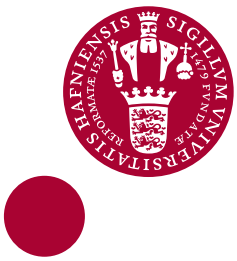

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Expanding the potential of NAI-107 for treating serious ESKAPE pathogens: synergistic combinations against Gram-negatives and bactericidal activity against non-dividing cells

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Objectives: To characterize NAI-107 and related lantibiotics for their *in vitro* activity against Gram-negative pathogens, alone or in combination with polymyxin, and against non-dividing cells or biofilms of *Staphylococcus aureus*. NAI-107 was also evaluated for its propensity to select or induce self-resistance in Gram-positive bacteria.

Methods: We used MIC determinations and chequerboard experiments to establish the antibacterial activity of the examined compounds against target microorganisms. Time–kill assays were used to evaluate killing of exponential and stationary-phase cells. The effects on biofilms (growth inhibition and biofilm eradication) were evaluated using biofilm-coated pegs. The frequency of spontaneous resistant mutants was evaluated by either direct plating or by continuous sub-culturing at 0.5 × MIC levels, followed by population analysis profiles.

Results: The results showed that NAI-107 and its brominated variant are highly active against *Neisseria gonorrhoeae* and some other fastidious Gram-negative pathogens. Furthermore, all compounds strongly synergized with polymyxin against *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, and showed bactericidal activity. Surprisingly, NAI-107 alone was bactericidal against non-dividing *A. baumannii* cells. Against *S. aureus*, NAI-107 and related lantibiotics showed strong bactericidal activity against dividing and non-dividing cells. Activity was also observed against *S. aureus* biofilms. As expected for a lipid II binder, no significant resistance to NAI-107 was observed by direct plating or serial passages.

Conclusions: Overall, the results of the current work, along with previously published results on the efficacy of NAI-107 in experimental models of infection, indicate that this lantibiotic represents a promising option in addressing the serious threat of antibiotic resistance.

Introduction

The increasing incidence of MDR pathogens has led to the dire prediction that humanity will soon enter the post-antibiotic era, when today's routine surgical procedures will become high-risk endeavours.[1,2] This worrisome scenario is exacerbated by the paucity of new antibiotics reaching the market, and in particular of new chemical classes suitable for systemic administration and not affected by prevailing resistance mechanisms. Indeed, since the beginning of the century most antibacterial drugs introduced into human use or under advanced clinical development represent improved analogues of marketed compounds.[3]

Of particular concern are infections caused by the ESKAPE pathogens, which include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.[4] In particular, infections by MRSA are associated with community and hospital infections, and up to 80% of all MRSA infections in the USA have been ascribed to the MRSA clone USA300, which is also highly resistant to other antibiotics.[5] Given the limited options available to treat ESKAPE pathogens, it is important to develop new antibiotics that are not affected by prevailing resistance mechanisms, while at the same time devising strategies that minimize the spread of antibiotic resistance.

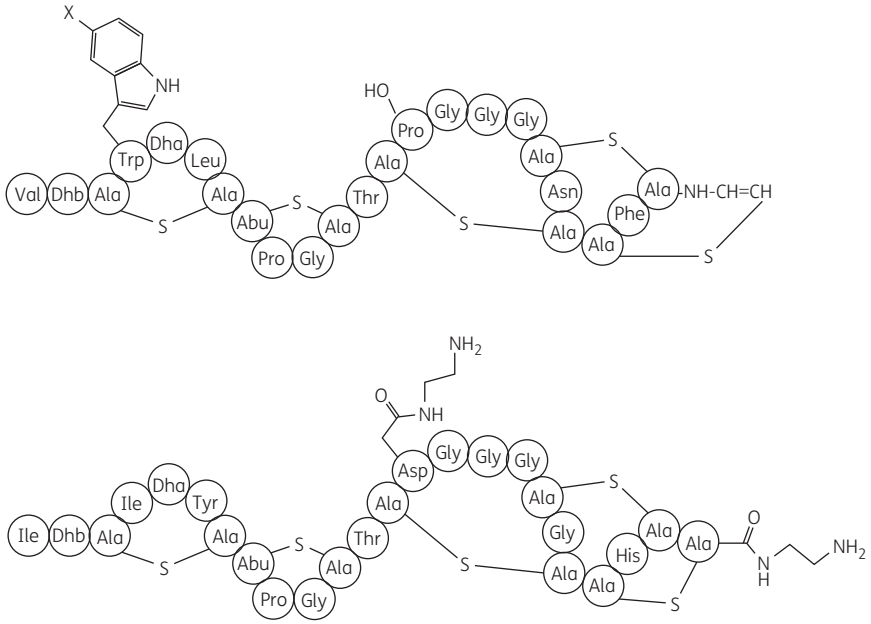

Figure 1. Structures of NAI-107 (top, where X = Cl), NAI-108 (top, where X = Br) and NAI-857DA (bottom). Note that NAI-107 and NAI-108 are obtained directly from fermentation, whereas NAI-857DA is obtained by converting the natural lantibiotic NAI-857 into its diamide.[8,10]

One class of antibiotics that has been receiving increasing attention is represented by the lantibiotics. These compounds, which belong to the growing family of ribosomally synthesized and post-translationally modified peptides, are characterized by the presence of (methyl)-lanthionine bridges that confer rigidity and stability on the peptide.[6] The prototype lantibiotic is nisin, which has been used for decades as a food preservative.

One of the most potent lantibiotics is NAI-107 (Figure 1), a chlorine-containing compound active against MDR Gram-positive pathogens, including MRSA, glycopeptide-intermediate *S. aureus* (GISA), VRE and penicillin-resistant *Streptococcus pneumoniae*.[7,8] Lantibiotics with similar antibacterial properties have been obtained after incorporation of Br into NAI-107, leading to NAI-108,[9] or after semi-synthetic conversion, leading to NAI-857DA and related compounds.[10] NAI-857DA and NAI-107/108 share a similar peptide scaffold and identical topology of thioether rings, with the first two N-terminal rings shared with nisin (Figure 1). A rapid bactericidal activity[9–11] and a prolonged half-life in plasma[12] are consistent with the efficacy of NAI-107 in different experimental models of infection in rodents[13] and insects.[14] The ratio between the area under the concentration–time curve and the MIC has been proposed as the pharmacodynamic index predictive of efficacy in the mouse.[12]

The scope of this study was to characterize the *in vitro* properties of NAI-107 with respect to the propensity to select for resistance, its activity against biofilms and non-dividing cells of *S. aureus*, and its activity against Gram-negative pathogens, alone or in combination with polymyxin B. Where appropriate, the properties of NAI-108 and NAI-857DA were also investigated.

Materials and methods

Materials and methods are available as Supplementary data at *JAC* Online.

Results

Activity against fastidious Gram-negative pathogens

Most antibiotics targeting lipid II are large molecules that cannot cross the outer membrane barrier present in Gram-negative bacteria.[15] However, in contrast to most clinically used drugs targeting Gram-positive pathogens, NAI-107 extends its antibacterial spectrum to some fastidious Gram-negative bacteria (Table 1), including *Neisseria meningitidis* (MIC range 0.06–1 mg/L), *Moraxella catarrhalis* (MIC range 0.25–1 mg/L) and *Haemophilus influenzae* (MIC range 8–16 mg/L). Although only a few strains were tested in comparison, NAI-107 was considerably more active than nisin (Table 1), suggesting that the activity against these bacterial species is not a general property of lantibiotics. No activity was seen against individual strains of *Salmonella* Enteritidis, *Enterobacter cloacae* and *Proteus mirabilis* (data not shown).

Interestingly, NAI-107 was highly active against *Neisseria gonorrhoeae*, including isolates with intermediate or high resistance to penicillin. Against 18 tested strains, the observed MICs ranged from 0.015 to 2 mg/L (Table 2), with no apparent correlation to a strain's susceptibility to penicillin. Similar results were observed with NAI-108, which, as previously observed with Gram-positive pathogens,[9] was usually twice as active as NAI-107 (Table 2). In contrast, NAI-857DA was substantially less active than the other two lantibiotics (Table 2).

NAI-107 was essentially inactive against three strains each of the Gram-negative pathogens *Escherichia coli*, *K. pneumoniae* and *P. aeruginosa*, with no measurable MICs at the highest concentration tested (Table 3). Some activity could, however, be observed against *A. baumannii*, with one strain showing an MIC of 32 mg/L (Table 4). In about half of 12 independent *A. baumannii* isolates, NAI-108 was slightly more active than NAI-107, with 16 mg/L as

Table 1. MIC (mg/L) or MIC ranges (mg/L) of NAI-107 for selected Gram-negative pathogens

Species	Number of tested strains	NAI-107	Vancomycin	Teicoplanin	Linezolid	Nisin
H. influenzae[a]	18	8–16	64 to >128	64–128	8–32	>128[d]
M. catarrhalis[b]	8	0.25–1	32–64	4–32	4–8	1–4[d]
N. meningitidis[c]	4	0.06–1	32 to >128	16–128	8–32	8[e]

[a]Tested strains include ATCC strains 49247, 9334, 19418 and 9006, and 14 clinical isolates collected in Italy and the UK.
[b]Tested strains include ATCC 8176 and seven clinical isolates collected in the USA and the UK.
[c]Tested strains include ATCC 13804, 13090, 13102 and 13113.
[d]Only two strains tested with this antibiotic.
[e]Only one strain tested with this antibiotic.

Table 2. MICs (mg/L) of NAI-107, NAI-108, NAI-857DA and penicillin for *N. gonorrhoeae*

Strain code[a]	NAI-107	NAI-108	NAI-857DA	Penicillin
ATCC 49226	0.25	0.125	2	1
L1596	0.125	0.125	4	≤0.015
L1599	0.015	0.015	0.25	0.25
L1601	0.5	0.25	8	>32
L1602	0.25	0.125	8	>32
L1603	1	1	16	>32
L1604	0.5	0.25	4	>32
L1605	0.25	0.125	8	>32
ND755	1	1	8	2
ND756	0.25	0.125	2	0.5
ND757	1	0.5	16	4
ND758	1	0.5	8	4
ND759	0.25	0.125	2	0.25
ND760	1	0.5	8	4
ND761	1	0.5	16	4
ND762	0.5	0.25	16	2
ND763	1	0.5	8	2
ND764	2	1	8	2

[a]Strains with an L or ND prefix are clinical isolates collected in Italy or the USA.

the lowest observed MIC (Table 4). It should be noted that none of the analysed strains was resistant to polymyxin (Table 4; see also below).

The acquisition of polymyxin resistance in Gram-negative species involves LPS modifications[16–18] and vancomycin is active against some LPS-deficient *A. baumannii* mutants.[19] We thus tested the LPS-deficient *A. baumannii* mutants AB167R and AB176R (defective in LPS formation) and CR17 (which adds phosphoethanolamine to LPS), as well as the polymyxin-susceptible WT ATCC 19606.[20] The LPS-deficient strains proved susceptible to NAI-107 (Table 4), with MICs of 2–4 mg/L, while the WT and the *pmrA* mutant were not susceptible (MIC >128 mg/L). Therefore, the activity of NAI-107 against Gram-negative species should be achievable via permeabilization of the outer membranes.

Synergism with polymyxin

Antibiotics (e.g. vancomycin, daptomycin) that are not effective against Gram-negative bacteria can become active if the permeability barrier provided by the outer membrane is weakened by sub-inhibitory concentrations of polymyxin or colistin.[21–24] These drugs have become the last options to treat infections by MDR Gram-negative pathogens, but they also have significant toxicity. If polymyxin or colistin concentrations could be substantially reduced in effective combinations with NAI-107, the toxic effect would be less severe.

We thus tested combinations of NAI-107 and polymyxin against three independent isolates each of the target pathogens *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*. Against *A. baumannii*, the fractional inhibitory concentrations (FICs) of polymyxin and NAI-107 were 0.03–0.125 and 0.016–0.06, respectively, with FIC indexes ranging from 0.09 to 0.14 (Table 3). Similarly, polymyxin and NAI-107 formed synergistic combinations against all the tested strains of the other examined species: the lowest FICs of polymyxin against *E. coli*, *K. pneumoniae* and *P. aeruginosa* were 0.03–0.06, 0.06–0.25 and 0.125–0.25, respectively, with growth inhibition requiring NAI-107 concentrations of 2–8, 2–16 and 4–32 mg/L, respectively (Table 3). Since NAI-107 was inactive against these strains, FIC indexes were essentially determined by the polymyxin FIC and ranged from 0.28 (for *K. pneumoniae* L3392) to 0.04 (for *E. coli* L47).

We also evaluated the lowest polymyxin concentration required to inhibit the growth of the analysed strains in the presence of 2 mg/L NAI-107, a concentration that can be readily maintained after systemic administration in rodents.[12,13] With the exception of *P. aeruginosa* ATCC 27853, for which the polymyxin concentration was 0.125 mg/L (i.e. 0.5 × MIC), in all other cases ≤0.06 mg/L polymyxin was sufficient to inhibit growth in the presence of 2 mg/L NAI-107 (Table 3).

Polymyxin is known to rapidly kill susceptible Gram-negative bacteria,[25] as does NAI-107 for Gram-positives.[11] We thus tested different growth-inhibiting combinations of polymyxin and NAI-107 for their ability to reduce the viable bacterial counts in dynamic chequerboards. We did observe a decrease in viable counts in combinations containing NAI-107 and sub-inhibitory concentrations of polymyxin but not in cultures treated with polymyxin alone. At 0.031 mg/L polymyxin (i.e. 0.25 × MIC), the viable count for *A. baumannii* L3030 was undistinguishable from that of the untreated control (Figure 2a). However, addition of as little as

Table 3. *In vitro* synergism with polymyxin B

		Polymyxin		NAI-107				
Species	Strain code	MIC (mg/L)	FIC	MIC (mg/L)	concentration of NAI-107 in the most synergistic combination (mg/L)	FIC	FIC index	Polymyxin (mg/L) at 2 mg/L NAI-107[a]
A. baumannii	L373[b]	0.5	0.03	128	8	0.06	0.09	0.03
	L3030[c]	0.125	0.06	128	4	0.03	0.09	0.016
	L2859[b]	0.25	0.125	32	0.5	0.016	0.14	0.06
E. coli	L47[d]	0.125	0.03	>256	4	≤0.008	0.04	0.008
	ATCC 25922	0.125	0.03	>256	8	≤0.016	0.05	0.004
	ND480[b]	0.25	0.06	>256	2	≤0.004	0.07	0.016
K. pneumoniae	L3392[b]	0.06	0.25	>256	16	≤0.03	0.28	0.03
	ND484[e]	0.25	0.06	>64	4	≤0.03	0.09	0.03
	ND443[b]	0.25	0.06	>256	2	≤0.004	0.07	0.016
P. aeruginosa	ATCC 10145	0.125	0.125	>256	4	≤0.008	0.13	0.03
	ATCC 25668	0.125	0.125	>256	32	≤0.06	0.19	0.06
	ATCC 27853	0.25	0.25	>256	8	≤0.016	0.27	0.125

The table reports the concentrations of polymyxin (as fractions of the MIC) and those of NAI-107 giving the lowest calculated FIC index for each strain.
[a]Lowest polymyxin concentration required for growth inhibition in the presence of 2 mg/L NAI-107.
[b]Clinical isolate collected in Italy.
[c]Clinical isolate collected in the UK.
[d]Strain from historical Lepetit collection.
[e]Clinical isolate collected in the USA.

Table 4. Antibacterial activities against *A. baumannii*

	MIC (mg/L)			
Strain code[a]	NAI-107	NAI-108	polymyxin	colistin
L256	128	128	1	1
L2831	128	32	≤0.06	1
L2859	32	16	≤0.06	0.125
L2860	128	128	0.125	0.5
L364	>128	>128	≤0.06	0.5
L373	128	64	0.25	1
L3030	128	128	0.25	0.25
ATCC 17904	128	128	≤0.06	≤0.06
L756	128	128	≤0.06	≤0.06
ND021808	128	128	≤0.06	≤0.06
ND045309	64	32	≤0.06	≤0.06
ND048710	128	64	≤0.06	0.25
ND049010	128	128	0.125	0.25
WT 19606	>128	NT	NT	0.5
AB167 (*lpxC*)[b]	2–4	NT	NT	>128
AB176 (*lpxD*)[b]	2–4	NT	NT	>128
CR17 (*pmrA*)[b]	>128	NT	NT	64

NT, not tested.
[a]Strains with an L or ND prefix are clinical isolates collected in Italy.
[b]Strains described by García-Quintanilla *et al.*[19]

≤0.5 mg/L NAI-107 was sufficient to decrease viable counts by at least four orders of magnitude (i.e. below the detection limit of 50 cfu/mL) for up to 20 h (Figure 2a and Table S1). Similarly, in the presence of 0.016 mg/L polymyxin, NAI-107 caused a concentration-dependent decrease in viable counts, with 2 mg/L NAI-107 being sufficient to decrease the viable counts below the detection limit, whereas 1 mg/L NAI-107, despite an initial decrease in viable counts, was unable to prevent full growth at 20 h (Figure 2a). In bactericidal combinations viable counts decreased in a time-dependent manner, with 1 h of incubation sufficient to decrease cfu/mL by 2.5–3.5 log, whereas a 3 h incubation was required to reduce cfu/mL below the detection limit (Figure 2a).

Next, we used *A. baumannii* L3030 to test the effects of the lantibiotics NAI-108 and NAI-857DA, and of vancomycin. As seen for NAI-107, NAI-108 showed time- and concentration-dependent killing, with 2 and ≤0.5 mg/L NAI-108 being sufficient to decrease viable counts below the detection limit in the presence of 0.016 and 0.031 mg/L polymyxin, respectively (Figure 2b). In contrast, at least 2 mg/L NAI-857DA (Figure 2c) or 2 mg/L vancomycin (Figure 2d) was necessary for reducing viable counts of *A. baumannii* L3030 below the detection limit in the presence of 0.031 mg/L polymyxin. Lowering the concentration of polymyxin to 0.016 mg/L required 4 and 64 mg/L NAI-857DA (Figure 2c) and of vancomycin (Table 5), respectively, for observing a complete bactericidal effect for the entire duration of the experiment. Only when polymyxin concentrations were raised to 0.062 mg/L (i.e. 0.5 × MIC) did we

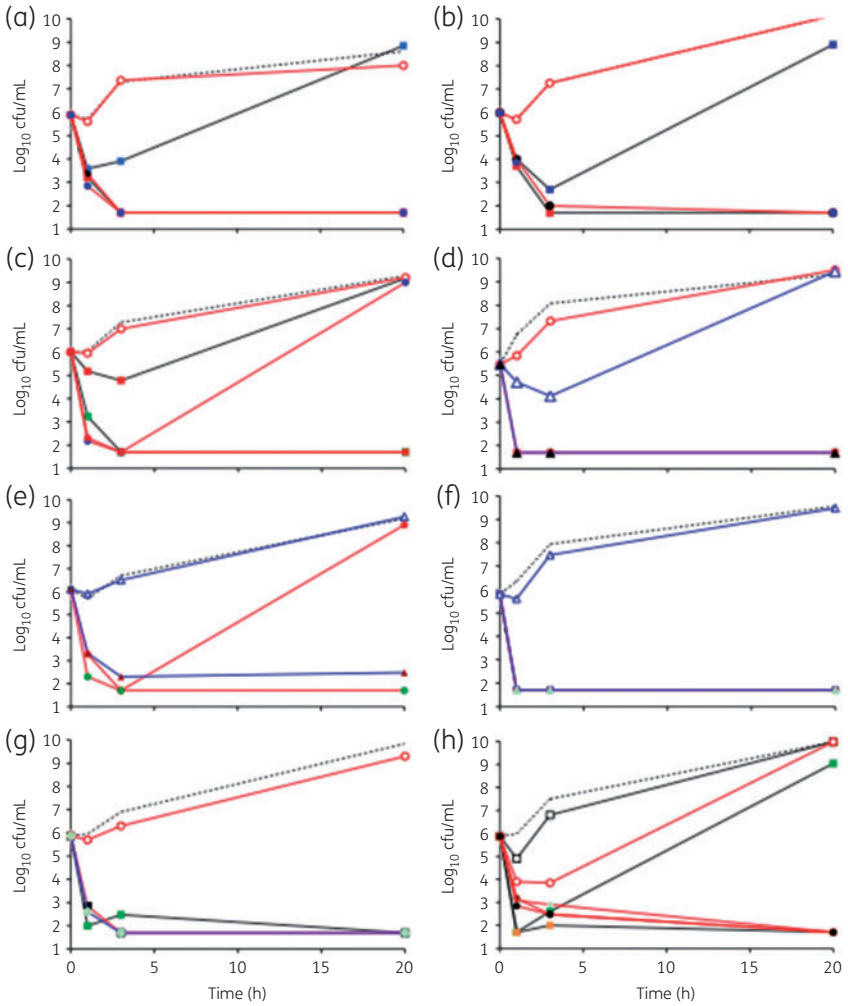

Figure 2. Killing of *A. baumannii* L3030 by NAI-107 (a), NAI-108 (b), NAI-857DA (c) or vancomycin (d), each in combination with polymyxin, and killing by NAI-107 in combination with polymyxin of *A. baumannii* L373 (e), *E. coli* ATCC 25922 (f), *K. pneumoniae* ND484 (g) or *P. aeruginosa* ATCC 10145 (h). Untreated controls are represented by broken lines. Polymyxin concentrations are represented as follows: 0.016 mg/L, squares and black lines; 0.031 mg/L, circles and red lines; and 0.062 mg/L, triangles and blue lines. Cultures containing polymyxin only are represented by open symbols, while filled symbols indicate the presence of a combination antibiotic (NAI-107, NAI-108, NAI-857DA or vancomycin) at the following concentrations: 0.125 mg/L, light green; 0.25 mg/L, purple; 0.5 mg/L, black; 1 mg/L, blue; 2 mg/L, red; 4 mg/L, green; and 8 mg/L, orange. The limit of detection was 50 cfu/mL.

observe a bactericidal effect at vancomycin concentrations of ≤0.5 mg/L (Figure 2d). It should be noted that 0.062 mg/L polymyxin alone did have a transient effect on cell viability, with a small decrease in viable cells up to 3 h, followed by full growth by 20 h (Figure 2d). Overall, these results are consistent with the general trend observed with the lantibiotics shown in Figure 1, with the hydrophobic lantibiotics NAI-107 and NAI-108 showing comparable activities, whereas the hydrophilic NAI-857DA was slightly less active than the other two compounds.[8] Vancomycin was less potent than NAI-107 in synergistic combinations with polymyxin. Table 5 describes a summary of the results observed with growth and viable counts in chequerboard experiments. Usually, in the presence of a given polymyxin FIC, the lowest concentration of antibiotic able to reduce viable counts below the detection limit at 3 h coincided with the concentration that was able to prevent growth (Table 5).

We next expanded the evaluation of NAI-107 using selected strains from Table 3. As seen with *A. baumannii* L3030, within 1–3 h NAI-107 was able to significantly reduce viable counts (below or close to the detection limit) of *A. baumannii* L373 (Figure 2e), *E. coli* ATCC 25922 (Figure 2f), *E. coli* L47 (data not shown), *K. pneumoniae* ND484 (Figure 2g) and *P. aeruginosa* ATCC 10145 (Figure 2h), most of the time at the same concentrations as those sufficient to cause growth inhibition (Table S2). With *P. aeruginosa* ATCC 10145, 0.5 × MIC polymyxin caused a transient decrease in viable counts, followed by regrowth. The addition of ≥0.125 mg/L NAI-107 prevented regrowth (Figure 2h).

Table 5. Effect of polymyxin-containing combinations on growth and viability of *A. baumannii* L3030

Polymyxin (mg/L)	NAI-107 (mg/L)				NAI-108 (mg/L)				NAI-857DA (mg/L)				Vancomycin (mg/L)			
	growth inhibition	killing at[a]			growth inhibition	killing at[a]			growth inhibition	killing at[a]			growth inhibition	killing at[a]		
		1 h	3 h	20 h		1 h	3 h	20 h		1 h	3 h	20 h		1 h	3 h	20 h
0.062	≤0.5	NT	NT	NT	≤0.25	NT	NT	NT	≤0.13	NT	NT	≤0.13	0.5	0.5	0.5	0.5
0.031	≤0.5	4	≤0.5	≤0.5	≤0.25	8	2	0.5	0.5	>2	1	2	2	≤4	≤4	≤4
0.016	2	8	2	2	0.5	8	2	2	4	>8	4	4	64	NT	NT	NT
0.008	8	16	4	8	4	16	4	8	8	>8	8	>8	128	NT	NT	NT
0.004	4	16	4	4	16	32	16	32	32	NT	NT	NT	128	NT	NT	NT
0.002	8	16	16	16	32	NT	NT	NT	64	NT	NT	NT	128	NT	NT	NT

NT, not tested.
At the indicated polymyxin concentration, the table reports for each compound the lowest concentration able to inhibit growth and the lowest concentration necessary to reduce the number of viable cells below the detection limit (50 cfu/mL) in replicated microtitre plate experiments.
[a]Lowest concentration required to reduce the number of viable counts below the detection limit after 1, 3 or 20 h. Note that the symbol ≤ is used only when cfu/mL was not determined from cultures that were fully inhibited at concentrations lower than indicated. cfu/mL at time zero ranged from 3×10^5 (vancomycin experiment) to 1×10^6 (NAI-857DA experiment).

Table 6. Activities against *S. aureus* biofilms (values in mg/L)

Microorganism	Parameter	NAI-107	NAI-108	NAI-857DA	Rifampicin	Amoxicillin
S. aureus L3988	MIC	1	NT	1	0.002	16
	MBIC	2	NT	2	0.007	>128
	MBEC	16	NT	2	0.03	>128
S. aureus L3797	MIC	4	8	8	0.002	128
	MBIC	8	16	32	0.002	>128
	MBEC	16	16	32	0.03	>128
S. aureus USA300	MIC	0.5	1	1	0.002	64
	MBIC	0.125	0.5	1	0.002	>128
	MBEC	16	32	32	0.03	>128
S. aureus L1400	MIC	1	2	2	0.002	128
	MBIC	1	2	2	0.002	128
	MBEC	32	16	16	0.06	>128

See the Materials and methods section for details.

Activity against S. aureus biofilms

NAI-107 and related lantibiotics are active against most Gram-positive pathogens, including MRSA and GISA.[7,9,10] In order to evaluate activity against biofilms, four *S. aureus* strains were selected for their ability to form biofilms (Table S1). Against these strains, NAI-107, NAI-108 and NAI-857DA were able to inhibit growth of planktonic cells detaching from the biofilms, with minimal biofilm-inhibiting concentrations (MBICs) comparable to MICs (Table 6). The minimal biofilm-eradicating concentration (MBEC) values were usually 16–32 mg/L, with the exception of NAI-857DA, which showed an MBEC of 2 mg/L against a single strain. Rifampicin and amoxicillin, used as positive and negative controls, respectively, behaved as expected (Table 6).

Table 7. MICs for the *S. aureus* strains used in time–kill experiments

	MIC (mg/L)			
	vancomycin	NAI-107	NAI-108	NAI-857DA
USA300	2	0.25	1–2*	1
Mu3	4*–8	4	8*–16	4*–8
Mu50	1	2	8	4

MICs were determined for three individual clones. When more than one value was found, the MIC values used for further study are those found in two of three determinations and are designated by an asterisk.

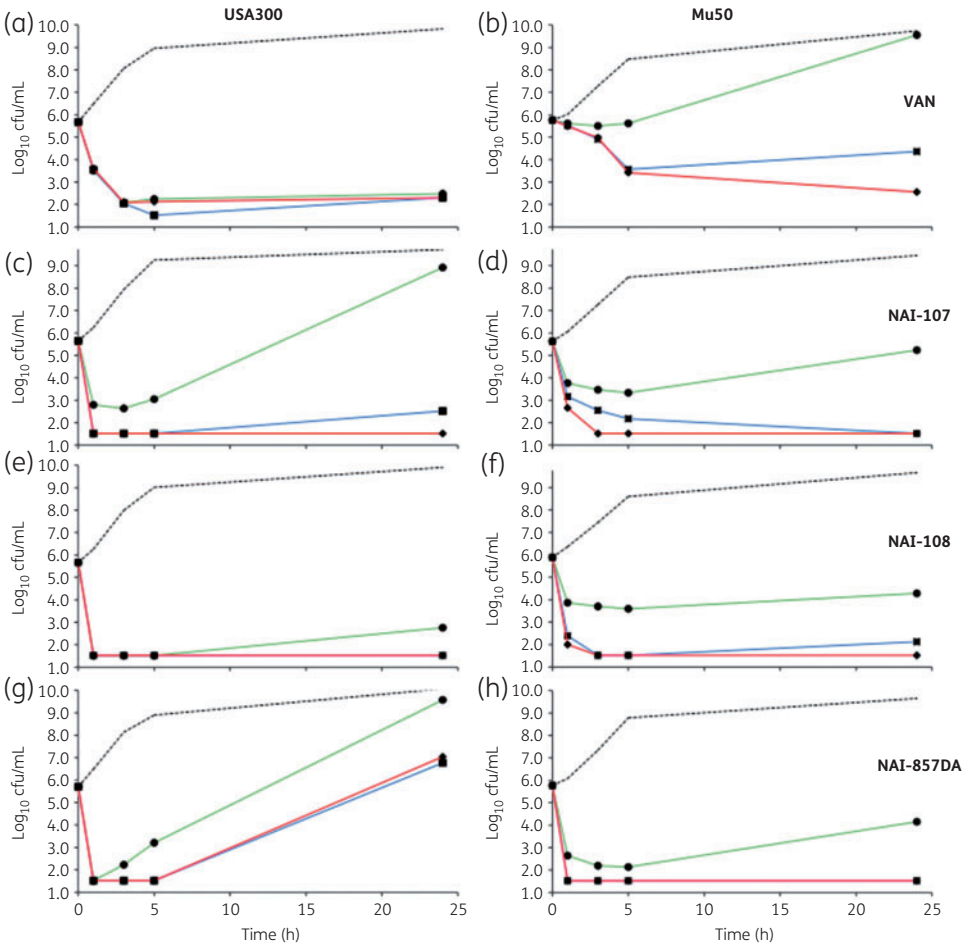

Figure 3. Killing of exponentially growing *S. aureus* USA300 (a, c, e and g) and Mu50 (b, d, f and h) by vancomycin (VAN; a and b), NAI-107 (c and d), NAI-108 (e and f) or NAI-857DA (g and h), as indicated. Compounds were added at 1 × MIC (green lines and circles), 3 × MIC (blue lines and squares) or 5 × MIC (red lines and diamonds). Broken lines denote untreated controls. Data represent the average of three independent treatments. Standard deviations are omitted for clarity. The limit of detection was 33 cfu/mL. Note that one of three USA300 cultures treated with 5 × MIC NAI-857DA showed no growth and these data were not considered in calculating the average cfu/mL in (h).

NAI-107 effectively kills non-dividing S. aureus cells

Daptomycin and oritavancin were shown to kill *S. aureus* cells in stationary phase, whereas vancomycin was inactive.[26,27] Therefore, we compared the effect of our lantibiotics on exponentially growing and non-dividing cells of *S. aureus*, using vancomycin as control. After determining the MICs of the compounds (Table 7), time–kill experiments were initially performed on the community-acquired MRSA strain USA300 and the glycopeptide-intermediate *S. aureus* (GISA) strain Mu50.[28]

Vancomycin demonstrated killing of exponentially growing USA300 by >99.9% within 3 h at 1 × MIC (2 mg/L), with no increase in viable counts seen up to 24 h (Figure 3a). Treatment of USA300 with the three lantibiotics resulted in reduction in bacterial titres of at least 99% within 1 h at all concentrations tested. However, viable counts at 24 h were comparable to untreated controls at 1 × MIC NAI-107 (0.25 mg/L), but not at higher concentrations (Figure 3c), and in the presence of all concentrations of NAI-857DA (1–5 mg/L; Figure 3g). This phenomenon was not observed with NAI-108 at all concentrations tested (2–10 mg/L; Figure 3e).

Against Mu50, vancomycin showed an effect pronouncedly different from that seen against USA300 (Figure 3b). At 1 × MIC (4 mg/L), there was no change in the number of viable cells for the first 5 h, and by 24 h the strain had reached the same density as the untreated control. Increasing vancomycin concentration to 3 × or 5 × MIC resulted in slow killing up to 5 h, but viable counts could still be observed at 24 h (Figure 3b). In contrast, the response of USA300 and Mu50 to the lantibiotics was similar: at 3 × or 5 × MIC, NAI-107, NAI-108 and NAI-857DA reduced viable counts of Mu50 (Figure 3d, f and h).

We next examined the activity of NAI-107, NAI-108 and NAI-857DA against non-dividing cells resuspended in PBS at ~5×10^5 cfu/mL. In these experiments, we also included the GISA strain Mu3.[28] NAI-107, NAI-108 and NAI-857DA were tested at 3 ×, 5 × and 10 × MIC, whereas vancomycin, which was expected to have little activity, was used as a control at 10 × MIC only. Against USA300, NAI-107 showed time- and concentration-dependent killing of non-dividing cells, with cfu/mL decreased by 99% within 5 h at 5 × MIC and within 1 h at 10 × MIC (Figure 4a).

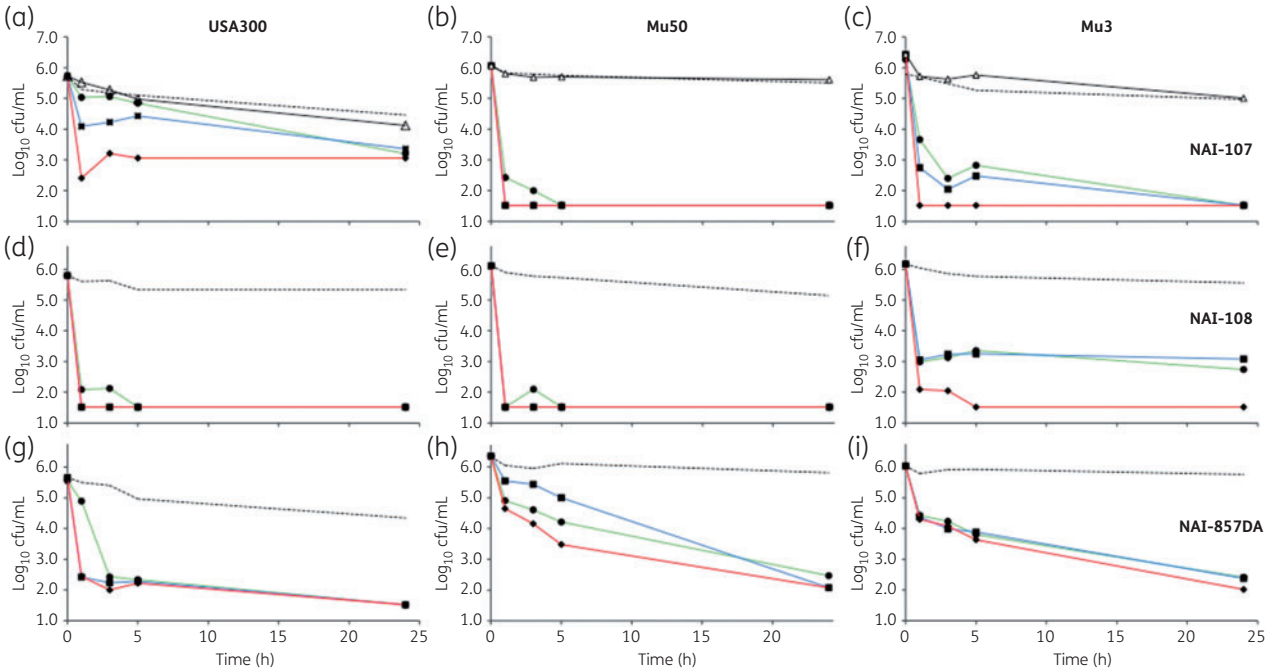

Figure 4. Killing of non-dividing cells of *S. aureus* strain USA300 (a, d and g), Mu50 (b, e and h) and Mu5 (c, f and i) by NAI-107 (a–c), NAI-108 (d–f) or NAI-857DA (g–i), as indicated. Antibiotics were added at 3 × MIC (green lines and circles), 5 × MIC (blue lines and squares) or 10 × MIC (red lines and diamonds). Broken lines denote untreated controls. Vancomycin was added at 10 × MIC and is shown only for (a), (b) and (c) (black lines and open triangles). Overnight cultures were diluted to ∼5×10^5 in pre-heated PBS (37 °C). Experiments were performed in triplicate. Standard deviations are omitted for clarity. The limit of detection was 33 cfu/mL. Note that one of three Mu50 cultures treated with NAI-857DA showed growth equivalent to controls and these data were not considered in calculating the average cfu/mL in (h).

Against Mu50 (Figure 4b) and Mu3 (Figure 4c), NAI-107 treatment resulted in ≥99% reduction of viable cells within 1–5 h, with the effect lasting the remainder of the experiment. Similar results were observed when USA300 was resuspended in spent medium rather than in PBS (data not shown).

When exposed to NAI-108, viable cells of USA300 (Figure 4d) and Mu50 (Figure 4e) dropped below the detection limit within 1 h at all concentrations tested. Only with Mu3 did we observe concentration-dependent killing, with 10 × MIC NAI-108 required to reduce viable counts below 0.1% of the starting titre (Figure 4f). When exposed to NAI-857DA, USA300 cells were killed within 3 h at all tested concentrations (Figure 4g), Mu50 cells were resistant to killing (Figure 4h) and strain Mu3 appeared to have an intermediate behaviour, with time-dependent and concentration-independent killing (Figure 4i).

Altogether, the above results indicate that dividing and non-dividing cells of the MRSA strain USA300 are rapidly killed by NAI-107 and by NAI-108 at equivalent multiples of the MIC, whereas this strain is able to eventually escape killing by NAI-857DA in growth medium but not in PBS. Under non-dividing conditions, the GISA strains Mu3 and Mu50 are rapidly killed by NAI-107 and by NAI-108, but only partially and slowly by NAI-857DA.

NAI-107 effectively kills non-dividing A. baumannii cells

Inspired by the above results, we exposed non-dividing cells of *A. baumannii* L3030 in PBS to combinations of NAI-107 and polymyxin. Under these conditions, the untreated strain was stable (just a few duplications observed during the incubation period) and killed by ≥0.5 mg/L polymyxin (Figure 5). Notwithstanding an MIC of 128 mg/L, NAI-107 alone was able to kill the strain within 3 h at ≥16 mg/L, with no detectable cells observed after 20 h. A similar killing effect by 16 mg/L NAI-107 was observed against non-dividing cells of *A. baumannii* L2859 (data not shown). It should be noted that the activities against dividing and non-dividing cells were measured under similar conditions, with the main difference represented by the presence of growth medium versus PBS. Furthermore, NAI-107 in PBS led to a decrease in cfu/mL after 3 h (Figure 5), at the same time that an increase in viable counts was observed in growth medium (Figure 2a). None of the other Gram-negatives tested (*E. coli* ATCC 25922, *K. pneumoniae* L3392 and *P. aeruginosa* ATCC 27853) was killed by NAI-107 under non-dividing conditions (data not shown).

Consistent with the data observed with growing cells, combinations of NAI-107 and polymyxin were highly effective in killing non-dividing *A. baumannii* L3030: at polymyxin concentrations of 0.125 or 0.06 mg/L, ≤2 mg/L NAI-107 was sufficient to reduce the number of viable counts below the detection limit (Figure 5). When 4 mg/L NAI-107 was used, killing was observed at 0.016 mg/L polymyxin, the lowest concentration tested (Figure 5).

Lack of in vitro resistance in Gram-positives

In contrast to molecules targeting cellular proteins, antibiotics binding to lipid II do not select resistant mutants by direct plating.[15,29] Consistently, no spontaneous resistance mutants of two GISA strains, two MRSA strains and one *E. faecalis* VanA strain

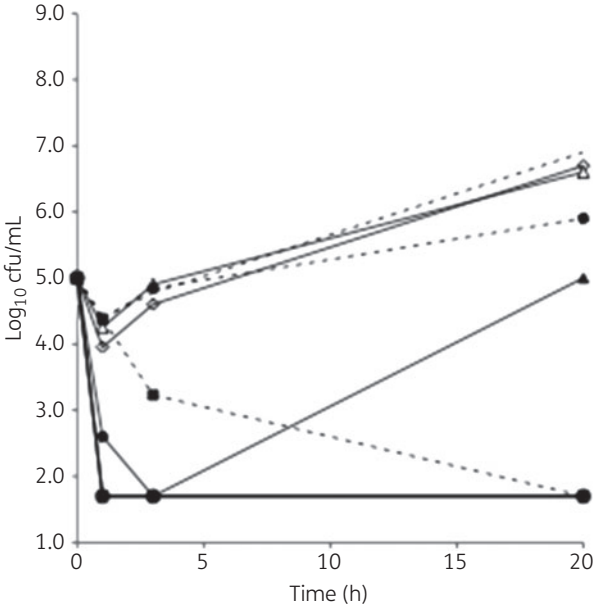

Figure 5. Killing of non-dividing *A. baumannii* L3030 by NAI-107 alone or in combination with polymyxin. Untreated controls are represented by broken lines with no symbols. Continuous lines and open symbols denote cultures treated with polymyxin alone at 1.0 (circles), 0.125 (diamonds) or 0.031 (triangles) mg/L. Broken lines and filled symbols denote cultures treated with NAI-107 alone at 16 (squares) or 8 (diamonds) mg/L. Continuous lines and filled symbols denote cultures treated with the following combinations: circles, 0.125 mg/L polymyxin and 2 mg/L NAI-107; triangles, 0.031 mg/L polymyxin and 4 mg/L NAI-107; and squares, 0.016 mg/L polymyxin and 4 mg/L NAI-107. Note that cells were resistant to DMSO (the solvent used to dissolve NAI-107) up to 3%.

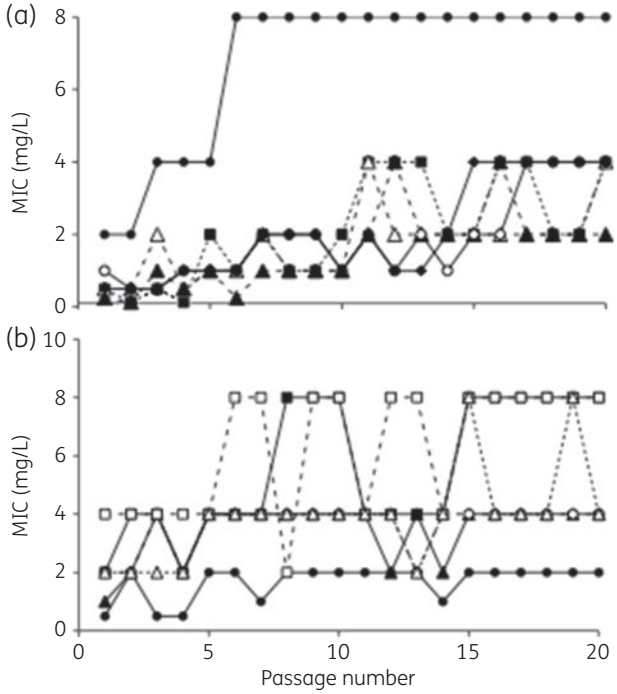

Figure 6. Variation in NAI-107 MICs after serial passages of cultures growing at 0.5 × MIC. (a) *S. aureus* strains: squares and broken line, MSSA strain L819; triangles and broken line, MRSA strains L1400 (open symbols) and L4064 (filled symbols); and continuous line, GISA strains L3797 (filled circles), L3798 (open circles) and L4062 (filled diamonds). (b) *Enterococcus* spp. VanA: filled symbols and broken line, *E. faecalis* strains A533 (circles), J1 (squares) and A8 (triangles); and open symbols and continuous line, *E. faecium* strains A6349 (circles), B518 (squares) and D561 (triangles).

were observed at 10 × MIC NAI-107, indicating a frequency lower than 10^{-10} (Table S3).

Using 12 strains (1 MSSA, 2 MRSA, 3 GISA, 3 *E. faecalis* VanA and 3 *E. faecium* VanA), 20 serial subcultures in the presence of subinhibitory concentrations yielded only modest increases in the NAI-107 MIC, which appeared to stabilize at 2- or 4-fold the initial value, with isolated spikes at 8-fold (Figure 6). Population profile analyses of cultures emerging after 1 passage, 10 passages or 20 passages indicated that the procedure had probably enriched for a subpopulation of cells already present in the initial culture, with no significant differences seen after 10 or 20 passages (Figure 7).

Discussion

Gram-negative bacteria can be sensitized to different antibiotics by sub-inhibitory concentrations of polymyxin or colistin.[30] We could find only a few reports addressing the ability of polymyxin-based combinations to kill the target pathogens: these studies were performed at 1 mg/L polymyxin and at a single, high concentration (20 mg/L) of vancomycin,[21] teicoplanin[31] or telavancin.[22] In this study, we show that, in addition its strong synergism, essentially the same combinations of NAI-107 and polymyxin that inhibit growth also reduce viable counts below the detection limit of the Gram-negative pathogens.

Few studies have investigated antibacterial activity against non-dividing *S. aureus* cells. Mascio *et al.*[27] demonstrated that daptomycin kills MRSA in stationary phase, with a mechanism that does not require energy or protein synthesis. In their elegant studies, Müller *et al.*[32] demonstrated that daptomycin binds to high-fluidity regions in the membrane, thus preventing the function of proteins that specifically localize to those regions. Combining the two observations, we surmise that high-fluidity regions are present in non-dividing cells. Unlike vancomycin, oritavancin kills non-dividing *S. aureus* cells, and septum staining of stationary phase cells is affected by oritavancin and not by vancomycin.[26]

NAI-107 forms 1:1 or 2:1 complexes with bactoprenol–pyrophosphate-coupled precursors of the bacterial cell wall, such as lipid II. In whole cells, NAI-107 does not form nisin-like pores in the bacterial membrane, but binding of NAI-107 to lipid II is followed by a slow membrane depolarization.[11] Current evidence indicates that NAI-107, like nisin, binds to the pyrophosphate moiety of lipid II,[11] a site distinct from the binding site of glycopeptides. Indeed, vancomycin does not kill non-dividing *S. aureus*,[26] as confirmed here, consistent with the fact that the D-Ala-D-Ala moiety (i.e. vancomycin's target) is not present in mature peptidoglycan.[33]

Altogether, the observed killing by NAI-107 of MRSA and GISA cells under non-dividing conditions is consistent with the

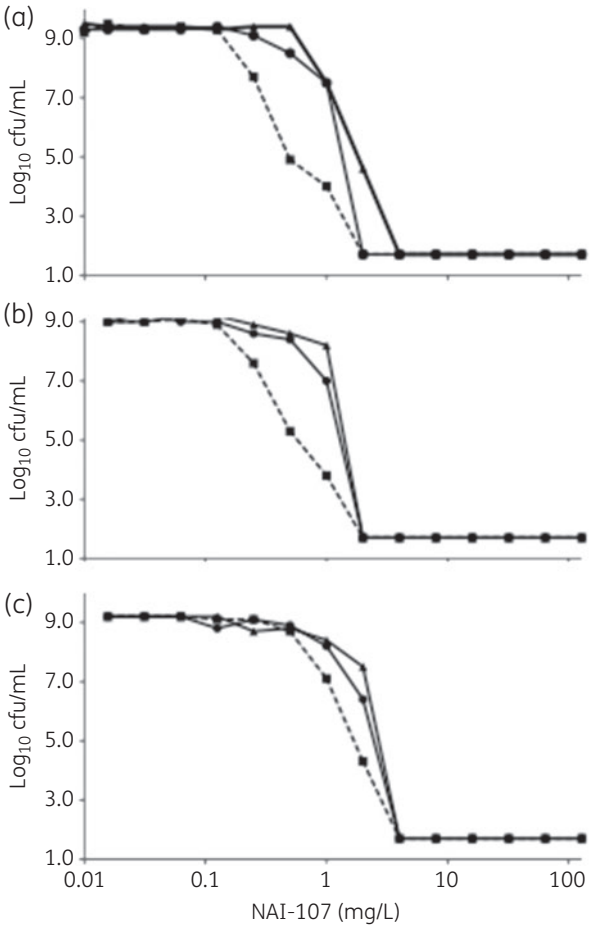

Figure 7. Population analysis profiles of cultures of strains MSSA L819 (a), GISA L4064 (b) and MRSA L4061 (c) emerging after 1 passage (squares, broken line), 10 passages (circles, continuous line) or 20 passages (triangles, continuous line). The limit of detection in these experiments was 50 cfu/mL.

hypothesis that killing requires interaction with the membrane. In the case of NAI-107, we believe this interaction occurs after an initial docking on lipid II or other bactoprenol–pyrophosphate-based intermediates. Indeed, it has been recently reported that peptidoglycan recycling is important for survival in the stationary phase of Gram-positive bacteria, including *S. aureus*.[34] Although not demonstrated by these authors, such recycling might involve pyrophosphate-based carriers similar to lipid II, and would explain NAI-107's activity against non-dividing cells. A similar explanation might apply also to the reported killing of non-dividing cells by oritavancin.[26]

We were surprised to observe that NAI-107 can also kill non-dividing cells of two *A. baumannii* strains at sub-MIC values. This phenomenon might be a general feature of this species, as it was not observed with *E. coli*, *K. pneumoniae* and *P. aeruginosa*. Thus, it is tempting to speculate that the permeability of the *A. baumannii* outer membrane might change as the cells go from a non-dividing to a dividing state, allowing NAI-107 to reach its target under the former conditions. Among the four pathogenic species in Table 3, *A. baumannii* is the only species for which NAI-107 showed occasionally a measured MIC. In this respect, it has been reported that *A. baumannii* is generally more permeable to large antibiotics such as novobiocin and erythromycin, possibly connected with the ability of *Acinetobacter* spp. to use long-chain fatty acids as growth substrates.[35]

A growing body of evidence indicates the importance of killing persisters and non-dividing cells in an infection setting.[36] Overall, the results of the current work, along with previously published results on the efficacy of NAI-107 in experimental models of infection,[12,13] indicate that this lantibiotic represents a promising option in addressing the serious threat of antibiotic resistance in Gram-positive pathogens, with rapid killing of actively dividing and non-dividing MRSA and GISA cells. In addition, the activity of NAI-107 against *N. gonorrhoeae*, its ability to kill non-dividing *A. baumannii* and its strong synergism with polymyxin might provide additional therapeutic options for treating infections by *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa*. From the limited comparisons reported here, it appears that NAI-108 shares the same properties as NAI-107.

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Transparency declarations

E. G., S. M., M. S. and S. D. are employees of NAICONS Srl. C. B., M. S. and S. D. are employees of KtedoGen Srl. S. M., M. S., D. J. and S. D. own shares of NAICONS Srl, which may be financially affected by the conclusions of the present article. T. T. T. and A. L.-O.: none to declare.

Supplementary data

Materials and methods and Tables S1 to S3 are available as Supplementary data at *JAC* Online.

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