Potentiation of Gram-positive specific antibiotics against Gram-negative bacteria through outer membrane disruption

Wesseling, C.M.J.

Chapter 5

Intrinsic antimicrobial activity of bis-amidines against Gram-positive bacteria

Charlotte M.J. Wesseling, Samantha Lok, Shanice Veraar, and Nathaniel I. Martin

1. Introduction

Antimicrobial resistance (AMR) is on the rise. In the 5 year period between 2014 and 2019, annual deaths worldwide due to AMR rose from 700k to 1.27 million.[1–4] The rightly coined "overlooked pandemic" requires attention, not only by controlling and reducing the use of antibiotics, but also by developing new antibiotics to revive the dried-up pipeline.[2,3,5] In addition to developing new antibiotics, another strategy for addressing the threat of AMR is by examining the potential for repurposing approved drugs as antimicrobial agents.[5–8]

Pentamidine (**1**, Figure 1) is a simple bis-amidine, was first synthesized in the 1930s, and is an effective antiparasitic drug. Pentamidine is on the WHO's list of essential medicines and it freely distributed in developing countries where parasitic diseases are endemic.[9,10] Beyond its antiparasitic activity, pentamidine has also been investigated as an anti-cancer agent and shows inhibition of *Escherichia coli* and *Staphylococcus aureus* growth, exhibiting moderate antimicrobial activity against Gram-positive bacteria.[11–18,10,19–24] Its adverse side-effects are also well-documented and include nephrotoxicity, tachycardia, hypotension, hypoglycaemia, and local reactions to the injection.[9,10,25,26] Efforts are being made to reduce the nephrotoxicity of pentamidine, such as employing nanotechnology.[27] Pentamidine is seen as a promising repurposing candidate as exemplified by the wide range of therapeutical applications proposed and/or approved.[27]

Recently, the possibility to repurpose pentamidine as a potentiator of Gram-positive antibiotics against Gram-negative bacteria was reported by Brown and coworkers.[11] Compelling *in vivo* data from systemic murine models with colistin-sensitive and -resistant *Acinetobacter baumannii* strains showed pentamidine to be highly effective in enhancing the antibacterial activity of novobiocin.[11] The results of this study highlight the potential of repurposing pentamidine as a combination treatment against Gram-negative bacteria.[5–8,11] In addition, this study explored the structure-activity relationship (SAR) of pentamidine and commercially available analogues.[11] Building on this work, our group recently conducted a follow-up SAR study wherein a number of novel bis-amidines inspired by pentamidine (Figure 1) were synthesized and assessed for antibiotic synergy (see Chapter 2 of this thesis).[12]

A number of the bis-amidines prepared in our earlier study showed strong potentiation of several Gram-positive analogues.[12] Disruption of the Gram-negative outer membrane (OM) was established as the mode of potentiation for these compounds and for this reason the selectivity of membrane disruption was also explored by means of a hemolysis assay.[11,12] Several analogues proved to be hemolytic when tested under stringent conditions including high concentrations and long incubation times (20 hours).[12] From these studies however, compounds **3** and **4** emerged as having potent OM disrupting ability and low hemolytic activity (Figure 1).[12] Still, a question remained, while compounds **3** and **4** showed the highest OM disruption, compounds **6**-**9** displayed lower OM disruption (data not shown), but potentiated the activity of antibiotics even stronger.[12] This finding prompted us to also perform a preliminary screen of these analogues for inherent antibacterial activity against a selected *S. aureus* strain.

In literature several SAR studies of pentamidine analogues against *S. aureus* have been reported.[21,28–30] As mentioned before, the development of new antibiotics is key in combating AMR.[3,31] A systematic analysis of the AMR numbers of 2019 revealed that of the Gram-positive bacteria *S. aureus* and *Streptococcus pneumoniae* were each responsible for more than 250.000 deaths in 2019, while deaths associated with AMR *Enterococcus faecium* lay between 100.000 and 250.000 deaths.[1] According to the WHO's priority list, vancomycin-resistant *E. faecium* and methicillin- and vancomycin-resistant *S. aureus* are classified as priority 2 "high", while *S. pneumoniae* is classified as priority 3 "medium".[31] The work described in this chapter work aimed to assess and improve the inherent activity of our new pentamidine analogues towards Gram-positive bacteria, specifically *S. aureus* and *E. faecium*.

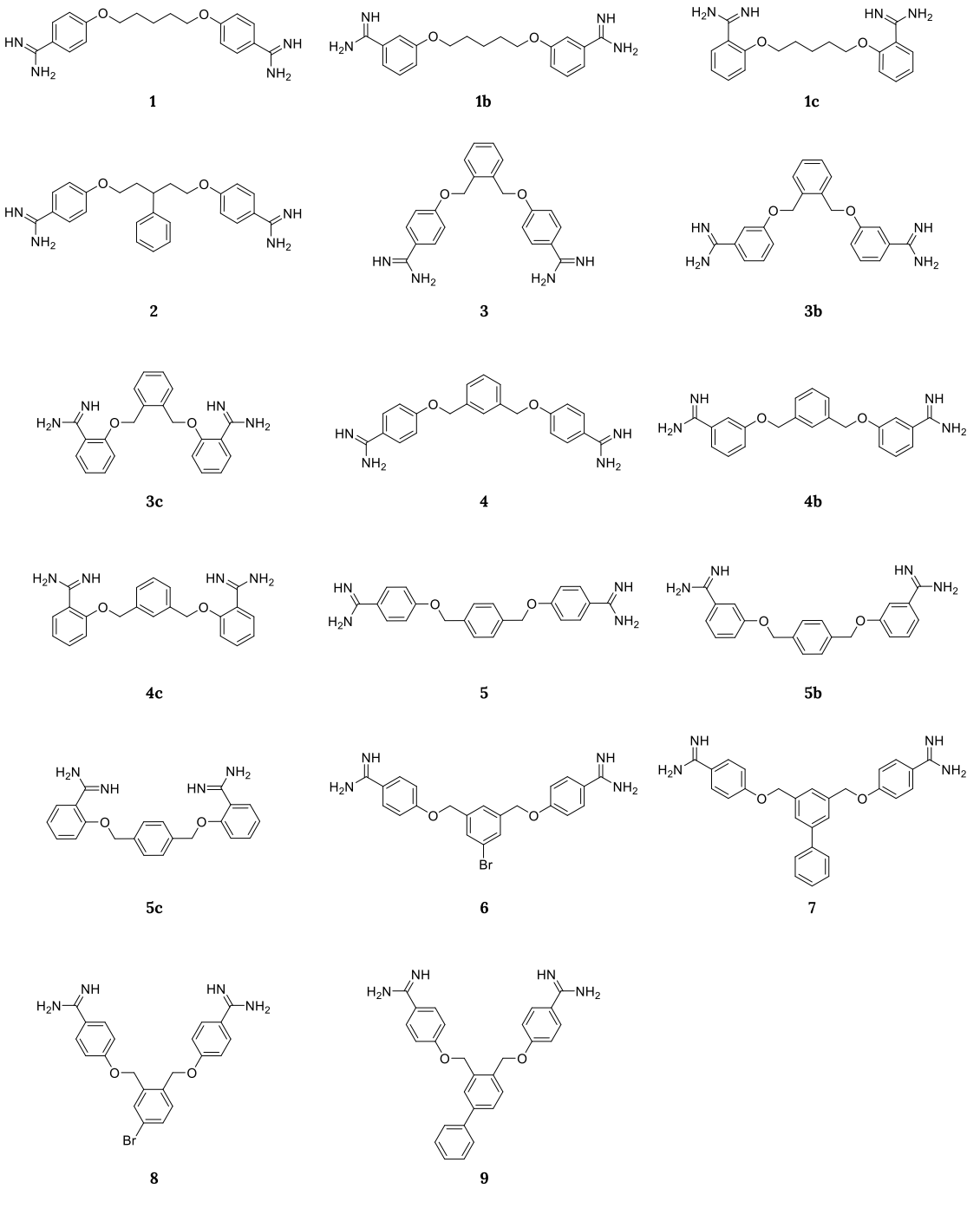


Figure 1. Overview of pentamidine (**1**) and the bis-amidines described as potentiators by the group of Brown (**2**)[11] and from our previous publication (**1b**, **1c**, and **3–9**) (see Chapter 2).[12]

2. Results and Discussion

The inherent antibacterial activity of the pentamidine-inspired bis-amidines from our previous study (Figure 1),[12] were establish by determining the minimum inhibitory concentration (MIC) of each compound against S. *aureus* (ATCC 29213) grown in lysogeny broth (LB). Table 1 provides a summary of the MIC values thus obtained, along with the FICI values for each compound (based on the synergy observed when combined with erythromycin and tested against *E. coli*) as well as the hemolytic activity determined for each compound.

Table 1. Overview of the antimicrobial and hemolytic activity of the previously described bis-amidines. The minimal inhibitory concentration (MIC) is determined against *S. aureus* ATCC 29213 (μg/mL). The hemolytic activity is determined at 128 μg/mL after 20 hours of incubation. The hemolytic activity is normalized to the positive control (0.1% Triton X-100) and a value of <10% is considered non-hemolytic.[32]

	MIC (μg/mL)	FICI	% Hemolysis
Pentamidine (1)	8-16	0.500	0.0
1b	16	≤0.375	0.0
1c	>128	>0.5[a]	0.0
2	2	≤0.063	6.6
3	16	≤0.125	0.0
3b	32	≤0.313	0.5
3c	>128	>0.5[a]	0.0
4	16	≤0.094	0.0
4b	4	≤0.250	0.9
4c	128	>0.5[a]	0.2
5	8	≤0.313	0.5
5b	8	≤0.250	0.3
5c	128	>0.5[a]	0.1
6	4	≤0.063	13
7	1	≤0.047	10
8	1	≤0.094	14
9	1	≤0.078	78
Vancomycin	1	-	<5%[33]

[a]The fractional inhibitory concentration index (FICI) values were taken from Table 1 of the previous SAR study with bis-amidines (See Chapter 2).[12] These values were calculated from checkerboard assays with erythromycin against *E. coli* BW25113 in LB.[12]

The effect of the bis-amidine geometries on inherent activity roughly reflected the trends observed for their synergistic activity.[12] For example, when the amidine moieities are located at the *ortho* position as **1c**, **3c**, **4c**, and **5c**, a significant reduction of inherent activity results is noted relative to the corresponding *para*-amidine analogues

1, **3**, **4**, and **5**. Similarly, these *ortho* analogues were found to also been largely devoid of synergistic potential with a fractional inhibitory concentration index (FICI) above 0.5.[12] Conversely, in the case of the *meta*-amidine analogues prepared (**1b**, **3b**, **4b** and **5b**), the antibacterial activities measured where found to be similar to *para*-substituted compounds. Notably, the compound with the most potent *in vitro* antibacterial activity among the *ortho*-, *meta*-, *para*- series of analogues prepared was *meta* analogue **4b** which was found to exhibit a 2- to 4-fold enhancement compared to pentamidine (Table 1).

Overall, the lowest MICs were observed for the bis-amidines **7**-**9** (Table 1), which showed a significant 16-fold improvement compared to pentamidine. Notable in this regard are a previously reported SAR studies that yielded pentamidine analogues which in some cases showed enhanced activity against MRSA strains.[21] A closer look at these reports revealed the use of Mueller-Hinton broth (MHB) as medium.[28] MHB is a medium that is normally adjusted with cations, Mg^{2+} and Ca^{2+}, and the absence of cations could hypothetically improve MIC values of positively charged bis-amidines. Re-evaluation of a selection of our analogues in MHB and cation-adjusted MHB (CAMHB) resulted in lower MIC for a majority of the analogues (Table 2). For instance, the MIC of pentamidine, originally 8-16 μg/mL in LB, dropped to 2 μg/mL in MHB and 4 μg/mL in CAMHB. By comparison, the MICs of the most potent compounds **7** and **9** dropped to 0.5 μg/mL in MHB, while for **8** the MIC did not change when screening in different media.

Table 2. The effect of different media on the antimicrobial activity of bis-amidines **1**-**4** and **6**-**9**. The minimal inhibitory concentration (MIC) is determined against *S. aureus* ATCC29213 (μg/mL).

	LB	**MHB**	**CAMHB**
Pentamidine (1)	8-16	2	4
2	2	1	1
3	16	4	8
4	16	4	8
6	4	1	1
7	1	0.5	0.5
8	1	1	1
9	1	0.5	1
Vancomycin	1	0.5	1

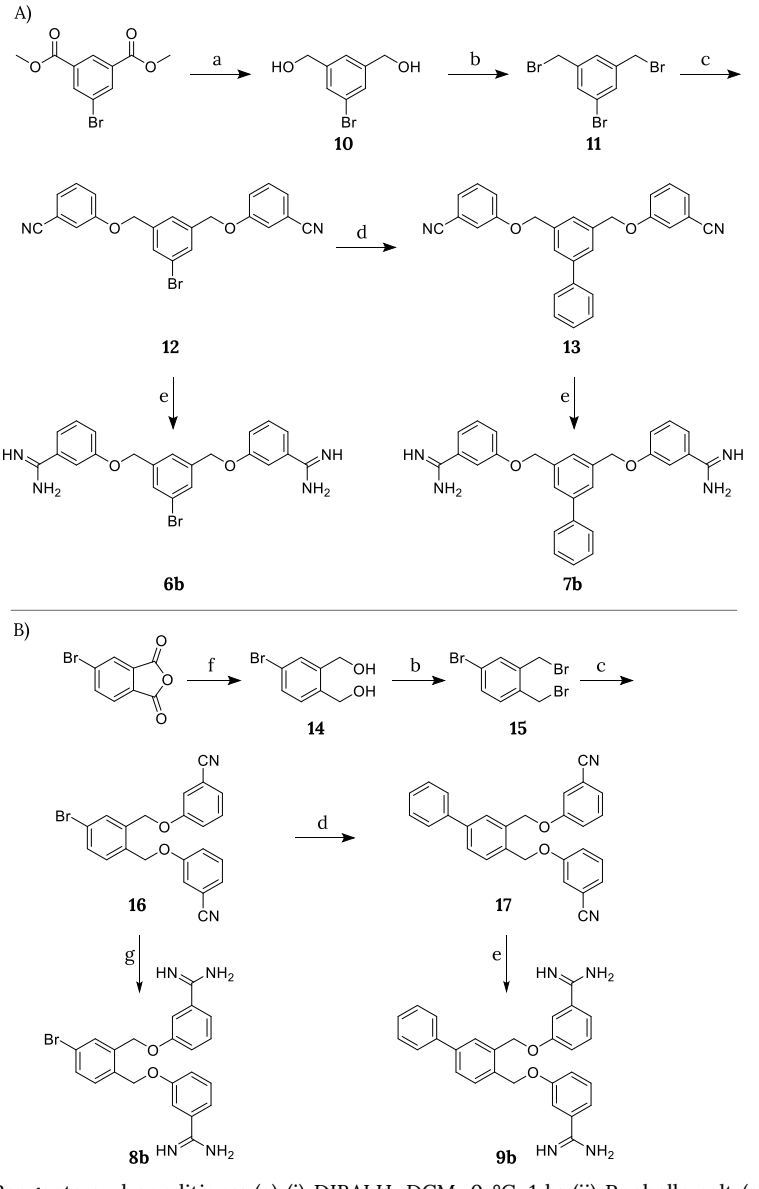
Analogues **7**-**9** exhibited the most potent antibacterial activity and were not impacted by the use of different growth media conditions. However, assessment of hemolytic activity clearly reveals bis-amidines **6**-**9** to be hemolytic (Table 1). As mentioned in our previous SAR study (Chapter 2), an increase in lipophilicity is often associated with an increase in hemolysis.[12] However, lipophilicity alone does not explain the high difference in hemolytic activity between **7** and **9** (10% and 78%, respectively, Table 1) with our data clearly showing that the geometry of the aromatic linker plays a role. To further explore this effect, the *meta*-oriented bis-amidine analogues **6b**, **7b**, **8b**,

and **9b** were synthesized (Scheme 1). The synthetic routes used in their preparation were largely based on the reported syntheses of analogues **6**, **7**, **8**, and **9** as described in Chapter 3.[12] In the case of **6b** and **7b** compound **11** served as a common intermediate.[12,34,35] In the subsequent Williamson ether synthesis, however, 3-cyanophenol instead of 4-cyanophenol was used to yield **12**.[12,36] Transformation to the bis-amidine products was then performed as for **6** and **7** as described in Chapter 2.[12] Likewise, the first three steps in the synthesis of **8b** and **9b** were identical to their parent compounds **8** and **9** with the only difference being the use the 4-cyanophenol for 3-cyanophenol.[12,37] As for bis-amidine **8**, the use of LHMDS resulted in the loss of the bromo-group in bis-amidine **8b**.[12] Therefore, the same three-step reaction used to obtain **8** was employed and yielded **8b**.[12,38] The aforementioned Suzuki coupling was performed on bis-nitrile **16** as for bis-nitrile **12**.[39–41] Bis-amidine **9b** was obtained from **16** using LHMDS as described before.[12]

The newly obtained analogues **6b**, **7b**, **8b**, and **9b** were then screened for antimicrobial activity against *S. aureus* (ATCC 29213) and their hemolytic activity also assessed. The compounds were compared to their parent compounds **6**, **7**, **8**, **9** (Table 3). Bis-amidine **2** was also included, due to its relatively low MIC values in all media (see Table 2) and its low hemolytic activity. Based on the values thus obtained for antibacterial and hemolytic activity, an estimate for the therapeutic window of the compounds was calculated (Table 3). The calculate therapeutic wind was based on the difference between MIC and the concentration at which the hemolytic activity was below 10% (see caption Table 3). It should be noted that these estimated therapeutic window values should only be see as indicative given that they are based on *in vitro* assays. Nonetheless, such an analysis can provide an indication of selectivity of the compounds for bacterial cells vs erythrocytes.

Table 3. MIC values of pentamidine analogues **2** and **6**-**9b** in LB broth for *S. aureus* ATCC 29213. The therapeutic window is calculated from the highest non-hemolytic concentration divided by the MIC (μg/ml). If the compound is still hemolytic at 32 μg/ml, the value ≤16 μg/ml was used in the calculation. The MIC values, the percentage of hemolysis at different concentrations, and the therapeutic window for the other bis-amidines can be found in the Supplementary data, Table S1.

	MIC (μg/ml)	% Hemolysis (128 μg/ml)	% Hemolysis (64 μg/ml)	% Hemolysis (32 μg/ml)	Therapeutic window
2	2	6.6	0.6	0.0	64
6	4	13	1.8	0.0	16
6b	1	4.2	0.6	0.0	128
7	1	10	3.6	2.3	64
7b	0.5	84	49	11	≤32
8	1	14	3.4	0.7	64
8b	1	4.8	0.8	0.0	128
9	1	78	54	22	≤16
9b	4	61	44	21	≤4



Scheme 1. Reagents and conditions: (a) (i) DIBALH, DCM, 0 °C, 1 h; (ii) Rochelle salt (quench), rt, overnight (96%); (b) PPh_3, CBr_4, DCM, rt, 2 h (55-74%); (c) 4-cyanophenol, NaH, DMF, 80 °C, 1 h (quant.); (d) phenylboronic acid, Pd(dppf)Cl_2·DCM, THF/Na_2CO_3(aq) (1:1), 65 °C, 18 h (81-83%); (e) (i) LHMDS, THF, rt, 48 h; (ii) HCl (dioxane), 0 °C-rt, overnight (21-93%); (f) (i) LAH, $ZnCl_2$, THF, rt, 6 h; (ii) Rochelle salt (quench), rt, overnight (95%); (g) (i) NH_2OH·HCl, DIPEA, EtOH, 85 °C, 6 h; (ii) Ac_2O, AcOH, rt, 4 h; (iii) Zn powder, AcOH, 35 °C, 6 h (10%).

Interestingly, the newly synthesized compounds **6b**, **7b**, **8b**, and **9b** exhibit both a greater range of hemolytic activity (4.2-84%) and antibacterial activity (0.5 – 4 μg/mL) compared to the *para*-amidine analogues **6**, **7**, **8**, and **9** (Table 3). Again, the relation between hemolytic activity and antibacterial activity doesn't seem to be directly correlated: **6b** and **8b** are non-hemolytic and have MIC values of 1 μg/mL, whereas **9b** is hemolytic and has a MIC of 4 μg/mL. In contrast, the more potent analogue, **7b**, with an MIC of 0.5 μg/mL also exhibits the highest hemolytic activity. The therapeutic window calculations here provide a convenient means of comparing all analogues revealing **6b** and **8b** as the most selective.

In addition to *S. aureus* ATCC 29213, several other strains of *S. aureus* and *E. faecium* were selected for MIC assays to establish the range of activity of the bis-amidines (Table 4). The selected strains include vancomycin-resistant strains (*S. aureus* LIM2 (intermediate-resistant) and VRS3b and *E. faecium* E155 and 7314) and methicillin-resistant *S. aureus* (MRSA) strains (COL and USA300) to further establish the activity of bis-amidines against drug resistant oragnisms. The MIC values shown in Table 4 were obtained using LB broth as growth media. The same assays were also run in MHB and CAMHB (Supplementary data, Table S3-4). In the case of *E. faecium* strains, TSB was also employed as medium for the MIC assays (Supplementary data Table S5). Based on the therapeutic window of the bis-amidines (Table 3), a selection of the bis-amidines is displayed in Table 4 (therapeutic window ≥ 64).

Table 4. MIC values (μg/ml) of pentamidine (**1**) and bis-amidines with a therapeutic window ≥ 64 (**2**, **6b**, **7**, **8**, and **8b**) in LB broth for *S. aureus* and *E. faecium* strains. The maximum concentration tested for pentamidine (**1**) was 64 μg/ml and for vancomycin 128 μg/ml. Results of the other bis-amidines is located in Supplementary data, Table S2.

	S. aureus					*E. faecium*	
	ATCC 29213	**LIM2**	**VRS3b**	**COL**	**USA300**	**E155**	**7314**
1	8-16	>64	8	8	16	>64	64
2	2	32	2	2	4	16	16
6b	1	16	0.5	0.5	2	32	16
7	1	4	0.25	0.5	1	4	16
8	1	16	1	1	2	16	16
8b	1	32	2	4	8	32	32
Vancomycin	1	4	128	2	<2	>128	128

Of this selection, bis-amidine **7** clearly displays the most potent overall antibacterial activity (Table 4). Only against the *E. faecium* strains and *S. aureus* LIM2, were MIC values for **7** above 4 μg/ml. This compound was assessed as beingh borderline hemolytic at 128 μg/ml (10%, Table 3). The most selective bis-amidines **6b** and **8b**, based on the therapeutic window as determined for *S. aureus* ATCC 29213, display even less activity against *E. faecium* and *S. aureus* LIM2 (≤16 μg/ml, Table 4). However, against the other strains, **6b** displays similar MIC values (maximum a 2-fold difference) to those

measured for **7**. Given that these values were obtained in LB, the effects of other growth media on the MIC values of bis-amidines **6b** and **7** were assessed. To this end, the activities of **6b** and **7** were established against *S. aureus* strains in LB, MHB, and CAMHB and for *E. faecium* also in TSB (Table 5). Interestingly, in the other growth media, the *S. aureus* LIM2 and *E. faecium* 7314 strains aooeared to more susceptible to bis-amidine **7**. While for **6b** no such enhancement of activity was observed. There was, however, an improvement seen for **6b** in CAMHB against the *E. faecium* strain E155. In some cases **6b** even shows 2-fold lower MIC values in MHB and CAMHB compared to **7**. Still, overall, bis-amidine **7** is clearly the more potent compound.

A final detail that does require some attention is the overall trend seen for *S. aureus* LIM2 and *E. faecium* E155 and 7314: in general these strains seem less susceptible to pentamidine and bis-amidine analogues compared to the other strains tested (Table 4 and 5, See supplementary data, Table S2-S5). In the case of *S. aureus* LIM2 this could be the results of a thickened cell wall, often seen for vancomycin intermediate-resistant *S. aureus* strains.[42–44] *E. faecium* E155 and 7314 carry the VanA and VanB resistance gene responsible for their vancomycin-resistance, similar to *S. aureus* VRS3b, which is susceptible to the bis-amidines.

Possibly, the *E. faecium* strains are harder to target because of other factors contributing to their inherent insensitivity to the bis-amidines. In terms of resistance, in the literature there is mention of MDR efflux pumps that result in increased resistance to pentamidine[45–48]. The chromosomally-encoded NorA or the plasmid-encoded QacA and QacB are most commonly reported for *S. aureus*, but a QacA/B plasmid has also been found in an isolated *E. faecium* strain.[45–55] Given that there is no literature reporting the presence of this gene or plasmids in the aforementioned strains, we cannot draw a firm conclusion as of yet. Still, future studies to address this point could include strains with either the QacA, QacB, and QacC plasmid or with the NorA gene.[47,50,51,55]

Table 5. MIC values (μg/ml) of bis-amidines **6b** and **7** for *S. aureus* and *E. faecium* strains in different broths. The maximum concentration tested for vancomycin was 128 μg/ml. Results of the other bis-amidines is located in Supplementary data, Table S2-S5.

	S. aureus					*E. faecium*	
	ATCC 29213	**LIM2**	**VRS3b**	**COL**	**USA300**	**E155**	**7314**
				LB			
6b	1	16	0.5	0.5	2	32	16
7	1	4	0.25	0.5	1	4	16
Vancomycin	1	4	128	2	<2	>128	128
				MHB			
6b	0.5	8	0.5	0.125	0.5	8	8
7	0.5	2	0.25	0.25	1	2	2
Vancomycin	0.5	4	128	2	1	>128	>128
				CAMHB			
6b	0.5	16	0.5	0.25	0.5	2	16
7	0.5	4	0.5	0.5	1	2	2
Vancomycin	1	4	128	2	1	>128	>128
				TSB			
6b	-	-	-	-	-	32	16
7	-	-	-	-	-	4	4
Vancomycin	-	-	-	-	-	64	128

3. Conclusion

In this study we examined the inherent antibacterial activity of the bis-amidines previously investigated as antibiotic synergists (see Chapter 2). This revealed bis-amidine **7** to have potent antibacterial activity against *S. aureus* and *E. faecium* strains (majority of the MIC values ≤2 μg/ml). An estimation of the selectivity of **7** and the other bis-amdine studied was achieved by comparing the concentrations at which they exhibit hemolytic activity versus their MIC values. This revealed compound **7** to have relatively large therapeutic window (64-fold difference between hemolytic activity concentration and MIC against *S. aureus* ATCC 29213 screened in LB).

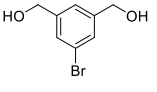
A focused SAR study also revealed the optimal positioning of the amidine group to be *para* and *meta*-in terms of antibacterial activity, while the *ortho*-amidine analogues displayed little-to-no activity. In addition, increasing the linker hydrophobicity, increased both the inherent activity and hemolytic activity. However, the positioning of the linker relative to the amidines clearly also plays a role given that a direct correlation of lipophilicity to hemolytic activity couldn't be made when comparing bis-amidines **6-9b**. These trends are similar to the trends observed for the synergistic potential of the same bis-amidines in the previous SAR study focused on potentiation of Gram-positive antibiotics against Gram-negative bacteria.[11,12]

Based on the results from the SAR study reported in Chapter 2, it appeared that the bis-amidines studied might have a secondary mode of action that contributed to their synergistic activity; the disruption of the OM alone, could not explain the trends seen in the FICI.[12] The trends observed for the inherent *in vitro* activity of the bis-amidines towards Gram-positive bacteria suggest that this is indeed the case, especially since Gram-positive bacteria don't have an OM.

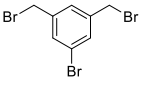
4. Materials and methods

General procedures. All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. For compound characterization, 1H NMR spectra were recorded at 400 MHz with chemical shifts reported in parts per million (ppm) downfield relative to CHCl3 (7.26) or DMSO (δ 2.50). ^{1}H NMR data are reported in the following order: multiplicity (s, singlet; d, doublet; t, triplet; q, quartet and m, multiplet), coupling constant (J) in hertz (Hz) and the number of protons. Where appropriate, the multiplicity is preceded by br, indicating that the signal was broad. ^{13}C NMR spectra were recorded at 101 MHz with chemical shifts reported relative to $CDCl_3$ (δ 77.16) or DMSO (δ 39.52). HRMS analysis was performed on a Shimadzu Nexera X2 UHPLC system with a Waters Acquity HSS C18 column (2.1 × 100 mm, 1.8 μm) at 30 °C and equipped with a diode array detector. The following solvent system, at a flow rate of 0.5 mL/min, was used: solvent A, 0.1 % formic acid in water; solvent B, 0.1 % formic acid in acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 1 min, 95:5 to 15:85 (A/B) over 6 min, 15:85 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 3 min, then reversion back to 95:5 (A/B) for 3 min. This system was connected to a Shimadzu 9030 QTOF mass spectrometer (ESI ionisation) calibrated internally with Agilent's API-TOF reference mass solution kit (5.0 mM purine, 100.0 mM ammonium trifluoroacetate and 2.5 mM hexakis(1H,1H,3Htetrafluoropropoxy) phosphazine) diluted to achieve a mass count of 10000. Purity of the final compounds **6b**, **7b**, **8b**, and **9b** was confirmed to be ≥ 95% by analytical RPHPLC using a Shimadzu Prominence-i LC-2030 system with a Dr. Maisch ReproSil Gold 120 C18 column (4.6 × 250 mm, 5 μm) at 30 °C and equipped with a UV detector monitoring at 214 nm. The following solvent system, at a flow rate of 1 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile, 95/5; solvent B, 0.1 % TFA in water/acetonitrile, 5/95. Gradient elution was as follows: 95:5 (A/B) for 2 min, 95:5 to 0:100 (A/B) over 30 min, 0:100 (A/B) for 1 min, then reversion back to 95:5 (A/B) over 1 min, 95:5 (A/B) for 3 min. The compounds were purified via preparative HPLC using a BESTA-Technik system with a Dr. Maisch Reprosil Gold 120 C18 column (25 × 250 mm, 10 μm) and equipped with a ECOM Flash UV detector monitoring at 214 nm. The following solvent system, at a flow rate of 12 mL/min, was used: solvent A, 0.1 % TFA in water/acetonitrile 95/5; solvent B, 0.1 % TFA in water/acetonitrile 5/95. Unless stated otherwise in the protocol, the gradient elution was as follows: 70:30 (A/B) to 0:100 (A/B) over 25 min, 0:100 (A/B) for 3 min, then reversion back to 70:30 (A/B) over 1 min, 70:30 (A/B) for 1 min.

4.1. Synthesis



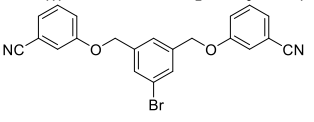
(5-bromo-1,3-phenylene)dimethanol (10) Protocol as described in literature.[34] Dimethyl 5-bromoisophthalate (2.3 g, 8.3 mmol) was dissolved in dry DCM (25 mL) under argon atmosphere. The solution was then cooled to 0 °C using an ice bath and DIBALH (40 mL, 1 M hexane solution, 4.8 eq.) was added dropwise. The mixture was stirred from 0 °C to room temperature for 1 hour. The reaction was quenched with Rochelle salt (60 mL, sat. aq.) and the biphasic mixture was stirred at room temperature overnight. The layers were separated and the aqueous layer was two times extracted with diethyl ether. The organic layers were combined, washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified using column chromatography (DCM/EtOAc = 1:1) and afforded compound **18** (1.8 g, 96%). ^{1}H NMR (400 MHz, MeOD) δ 7.42 (s, 2H), 7.28 (s, 1H), 4.58 (s, 4H), 3.35 (s, 2H). ^{13}C NMR (101 MHz, MeOD) δ 145.51, 129.42, 124.82, 123.31, 64.29.



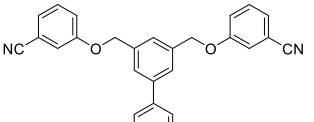
1-bromo-3,5-bis(bromomethyl)benzene (11) Protocol as described in literature.[56] To a solution of compound **10** (1.0 g, 4.6 mmol) in dry DCM (50 mL) was added PPh_3 (2.5 g, 9.7 mmol, 2.1 eq.) and CBr_4 (3.2 g, 9.7 mmol, 2.1 eq.) and the mixture was stirred at room temperature for two hours under argon atmosphere. The reaction was quenched

with water (30 mL) and the product was extracted from the aqueous layer with DCM three times. The combined organic layers were washed with water and brine, dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by column chromatography (petroleum ether 100%) to give compound **19** (0.87 g, 55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 – 7.45 (m, 2H), 7.34 (s, 1H), 4.53 (d, J = 4.0 Hz, 1H), 4.41 (d, J = 4.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.42, 140.11, 132.11, 132.09, 131.64, 128.40, 127.90, 122.83, 44.89, 31.64, 31.59.

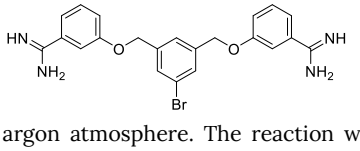
3,3'-(((5-bromo-1,3-phenylene)bis(methylene))bis(oxy))dibenzonitrile (12) These conditions were based on literature protocols.[36] 3-cyanophenol (0.49 g, 4.1 mmol, 2.4 eq.) was suspended in dry DMF (6 mL) under argon atmosphere. The suspension was cooled to 0 °C using an ice bath and NaH (160 mg, 60% dispersion in mineral oil, 2.4 eq.) was slowly added. The reaction was stirred for 30 minutes, the ice bath was removed and compound **11** (0.59 g, 1.7 mmol) was added. The reaction mixture was heated to 80 °C, stirred for 1 hour, and then cooled to room temperature. Water (18 mL) was added to the mixture to obtain precipitation and give compound **12** (0.72 g, quant.). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 – 7.54 (m, 2H), 7.43 – 7.37 (m, 3H), 7.29 (dt, J = 7.6, 1.2 Hz, 2H), 7.21 – 7.17 (m, 4H), 5.07 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.45, 138.79, 130.69, 130.23, 125.33, 124.61, 123.34, 120.16, 118.68, 117.88, 113.54, 69.27.



3,3'-(([1,1'-biphenyl]-3,5-diylbis(methylene))bis(oxy))dibenzonitrile (13) Conditions were based on protocols described in literature.[40,41] Dibenzonitrile intermediate **12** (0.15 g, 0.36 mmol) was dissolved in a 3:1 mixture of THF and 2 M Na_2CO_3 (aq.) of 4 mL, respectively. Phenylboronic acid (65 mg, 0.54 mmol, 1.5 eq.) and $Pd(dppf)Cl_2$·DCM (26 mg, 0.03 mmol, 0.1 eq.) were added. The reaction mixture was heated to 65 °C for 18 hours and then partitioned between DCM and $NaHCO_3$ (sat. aq.). The aqueous layer was three times extracted with DCM, the organic layers were combined and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the crude product was purified using column chromatography (petroleum ether/EtOAc = 4:1) to obtain compound **13** (0.12 g, 83%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 – 7.61 (m, 4H), 7.54 – 7.48 (m, 3H), 7.47 – 7.40 (m, 3H), 7.34 – 7.24 (m, 6H), 5.21 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.77, 142.63, 140.34, 137.22, 130.63, 129.07, 128.01, 127.38, 126.33, 125.23, 125.14, 120.25, 118.79, 117.96, 113.47, 70.22.



3,3'-(((5-bromo-1,3-phenylene)bis(methylene))bis(oxy))dibenzimidamide (6b) This protocol was based on the synthesis of structurally similar amidine containing compounds previously described in literature.[12,57–60] Compound **12** (100 mg, 0.24 mmol) was dissolved in a solution of LHMDS (2.0 mL, 1 M THF solution, 8.0 eq.)under argon atmosphere. The reaction was stirred at room temperature for 48 hours or longer until complete conversion to the bis-amidine (monitored by LCMS). The solution was cooled to 0 °C and quenched with HCl (3.6 mL, 4 M dioxane solution, 60 eq.). The mixture was stirred at room temperature for 30 minutes, then diluted with diethyl ether and filtered. The precipitate was purified by preparative HPLC with the gradient 30-100% in 30 minutes to give compound **6b** (23 mg, 21%). ^{1}H NMR (400 MHz, DMSO) δ 9.28 (s, 4H), 9.16 (s, 4H), 7.62 (d, J = 1.4 Hz, 2H), 7.56 – 7.48 (m, 3H), 7.43 (t, J = 2.1 Hz, 2H), 7.39 – 7.29 (m, 4H), 5.18 (s, 4H). ^{13}C NMR (101 MHz, DMSO) δ 165.26, 158.15, 139.57, 130.51, 129.92, 129.52, 125.78, 121.87, 120.64, 119.92, 114.66, 68.65. HRMS (ESI): calculated for $C_{22}H_{21}BrN_4O_2$ [M+H]+ 453.0926, found 453.0921.



3,3'-(([1,1'-biphenyl]-3,5-diylbis(methylene))bis(oxy))dibenzimidamide (7b) Following the procedure as described above for compound **6b**, using compound **13** (110 mg, 0.26 mmol), LHMDS (2.5 mL, 1 M THF solution, 9.4 eq.) and HCl (4.5 mL, 4 M dioxane solution, 68 eq.), afforded the crude product. The crude product was purified using HPLC with a 30-100% gradient for 30 minutes to obtain the pure compound **7b** (112 mg, 93%). ^{1}H NMR (400 MHz, DMSO) δ 9.36 (s, 4H), 9.28 (s, 4H), 7.68 (d, J = 1.6 Hz, 2H), 7.64 – 7.58 (m, 2H), 7.52 – 7.39 (m, 7H), 7.38 – 7.31 (m, 5H), 5.22 (s, 4H). 13C NMR (101 MHz, DMSO) δ 165.46, 158.42, 140.80, 139.61, 137.69, 130.49, 129.56, 129.15, 127.93, 126.84, 126.12, 125.93, 120.49, 120.06, 114.57, 69.59. HRMS (ESI): calculated for $C_{28}H_{26}N_4O_2$ [M+H]+ 451.2135, found 451.2129.

(4-bromo-1,2-phenylene)dimethanol (14) Conditions were based on protocol reported in literature.[61] LAH (15 mL, 1 M THF solution, 2 eq.) and $ZnCl_2$ (0.61 g, 4.5 mmol, 0.6 eq.) were suspended in dry THF (30 mL) and cooled to 0 °C, then 4-bromophthalic anhydride (1.7 g, 7.5 mmol) was slowly added. The mixture was stirred at room temperature for 6 hours under argon atmosphere. The mixture was cooled to 0 □C and quenched with Rochelle salt (30 mL, sat. aq.) and the biphasic mixture was stirred at room temperature overnight. The layers were separated and the aqueous layer was extracted with diethyl ether two times and the combined organic layers were washed with water and brine, dried over Na2SO4 and concentrated in vacuo. The crude product was purified by column chromatography (DCM/EtOAc = 1:1) to give compound **24** (1.5 g, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, J = 2.1 Hz, 1H), 7.42 (dd, J = 8.0, 2.1 Hz, 1H), 7.18 (d, J = 8.0 Hz, 1H), 4.62 (d, J = 2.6 Hz, 4H), 3.20 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.49, 138.18, 129.88, 128.77, 127.92, 122.30, 64.53, 64.40, 64.31, 63.49, 63.47, 31.08, 23.80.

4-bromo-1,2-bis(bromomethyl)benzene (15) Following the procedure described for compound **11**, using compound **14** (1.5 g, 7.0 mmol) as starting material, afforded compound 40 (1.8 g, 74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 (d, J = 2.1 Hz, 1H), 7.43 (dd, J = 8.2, 2.1 Hz, 1H), 7.24 (d, J = 8.2 Hz, 1H), 4.59 (s, 2H), 4.58 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.65, 135.67, 134.02, 132.69, 132.58, 131.24, 129.60, 123.17, 66.00, 42.46, 42.32, 30.14, 29.32, 29.12, 29.00, 28.83, 15.43.

3,3'-(((4-bromo-1,2-phenylene)bis(methylene))bis(oxy))dibenzonitrile (16) Following the procedure as described above for compound **13**, using compound **15** (0.84 g, 2.4 mmol), afforded compound **16** as a crude product. After the precipitation, the weight of the precipitate was too high. The precipitate was then dissolved in DMF, water was added, and the precipitated compound **16** was collected (1.2 g, quant.). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (d, J = 2.1 Hz, 1H), 7.55 (dd, J = 8.1, 2.1 Hz, 1H), 7.44 – 7.34 (m, 4H), 7.31 – 7.25 (m, 3H), 7.20 – 7.14 (m, 2H), 5.12 (d, J = 6.6 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.38, 136.48, 132.95, 132.13, 132.07, 131.07, 130.77, 130.75, 129.53, 129.21, 125.50, 125.44, 123.21, 120.12, 120.11, 118.60, 117.71, 117.70, 113.60, 113.58, 67.95, 67.62.

3,3'-(([1,1'-biphenyl]-3,4-diylbis(methylene))bis(oxy))dibenzonitrile (17) Following the procedure as described above for compound **13**, using compound **16** (150 mg, 0.36 mmol) afforded compound **17** (0.12 g, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (s, 1H), 7.73 – 7.63 (m, 3H), 7.59 – 7.42 (m, 5H), 7.37 – 7.32 (m, 3H), 7.30 – 7.22 (m, 4H), 5.37 – 5.22 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.65, 142.30, 140.16, 134.77, 134.27, 133.08, 130.70, 130.66, 130.17, 129.54, 129.21, 129.07, 128.36, 127.99, 127.78, 127.30, 125.28, 125.25, 125.21, 120.20, 120.16, 118.71, 117.78, 117.75, 113.52, 77.48, 77.16, 76.84, 68.63, 68.55, 68.37.

3,3'-(((4-bromo-1,2-phenylene)bis(methylene))bis(oxy))dibenzimidamide (8b) **C**onditions were based on literature.[38] To a suspension of compound **16** (130 mg, 0.31 mmol) and DIPEA (0.28 mL, 1.6 mmol, 5 eq.) in EtOH (5.0 mL) was added $NH_2OH{\cdot}HCl$ (104 mg, 1.5 mmol, 4.8 eq.). The reaction mixture was stirred at 85 °C overnight. The mixture was concentrated in vacuo and the residue was dissolved in AcOH (3.0 mL) and Ac_2O (0.14 mL, 1.5 mmol, 4.8 eq.) was added. The reaction was stirred overnight and then concentrated in vacuo. The residue was co-evaporated with toluene three times and then suspended in AcOH (4.0 mL) under argon atmosphere. Zinc powder (30 mg, 0.92 mmol, 1.5 eq.) was added and the mixture was stirred at 35 °C for 6 hours. Upon completion, the reaction mixture was filtered through Celite®, the Celite® was rinsed with acetone and all collected fractions were concentrated in vacuo. The crude product purified by preparative HPLC (gradient 30-100%, 30 minutes) to afford the final compound **8b** (14.7 mg, 10%). ^{1}H NMR (400 MHz, DMSO) δ 9.25 (s, 4H), 8.99 (s, 4H), 7.72 (d, J = 2.1 Hz, 1H), 7.62 – 7.40 (m, 6H), 7.40 – 7.32 (m, 4H), 5.28 (d, J = 10.6 Hz, 4H). HRMS (ESI): calculated for $C_{22}H_{21}BrN_4O_2$ [M+H]+ 453.0926, found 453.0919.

3,3'-(([1,1'-biphenyl]-3,4-diylbis(methylene))bis(oxy))dibenzimidamide (9b) Following the procedure as described above for compound **6b**, using compound **17** (110 mg, 0.26 mmol), LHMDS (2.5 mL, 1 M THF solution, 9.4 eq.) and HCl (4.6 mL, 4 M dioxane solution, 68 eq.), afforded the crude product. The crude product was purified using HPLC with a 30-100% gradient for 30 minutes to obtain the pure compound **9b** (64 mg, 53%). ^{1}H NMR (400 MHz, DMSO) δ 9.27 (dd, J = 15.0, 2.7 Hz, 8H), 7.80 (d, J = 1.9 Hz, 1H), 7.68 – 7.54 (m, 4H), 7.49 – 7.38 (m, 6H), 7.37 – 7.28 (m, 5H), 5.32 (d, J = 9.1 Hz, 4H). ^{13}C NMR (101 MHz, DMSO) δ 165.42, 165.39, 158.28, 140.24, 139.43, 135.39, 134.01, 130.45, 129.51, 129.11, 127.87, 127.28, 126.77, 126.64, 120.52, 119.99, 114.78, 114.68, 67.67, 67.31. HRMS (ESI): calculated for $C_{28}H_{26}N_4O_2$ [M+H]+ 451.2135, found 451.2128.

4.2. Antimicrobial assays

All compounds were screened for antimicrobial activity against *S. aureus* ATCC29213. A select group of the pentamidine analogues was further tested against *S. aureus* LIM2, *S. aureus* VRS3b, *S. aureus* MRSA Col, *S. aureus* USA300, *E. faecium* E155, and *E. faecium* 7314. The antimicrobial assay was performed according to CLSI guidelines. Bacteria were plated out directly from their glycerol stocks on blood agar plates, incubated overnight at 37 °C, and then kept in the fridge. The blood agar plates were only used for 2 weeks and then replaced.

4.3. Minimal inhibitory concentration (MIC) assay using Lysogeny Broth (LB)

A single colony from a blood agar plate was inoculated in LB at 37 °C until a 0.5 optical density at 600nm (OD_{600}) was reached (compared to the sterility control of LB). The bacterial suspension was diluted in fresh LB to 2.0 x 10^6 CFU/mL. The serial dilutions were prepared in polypropylene microtiter plates: a stock of the test compounds was prepared with a 2x final concentration in LB. 100 µl of the stock was added to the wells of the top row of which 50 µl was used for the serial dilution. The bottom row of each plate was used as the positive (50 µl of LB) and negative controls (100 µl of LB) (6 wells each). 50 µl of the 2.0 x 10^6 CFU/mL bacterial stock was added to each well except for the negative controls, adding up to a total volume of 100 µl per well. The plates were sealed with a breathable seal and incubated for 20 hours at 37 °C and 600 rpm. The MIC was visually determined after centrifuging the plates for 2 minutes at 3000 rpm.

4.4. MIC using Mueller Hinton Broth (MHB), cation-adjusted MHB (CAMHB), and tryptic soy broth (TSB).

Protocol identical to the MIC assays with LB except for the use of TSB as broth for the inoculation and the use of MHB, CAMHB, or TSB for the bacterial suspensions and serial dilutions.

4.5. Hemolysis assays.

The hemolytic activity of each analogue was assessed in triplicate. Red blood cells from defibrinated sheep blood obtained from Thermo Fisher were centrifuged (400 g for 15 minutes at 4°C) and washed with Phosphate-Buffered Saline (PBS) containing 0.002% Tween20 (buffer) for five times. Then, the red blood cells were normalized to obtain a positive control read-out between 2.5 and 3.0 at 415 nm to stay within the linear range with the maximum sensitivity. A serial dilution of the compounds (128 – 4 μg/mL, 75 μL) was prepared in a 96-well plate. The outer border of the plate was filled with 75 μL buffer. Each plate contained a positive control (0.1% Triton-X final concentration, 75 μL) and a negative control (buffer, 75 μL) in triplicate. The normalized blood cells (75 μL) were added and the plates were incubated at 37 °C for 1 hour or 20 hours while shaking at 500 rpm. After incubation, the plates were centrifuged (800 g for 5 minutes at room temperature). A flat-bottom plate of polystyrene with 100 μL buffer in each well was prepared. To this plate 25 μL of the supernatant was transferred to their respective wells in the flat-bottom plate. The values obtained from a read-out at 415 nm were corrected for background (negative control) and transformed to a percentage relative to the positive control.

Supplementary data

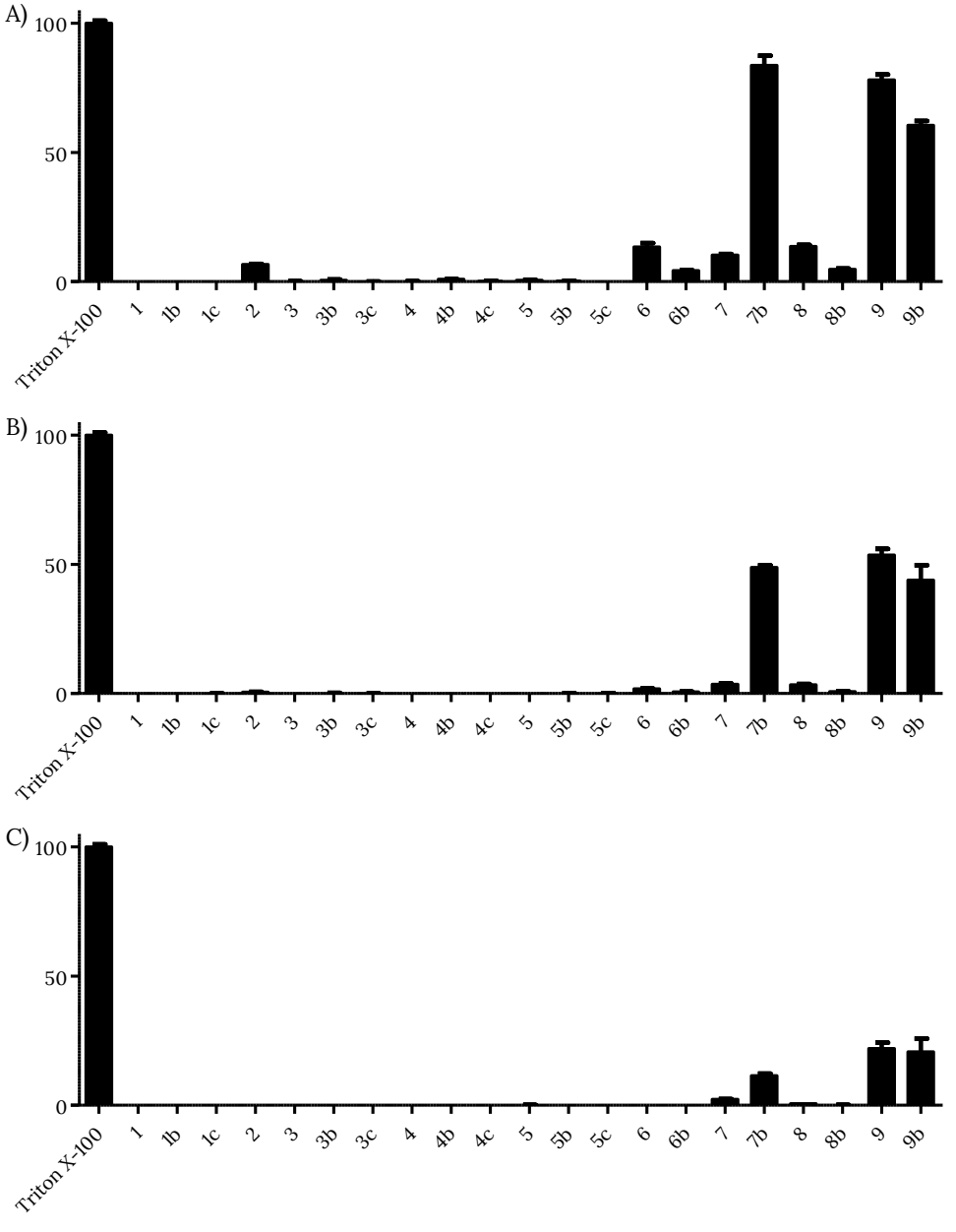


Figure S1. Hemolytic activity of all compounds after 20 hours of incubation. Concentration tested: A) 128 μg/mL; B) 64 μg/mL; C) 32 μg/mL. The hemolysis assay was performed as described in materials and methods. Values below 10% were defined as non-hemolytic.[32] Error bars represent the standard deviation based on n=3 technical replicates.

Table S1. MIC values (μg/ml) of pentamidine analogues in LB broth for *S. aureus* ATCC29213. Hemolytic activity of all compounds (128-32 μg/mL). The hemolysis assay was performed as described in materials and methods. Values below 10% were defined as non-hemolytic (see gray background for ≥ 10%).32 Values are the average of on n=3 technical replicates. The therapeutic window is calculated by dividing the highest non-hemolytic concentration by the MIC.

	MIC (μg/ml)	% Hemolysis (128 μg/ml)	% Hemolysis (64 μg/ml)	% Hemolysis (32 μg/ml)	Therapeutic window
1	8-16	0.0	0.0	0.0	16-8
1b	16	0.0	0.0	0.0	8
1c	>128	0.0	0.0	0.0	-
2	2	6.6	0.6	0.0	64
3	16	0.0	0.0	0.0	8
3b	32	0.5	0.1	0.0	4
3c	>128	0.0	0.0	0.0	-
4	16	0.0	0.0	0.0	8
4b	4	0.9	0.0	0.0	32
4c	128	0.2	0.0	0.0	1
5	8	0.5	0.0	0.1	16
5b	8	0.3	0.1	0.0	16
5c	128	0.1	0.1	0.0	1
6	4	13	1.8	0.0	16
6b	1	4.2	0.6	0.0	128
7	1	10	3.6	2.3	64
7b	0.5	84	49	11	≤32
8	1	14	3.4	0.7	64
8b	1	4.8	0.8	0.0	128
9	1	78	54	22	≤16
9b	4	61	44	21	≤4

Table S2. MIC values (μg/ml) of pentamidine analogues **1-4** and **6-9b** in LB broth for *S. aureus* and *E. faecium* strains. The maximum concentration tested for the bis-amidines was 32 μg/ml and vancomycin 128 μg/ml.

	S. aureus					*E. faecium*	
	ATCC 29213	**LIM2**	**VRS3b**	**COL**	**USA300**	**E155**	**7314**
1	8-16	>64	8	8	16	>64	64
2	2	32	2	2	4	16	16
3	16	>32	8	8	16	>32	>32
4	16	>32	16	16	16	16	32
6	4	16	2	2	2	16	32
6b	1	16	0.5	0.5	2	32	16
7	1	4	0.25	0.5	1	4	16
7b	0.5	4	0.125	0.25	1	8	8
8	1	16	1	1	2	16	16
8b	1	32	2	4	8	32	32
9	1	4	0.25	<0.5	1	2	4
9b	4	8	1	0.5	<2	4	8
Vancomycin	1	4	128	2	<2	>128	128

Table S3. MIC values (μg/ml) of pentamidine analogues **1-4** and **6-9b** in MHB broth for *S. aureus* and *E. faecium* strains. The maximum concentration tested for the pentamidine analogues was 32 μg/ml and vancomycin 128 μg/ml.

	S. aureus					*E. faecium*	
	ATCC 29213	**LIM2**	**VRS3b**	**COL**	**USA300**	**E155**	**7314**
1	2	>32	8	4	8	32	32
2	1	16	2	0.5	1	8	16
3	4	>32	4	4	8	>32	>32
4	4	32	4	4	8	8	>32
6	1	4	0.5	0.5	1	2	8
6b	0.5	8	0.5	0.125	0.5	8	8
7	0.5	2	0.25	0.25	1	2	2
7b	0.5	2	0.25	0.125	0.5	2	2
8	1	4	0.25	0.5	1	4	8
8b	0.5	2	0.5	0.25	1	2	4
9	0.5	2	0.5	0.5	1	1	1
9b	1	8	1	1	2	16	16
Vancomycin	0.5	4	128	2	1	>128	>128

Table S4. MIC values (μg/ml) of pentamidine analogues **1-4** and **6-9b** in CAMHB broth for *S. aureus* and *E. faecium* strains. The maximum concentration tested for the bis-amidines was 32 μg/ml (except for pentamidine with the *E. faecium* strains) and vancomycin 128 μg/ml.

	S. aureus					*E. faecium*	
	ATCC 29213	**LIM2**	**VRS3b**	**MRSA Col**	**USA300**	**E155**	**7314**
1	4	>32	8	4	8	>64	64
2	1	32	1	1	2	16	32
3	8	>32	8	4	8	32	32
4	8	>32	4	4	8	32	32
6	1	8	1	1	1	>32	16
6b	0.5	16	0.5	0.25	0.5	2	16
7	0.5	4	0.5	0.5	1	2	2
7b	0.5	4	0.25	0.5	1	2	4
8	1	16	0.5	0.25	1	8	8
8b	1	32	1	1	2	2	16
9	1	4	0.5	0.5	1	2	2
9b	0.5	4	0.5	0.5	2	1	4
Vancomycin	1	4	128	2	1	>128	>128

Table S5. MIC values (μg/ml) of pentamidine analogues **1-4** and **6-9b** in TSB broth for *E. faecium* E155 and 7314. The maximum concentration tested for the bis-amidines was 128 μg/ml and vancomycin 128 μg/ml.

	E. faecium	
	E155	**7314**
1	128	128
2	>2	>2
3	>128	>128
4	64	128
6	16	32
6b	32	16
7	4	4
7b	4	8
8	16	16
8b	8	8
9	<2	<2
9b	64	32
Vancomycin	64	128

References

(1) Murray, C. J.; Ikuta, K. S.; Sharara, F.; Swetschinski, L.; Aguilar, G. R.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; *et al.* Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis. *The Lancet* **2022**, *399* (10325), 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0.

(2) Laxminarayan, R. The Overlooked Pandemic of Antimicrobial Resistance. *The Lancet* **2022**, 399 (10325), 606–607. https://doi.org/10.1016/S0140-6736(22)00087-3.

(3) O'Neill, J. *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations*; Report; Government of the United Kingdom, 2016.

(4) O'Neill, J. *Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations*; Report; Government of the United Kingdom, 2014.

(5) Thakare, R.; Kaul, G.; Shukla, M.; Kesharwani, P.; Srinivas, N.; Dasgupta, A.; Chopra, S. Chapter 5 - Repurposing Nonantibiotic Drugs as Antibacterials. In *Drug Discovery Targeting Drug-Resistant Bacteria*; Kesharwani, P., Chopra, S., Dasgupta, A., Eds.; Academic Press, 2020; pp 105–138. https://doi.org/10.1016/B978-0-12-818480-6.00005-9.

(6) Farha, M. A.; Brown, E. D. Drug Repurposing for Antimicrobial Discovery. *Nature Microbiology* **2019**, 4 (April), 565–577. https://doi.org/10.1038/s41564-019-0357-1.

(7) Schweizer, F. Repurposing Antibiotics to Treat Resistant Gram-Negative Pathogens. In *Antibiotic Drug Resistance*; John Wiley & Sons, Ltd, 2019; pp 453–476. https://doi.org/10.1002/9781119282549.ch18.

(8) Bean, D. C.; Wareham, D. W. Pentamidine: A Drug to Consider Re-Purposing in the Targeted Treatment of Multi-Drug Resistant Bacterial Infections? *Journal of Laboratory and Precision Medicine* **2017**, 2, 49–49. https://doi.org/10.21037/jlpm.2017.06.18.

(9) Trypanosomiasis, human African (sleeping sickness) https://www.who.int/news-room/fact-sheets/detail/trypanosomiasis-human-african-(sleeping-sickness) (accessed 2022 -02 -15).

(10) Sands, M.; Kron, M. A.; Brown, R. B. Pentamidine: A Review. *Reviews of infectious diseases* **1985**, 7 (5), 625–634. https://doi.org/10.1093/clinids/7.5.625.

(11) Stokes, J. M.; MacNair, C. R.; Ilyas, B.; French, S.; Côté, J.-P.; Bouwman, C.; Farha, M. A.; Sieron, A. O.; Whitfield, C.; Coombes, B. K.; Brown, E. D. Pentamidine Sensitizes Gram-Negative Pathogens to Antibiotics and Overcomes Acquired Colistin Resistance. *Nat Microbiol* **2017**, 2 (5), 1–8. https://doi.org/10.1038/nmicrobiol.2017.28.

(12) Wesseling, C. M. J.; Slingerland, C. J.; Veraar, S.; Lok, S.; Martin, N. I. Structure–Activity Studies with Bis-Amidines That Potentiate Gram-Positive Specific Antibiotics against Gram-Negative Pathogens. *ACS Infect. Dis.* **2021**. https://doi.org/10.1021/acsinfecdis.1c00466.

(13) Herrera-Espejo, S.; Cebrero-Cangueiro, T.; Labrador-Herrera, G.; Pachón, J.; Pachón-Ibáñez, M. E.; Álvarez-Marín, R. In Vitro Activity of Pentamidine Alone and in Combination with Antibiotics against Multidrug-Resistant Clinical Pseudomonas Aeruginosa Strains. *Antibiotics* **2020**, 9 (12), 885. https://doi.org/10.3390/antibiotics9120885.

(14) Wu, C.; Xia, L.; Huang, W.; Xu, Y.; Gu, Y.; Liu, C.; Ji, L.; Li, W.; Wu, Y.; Zhou, K.; Feng, X. Pentamidine Sensitizes FDA-Approved Non-Antibiotics for the Inhibition of Multidrug-Resistant Gram-Negative Pathogens. *Eur J Clin Microbiol Infect Dis* **2020**, 39 (9), 1771–1779. https://doi.org/10.1007/s10096-020-03881-0.

(15) Edwards, K. J.; Jenkins, T. C.; Neidle, S. Crystal Structure of a Pentamidine-Oligonucleotide Complex: Implications for DNA-Binding Properties. *Biochemistry* **1992**, 31 (31), 7104–7109. https://doi.org/10.1021/bi00146a011.

(16) Sun, T.; Zhang, Y. Pentamidine Binds to TRNA through Non-Specific Hydrophobic Interactions and Inhibits Aminoacylation and Translation. *Nucleic Acids Research* **2008**, 36 (5), 1654–1664. https://doi.org/10.1093/nar/gkm1180.

(17) Paracini, N.; Clifton, L. A.; Skoda, M. W. A.; Lakey, J. H. Liquid Crystalline Bacterial Outer Membranes Are Critical for Antibiotic Susceptibility. **2018**. https://doi.org/10.1073/pnas.1803975115.

(18) Cavalier, M. C.; Ansari, M. I.; Pierce, A. D.; Wilder, P. T.; McKnight, L. E.; Raman, E. P.; Neau, D. B.; Bezawada, P.; Alasady, M. J.; Charpentier, T. H.; Varney, K. M.; Toth, E. A.; MacKerell, A. D.; Coop, A.; Weber, D. J. Small Molecule Inhibitors of Ca2+-S100B Reveal Two Protein Conformations. *Journal of Medicinal Chemistry* **2016**, 59 (2), 592–608. https://doi.org/10.1021/acs.jmedchem.5b01369.

(19) Amos, H.; Vollmayer, E. Effect of pentamindine on the growth of Escherichia coli. *J Bacteriol* **1957**, 73 (2), 172–177.

(20) Libman, M. D.; Miller, M. A.; Richards, G. K. Antistaphylococcal Activity of Pentamidine. *Antimicrobial Agents and Chemotherapy* **1990**, 34 (9), 1795–1796. https://doi.org/10.1128/AAC.34.9.1795.

(21) Maciejewska, D.; Zabiński, J.; Kaźmierczak, P.; Wójciuk, K.; Kruszewski, M.; Kruszewska, H. In Vitro Screening of Pentamidine Analogs against Bacterial and Fungal Strains. *Bioorganic and Medicinal Chemistry Letters* **2014**, 24 (13), 2918–2923. https://doi.org/10.1016/j.bmcl.2014.04.075.

(22) Bichowsky-Slomnitzki, L. The Effect of Aromatic Diamidines on Bacterial Growth. *J Bacteriol* **1948**, 55 (1), 27–31.

(23) Rolain, J.-M.; Fenollar, F.; Raoult, D. In Vitro Activity of Pentamidine against Tropheryma Whipplei. *International Journal of Antimicrobial Agents* **2011**, 38 (6), 545–547. https://doi.org/10.1016/j.ijantimicag.2011.07.015.

(24) Minnick, M. F.; Hicks, L. D.; Battisti, J. M.; Raghavan, R. Pentamidine Inhibits Coxiella Burnetii Growth and 23S RRNA Intron Splicing in Vitro. *International Journal of Antimicrobial Agents* **2010**, 36 (4), 380–382. https://doi.org/10.1016/j.ijantimicag.2010.05.017.

(25) Waller, D. G.; Sampson, A. P. Chemotherapy of Infections. In *Medical pharmacology and therapeutics*; Elsevier, 2018; pp 581–629. https://doi.org/10.1016/B978-0-7020-7167-6.00051-8.

(26) Goa, K. L.; Campoli-Richards, D. M. Pentamidine Isethionate: A Review of Its Antiprotozoal Activity, Pharmacokinetic Properties and Therapeutic Use in Pneumocystis Carinii Pneumonia. *Drugs* **1987**, 33 (3), 242–258. https://doi.org/10.2165/00003495-198733030-00002.

(27) Andreana, I.; Bincoletto, V.; Milla, P.; Dosio, F.; Stella, B.; Arpicco, S. Nanotechnological Approaches for Pentamidine Delivery. *Drug Deliv. and Transl. Res.* **2022**. https://doi.org/10.1007/s13346-022-01127-4.

(28) Liu, Y.; Hu, X.; Wu, Y.; Zhang, W.; Chen, X.; You, X.; Hu, L. Synthesis and Structure-Activity Relationship of Novel Bisindole Amidines Active against MDR Gram-Positive and Gram-Negative Bacteria. *European Journal of Medicinal Chemistry* **2018**, 150, 771–782. https://doi.org/10.1016/j.ejmech.2018.03.031.

(29) Donkor, I. O.; Clark, A. M. In Vitro Antimicrobial Activity of Aromatic Diamidines and Diimidazolines Related to Pentamidine. *European Journal of Medicinal Chemistry* **1999**, 34 (7), 639–643. https://doi.org/10.1016/S0223-5234(00)80032-X.

(30) Bistrović, A.; Krstulović, L.; Stolić, I.; Drenjančević, D.; Talapko, J.; Taylor, M. C.; Kelly, J. M.; Bajić, M.; Raić-Malić, S. Synthesis, Anti-Bacterial and Anti-Protozoal Activities of Amidinobenzimidazole Derivatives and Their Interactions with DNA and RNA. *Journal of Enzyme Inhibition and Medicinal Chemistry* **2018**, 33 (1), 1323–1334. https://doi.org/10.1080/14756366.2018.1484733.

(31) WHO. Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics. **2017**, 7.

(32) Amin, K.; Dannenfelser, R.-M. In Vitro Hemolysis: Guidance for the Pharmaceutical Scientist. *Journal of Pharmaceutical Sciences* **2006**, 95 (6), 1173–1176. https://doi.org/10.1002/JPS.20627.

(33) van Groesen, E.; Slingerland, C. J.; Innocenti, P.; Mihajlovic, M.; Masereeuw, R.; Martin, N. I. Vancomycins: Vancomycin-Polymyxin Nonapeptide Conjugates That Retain Anti-Gram-Positive Activity with Enhanced Potency against Gram-Negative Strains. *ACS Infect. Dis.* **2021**, 7 (9), 2746–2754. https://doi.org/10.1021/acsinfecdis.1c00318.

(34) Kong, N.; Liu, E. A.; Vu, B. T. Cis-Imidazolines. U.S. patent US 6617346 B1, 2003.

(35) Huang, H.; Li, H.; Martásek, P.; Roman, L. J.; Poulos, T. L.; Silverman, R. B. Structure-Guided Design of Selective Inhibitors of Neuronal Nitric Oxide Synthase. *Journal of Medicinal Chemistry* **2013**, *56* (7), 3024–3032. https://doi.org/10.1021/jm4000984.

(36) Gromyko, A. V.; Popov, K. V.; Mosoleva, A. P.; Streltsov, S. A.; Grokhovsky, S. L.; Oleinikov, V. A.; Zhuze, A. L. DNA Sequence-Specific Ligands: XII. Synthesis and Cytological Studies of Dimeric Hoechst 33258 Molecules. *Russian Journal of Bioorganic Chemistry* **2005**, *31* (4), 344–351. https://doi.org/10.1007/s11171-005-0047-z.

(37) García, D.; Foubelo, F.; Yus, M. Selective Lithiation of 4- and 5-Halophthalans. *Heterocycles* **2009**, *77* (2), 991–1005. https://doi.org/10.3987/COM-08-S(F)85.

(38) Goswami, R.; Mukherjee, S.; Wohlfahrt, G.; Ghadiyaram, C.; Nagaraj, J.; Chandra, B. R.; Sistla, R. K.; Satyam, L. K.; Samiulla, D. S.; Moilanen, A.; Subramanya, H. S.; Ramachandra, M. Discovery of Pyridyl Bis(Oxy)Dibenzimidamide Derivatives as Selective Matriptase Inhibitors. *ACS Medicinal Chemistry Letters* **2013**, *4* (12), 1152–1157. https://doi.org/10.1021/ml400213v.

(39) Suzuki, A. Organoborane Coupling Reactions (Suzuki Coupling). *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences* **2004**, *80* (8), 359–359.

(40) Martino, G.; Muzio, L.; Riva, N.; Gornati, D.; Seneci, P.; Eleuteri, S. Aminoguanidine Hydrazones as Retromer Stabilizers Useful for Treating Neurological Diseases. WO 2020/201326 A1, 2020.

(41) Innocenti, P.; Woodward, H.; O'Fee, L.; Hoelder, S. Expanding the Scope of Fused Pyrimidines as Kinase Inhibitor Scaffolds: Synthesis and Modification of Pyrido[3,4-d]Pyrimidines. *Organic & Biomolecular Chemistry* **2014**, *13* (3), 893–904. https://doi.org/10.1039/C4OB02238F.

(42) G C, B.; Sahukhal, G. S.; Elasri, M. O. Role of the MsaABCR Operon in Cell Wall Biosynthesis, Autolysis, Integrity, and Antibiotic Resistance in Staphylococcus Aureus. *Antimicrobial Agents and Chemotherapy* *63* (10), e00680-19. https://doi.org/10.1128/AAC.00680-19.

(43) Samanta, D.; Elasri, M. O. The MsaABCR Operon Regulates Resistance in Vancomycin-Intermediate Staphylococcus Aureus Strains. *Antimicrobial Agents and Chemotherapy* **2014**, *58* (11), 6685–6695. https://doi.org/10.1128/AAC.03280-14.

(44) Sieradzki, K.; Tomasz, A. Alterations of Cell Wall Structure and MetabolismAccompany Reduced Susceptibility to Vancomycin in an IsogenicSeries of Clinical Isolates of Staphylococcusaureus. *Journal of Bacteriology* **2003**, *185* (24), 7103–7110. https://doi.org/10.1128/JB.185.24.7103-7110.2003.

(45) Schindler, B. D.; Kaatz, G. W. Multidrug Efflux Pumps of Gram-Positive Bacteria. *Drug Resistance Updates* **2016**, *27*, 1–13. https://doi.org/10.1016/j.drup.2016.04.003.

(46) Costa, S. S.; Viveiros, M.; Amaral, L.; Couto, I. Multidrug Efflux Pumps in Staphylococcus Aureus: An Update. *Open Microbiol J* **2013**, *7*, 59–71. https://doi.org/10.2174/1874285801307010059.

(47) Paulsen, I. T.; Brown, M. H.; Skurray, R. A. Characterization of the Earliest KnownStaphylococcus Aureus Plasmid Encoding a Multidrug Efflux System. *Journal of Bacteriology* **1998**, *180* (13), 3477–3479. https://doi.org/10.1128/JB.180.13.3477-3479.1998.

(48) Mitchell, B. A.; Brown, M. H.; Skurray, R. A. QacA Multidrug Efflux Pump FromStaphylococcus Aureus: Comparative Analysis of Resistance to Diamidines, Biguanidines, and Guanylhydrazones. *Antimicrobial Agents and Chemotherapy* **1998**, *42* (2), 475–477. https://doi.org/10.1128/AAC.42.2.475.

(49) Hassan, K. A.; Skurray, R. A.; Brown, M. H. Transmembrane Helix 12 of the Staphylococcus Aureus Multidrug Transporter QacA Lines the Bivalent Cationic Drug Binding Pocket. *Journal of Bacteriology* **2007**, *189* (24), 9131–9134. https://doi.org/10.1128/JB.01492-07.

(50) Hsieh, P.-C.; Siegel, S. A.; Rogers, B.; Davis, D.; Lewis, K. Bacteria Lacking a Multidrug Pump: A Sensitive Tool for Drug Discovery. *PNAS* **1998**, *95* (12), 6602–6606. https://doi.org/10.1073/pnas.95.12.6602.

(51) Chan, K.-F.; Zhao, Y.; Chow, L. M. C.; Chan, T. H. Synthesis of (±)-5′-Methoxyhydnocarpin-D, an Inhibitor of the Staphylococcus Aureus Multidrug Resistance Pump. *Tetrahedron* **2005**, *61* (16), 4149–4156. https://doi.org/10.1016/j.tet.2005.02.024.

(52) Dashtbani-Roozbehani, A.; Brown, M. H. Efflux Pump Mediated Antimicrobial Resistance by Staphylococci in Health-Related Environments: Challenges and the Quest for Inhibition. *Antibiotics* **2021**, *10* (12), 1502. https://doi.org/10.3390/antibiotics10121502.

(53) Rizzotti, L.; Rossi, F.; Torriani, S. Biocide and Antibiotic Resistance of Enterococcus Faecalis and Enterococcus Faecium Isolated from the Swine Meat Chain. *Food Microbiology* **2016**, *60*, 160–164. https://doi.org/10.1016/j.fm.2016.07.009.

(54) Alotaibi, S. M. I.; Ayibiekea, A.; Pedersen, A. F.; Jakobsen, L.; Pinholt, M.; Gumpert, H.; Hammerum, A. M.; Westh, H.; Ingmer, H. 2017. Susceptibility of Vancomycin-Resistant and -Sensitive Enterococcus Faecium Obtained from Danish Hospitals to Benzalkonium Chloride, Chlorhexidine and Hydrogen Peroxide Biocides. *Journal of Medical Microbiology* 66 (12), 1744–1751. https://doi.org/10.1099/jmm.0.000642.

(55) Mayer, S.; Boos, M.; Beyer, A.; Fluit, A. C.; Schmitz, F.-J. Distribution of the Antiseptic Resistance Genes QacA, QacB and QacC in 497 Methicillin-Resistant and -Susceptible European Isolates of Staphylococcus Aureus. *Journal of Antimicrobial Chemotherapy* **2001**, 47 (6), 896–897. https://doi.org/10.1093/jac/47.6.896.

(56) Huang, H.; Li, H.; Martásek, P.; Roman, L. J.; Poulos, T. L.; Silverman, R. B. Structure-Guided Design of Selective Inhibitors of Neuronal Nitric Oxide Synthase. *Journal of Medicinal Chemistry* **2013**, 56 (7), 3024–3032. https://doi.org/10.1021/jm4000984.

(57) Bruncko, M.; McClellan, W. J.; Wendt, M. D.; Sauer, D. R.; Geyer, A.; Dalton, C. R.; Kaminski, M. A.; Weitzberg, M.; Gong, J.; Dellaria, J. F.; Mantei, R.; Zhao, X.; Nienaber, V. L.; Stewart, K.; Klinghofer, V.; Bouska, J.; Rockway, T. W.; Giranda, V. L. Naphthamidine Urokinase Plasminogen Activator Inhibitors with Improved Pharmacokinetic Properties. *Bioorganic and Medicinal Chemistry Letters* **2005**, *15* (1), 93–98. https://doi.org/10.1016/j.bmcl.2004.10.026.

(58) Zhang, J.; Qian, K.; Yan, C.; He, M.; Jassim, B. A.; Ivanov, I.; Zheng, Y. G. Discovery of Decamidine as a New and Potent PRMT1 Inhibitor. *MedChemComm* **2017**, 8 (2), 440–444. https://doi.org/10.1039/c6md00573j.

(59) Wendt, M. D.; Rockway, T. W.; Geyer, A.; McClellan, W.; Weitzberg, M.; Zhao, X.; Mantei, R.; Nienaber, V. L.; Stewart, K.; Klinghofer, V.; Giranda, V. L. Identification of Novel Binding Interactions in the Development of Potent, Selective 2-Naphthamidine Inhibitors of Urokinase. Synthesis, Structural Analysis, and SAR of N-Phenyl Amide 6-Substitution. *Journal of Medicinal Chemistry* **2004**, 47 (2), 303–324. https://doi.org/10.1021/jm0300072.

(60) Abou-Elkhair, R. A. I.; Hassan, A. E. A.; Boykin, D. W.; Wilson, W. D. Lithium Hexamethyldisilazane Transformation of Transiently Protected 4-Aza/Benzimidazole Nitriles to Amidines and Their Dimethyl Sulfoxide Mediated Imidazole Ring Formation. *Organic Letters* **2016**, 18 (18), 4714–4717. https://doi.org/10.1021/acs.orglett.6b02359.

(61) García, D.; Foubelo, F.; Yus, M. Regioselective Reductive Opening of Substituted Phthalans: Synthetic Applications. *Tetrahedron* **2008**, 64 (19), 4275–4286. https://doi.org/10.1016/j.tet.2008.02.072.