50}, 80 µM).

Interestingly, by aligning the VIM-1-like and VIM-2-like protein sequences, an identity of ca. 90% was observed overall (Fig. 2). Particularly, all VIM-1-like sequences differed from VIM-2-like ones by an Ala residue in the VIM-1 sequence at position 148 (Val148 in VIM-2, Ala148 in the VIM-1 sequences). We therefore hypothesized that this amino acid substitution might be responsible for the differences observed in TAN inhibitory activity. Therefore, site-directed mutagenesis was performed using the Q5 Site-Directed Mutagenesis kit (ref. E0554S; New England Biolabs, Ipswich, MA) to substitute Val to Ala at position 148 in the VIM-2 sequence and conversely, to substitute Ala to Val at position

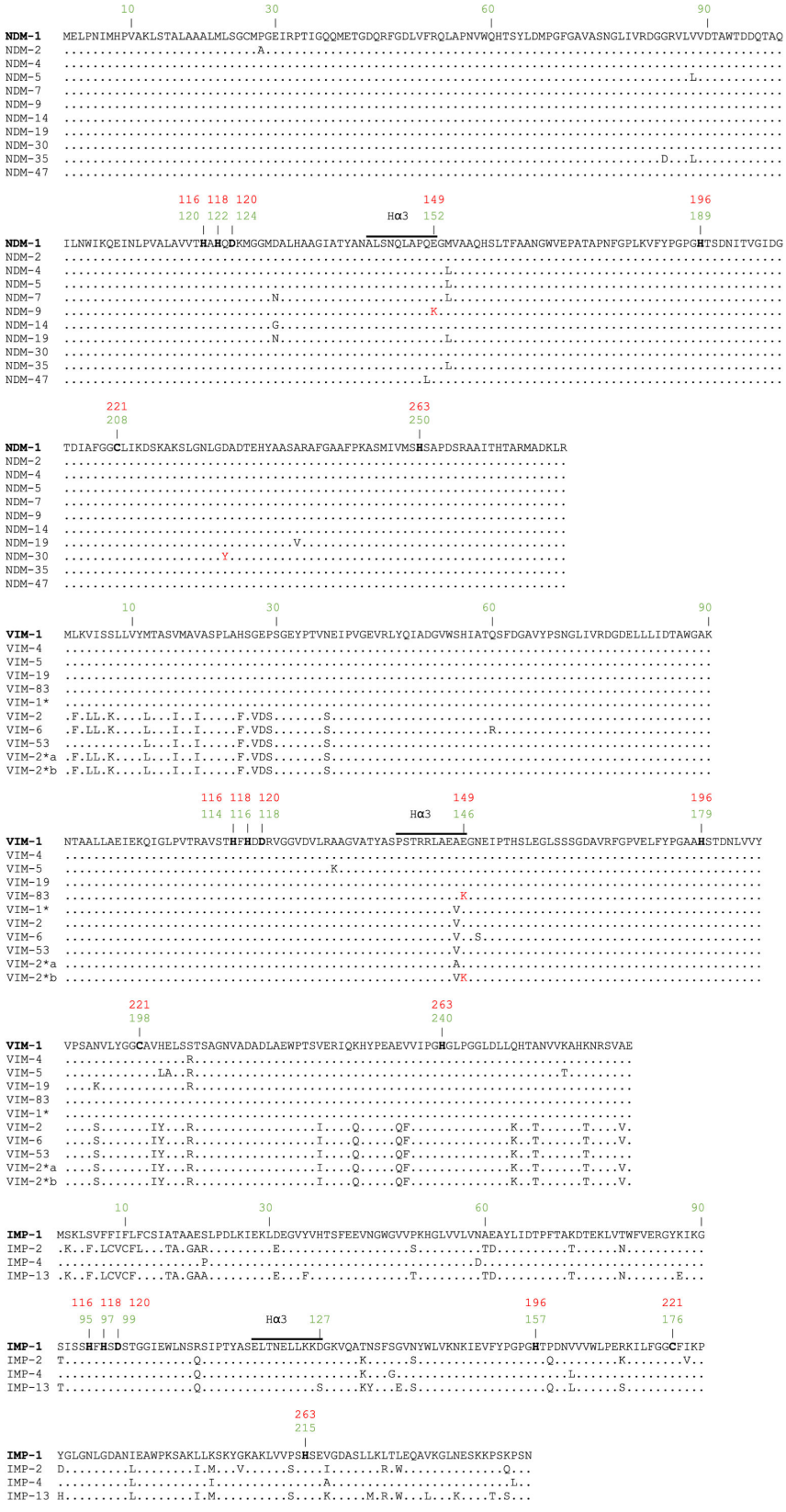

FIG 2 Sequence alignment of NDM-like, VIM-like, and IMP-like enzymes. Nomenclature is either marked in green according to the respective subclass B1 β-lactamase sequence numbering or in red according to BBL numbering. Residues in bold are zinc ligands conserved in the subclass B1 MBL enzymes defining

(Continued on next page)

FIG 2 (Continued)

the BBL numbering. (.), identical residue compared to the first described variant of the corresponding β-lactamase subgroup; the Hα3 helix is overlined. VIM-1* corresponds to VIM-1 Ala148Val; VIM-2*[a] corresponds to VIM-2 Val148Ala; VIM-2*[b] corresponds to VIM-2 Glu149Lys. Key amino acids with respect to taniborbactam resistance are red-highlighted.

148 in the VIM-1 sequence. For that purpose, the following primers were used, namely VIM-2-For (5′-GCT AGC CGA GGT GGA GGG GAA CGA G-3′), VIM-2-Rv (5′-CGG CGT GTC GAC GGT GAT-3′), VIM-1-For (5′-GCT AGC CGA GGC GGA GGG GAA CG), and VIM-1-Rv (5′-CGG CGT GTC GAC GGT GAT-3′) (Fig. 2). Actually, the Ala148Val substitution in the VIM-1 sequence resulted in a twofold increase in the MIC value of CAZ but did not

TABLE 1 Susceptibility testing of β-lactams for metallo-β-lactamase-producing *E. coli* TOP10 recombinant strains[a]

Strain (β-lactamase produced)	Ambler class[b]	Minimal inhibitory concentrations (µg/mL)			
		Ceftazidime	Ceftazidime-TAN	Cefepime	Cefepime-TAN
E. coli TOP10	–	≤0.06	≤0.03	≤0.06	≤0.03
E. coli ATCC 25922	–	≤0.06	≤0.03	≤0.06	≤0.03
E. coli NCTC 13353	A	>64	1	>64	1
E. coli NDM-1	B1	>256	256	16	0.5
E. coli NDM-2	B1	>256	256	8	0.25
E. coli NDM-4	B1	>256	256	16	0.5
E. coli NDM-5	B1	>256	256	16	0.5
E. coli NDM-7	B1	>256	128	8	0.25
E. coli NDM-9	B1	>256	>256	16	16
E. coli NDM-14	B1	>256	256	32	0.5
E. coli NDM-19	B1	>256	128	4	≤0.03
E. coli NDM-30	B1	>256	>256	2	2
E. coli NDM-35	B1	>256	64	8	0.5
E. coli NDM-47	B1	>256	128	8	0.5
E. coli VIM-1	B1	64	2	16	1
E. coli VIM-1-Ala148Val	B1	256	2	8	0.5
E. coli VIM-2	B1	32	0.06	0.25	≤0.03
E. coli VIM-2-Val148Ala	B1	32	0.5	0.25	0.06
E. coli VIM-2-Glu149Lys	B1	128	128	4	4
E. coli VIM-4	B1	128	0.25	0.5	≤0.03
E. coli VIM-5	B1	64	0.125	≤0.06	≤0.03
E. coli VIM-6	B1	16	1	0.25	≤0.03
E. coli VIM-19	B1	64	0.25	1	≤0.03
E. coli VIM-53	B1	16	0.06	0.125	≤0.03
E. coli VIM-83	B1	256	256	64	64
E. coli IMP-1	B1	>256	>256	16	16
E. coli IMP-2	B1	>256	>256	16	16
E. coli IMP-4	B1	>256	>256	16	16
E. coli IMP-13	B1	64	64	2	2
E. coli SIM-1	B1	2	2	≤0.06	≤0.03
E. coli DIM-1	B1	2	0.125	≤0.06	≤0.03
E. coli GIM-1	B1	8	0.25	≤0.06	≤0.03
E. coli SPM-1	B1	128	4	4	≤0.03
E. coli PFM-1	B2	≤0.06	≤0.03	≤0.06	≤0.03
E. coli AIM-1	B3	4	4	≤0.06	≤0.03
		Imipenem	**Imipenem-TAN**		
E. coli ATCC 25922	–	0.25	0.25		
E. coli PFM-1	B2	2	2		

[a]Data of minimal inhibitory concentrations by microdilution; TAN, taniborbactam at 4 µg/mL.
[b](-) Not applicable.

TABLE 2 Determination of the IC_{50} of taniborbactam according to different subclasses and variants of metallo-β-lactamases

MBL enzyme	Ambler class	MBL variant	IC_{50} taniborbactam (μM)
NDM-like	B1	NDM-1	0.1
		NDM-2	2.1
		NDM-4	2.9
		NDM-5	0.1
		NDM-7	0.002
		NDM-9	53
		NDM-14	0.04
		NDM-19	2.4
		NDM-30	17
		NDM-35	1.7
		NDM-47	0.4
VIM-1-like	B1	VIM-1	0.3
		VIM-4	0.004
		VIM-5	0.4
		VIM-19	0.4
		VIM-1-Ala148Val	0.1
		VIM-83	80
VIM-2-like	B1	VIM-2	0.04
		VIM-6	0.02
		VIM-53	0.04
		VIM-2-Val148Ala	0.03
		VIM-2-Glu149Lys	46
IMP-like	B1	IMP-1	>100
		IMP-2	>100
		IMP-4	>100
		IMP-13	>100
SIM-like	B1	SIM-1	>100
SPM-like	B1	SPM-1	2.4
DIM-like	B1	DIM-1	0.3
GIM-like	B1	GIM-1	0.9
PFM-like	B2	PFM-1	>100
AIM-like	B3	AIM-1	>100

change the MIC of CAZ-TAN for the corresponding mutated strain, which is consistent with slightly better TAN activity in this VIM-1-Ala148Val producer. Nevertheless, this substitution led to a threefold reduction of the IC_{50} of TAN against the VIM-1-Ala148Val mutant (Table 2). That single amino acid substitution might therefore contribute to the discrepancies observed between the VIM-1-like and VIM-2-like enzymes.

Our results highlighted that several amino acid positions located in different sites of the MBL sequences might have a critical impact on the inhibitory activity of TAN. Hence, significantly variable IC_{50} values were found among NDM-like enzymes. Noticeably, after having previously identified position 149 (BBL numbering) as being critical when comparing NDM-1 and NDM-9, we identified here position 236 as also being of major relevance for NDM enzymes by identifying NDM-30, differing from NDM-1 by an Asp236Tyr substitution, as being resistant to the inhibitory action of TAN. This observed feature could likely be attributed to a reduced affinity of TAN to the targeted enzyme, in line with a previous study that reported specific binding regions to this inhibitor (18). Finally, the Glu to Lys substitution that was identified at position 149 in the VIM-83 sequence when compared to VIM-1 was newly identified as a source of TAN resistance.

This report first evaluated the relative activity of TAN on a wide range of acquired MBL enzymes. This work highlighted that VIM-1-like enzymes are less inhibited by TAN compared to VIM-2-like enzymes, in part promoted by a specific amino acid substitution

at position 148 (Ala148Val) (Fig. 2). Furthermore, we identified here three other B1 MBLs being refractory to the inhibitory action of TAN, including NDM-30, VIM-83, and SIM-1 (Fig. 1). Interestingly, those specific enzymes have been reported in diverse Gram-negative species, with NDM-30 reported from *Klebsiella oxytoca* isolate in South Korea (19), VIM-83 from *Enterobacter cloacae* in Spain (Genbank access number: OP353772), and SIM-1 reported in *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* (20).

It is important to underscore that VIM-83, NDM-9, and NDM-30 differ by only a single amino acid substitution from the widespread VIM-1 and NDM-1 enzymes, respectively, therefore highlighting that the future efficacy of TAN against B1 MBLs might be compromised by the selection of such variants upon selective pressure. Considering that those "resistant" enzymes are already circulating, it is likely that their further emergence might occur more rapidly.

To conclude, this work contributed to a better appreciation of the real potential of TAN as an effective subclass B1 MBL inhibitor, supposed to be associated with cefepime to eventually target infections caused by producers of NDM-like and VIM-like enzymes.

ACKNOWLEDGMENTS

This work was financed by the University of Fribourg, Switzerland, the NARA, and the Swiss National Science Foundation (grant FNS 310030_1888801).

C.L.T., P.N., and L.P. designed the study. C.L.T. and C.V. performed the experiments. All authors contributed to data interpretation. P.N. provided financial support. C.L.T., C.V., P.N., A.J.V., and L.P. drafted the manuscript.

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FUNDING

Funder	Grant(s)	Author(s)
University of Fribourg		Patrice Nordmann

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Christophe Le Terrier, Conceptualization, Investigation, Methodology, Validation, Writing – original draft | Clément Viguier, Investigation, Validation | Patrice Nordmann, Project administration, Supervision, Validation, Writing – review and editing | Alejandro J. Vila,

Supervision, Validation, Writing – review and editing | Laurent Poirel, Conceptualization, Supervision, Validation, Writing – review and editing

DATA AVAILABILITY

All data from this study can be made available upon request, without limitation in time.

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