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

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Chapter 6

Summary and Future Outlook

1. Summary

Extending our current arsenal of antibiotics is key in winning the arms-race between humans and resistant bacteria. Classes of antibiotics otherwise limited to the treatment of Gram-positive pathogens may be potentiated to Gram-negative bacteria by disruption of their additional outer membrane. The work described in this thesis focusses on the development of novel synergists designed to selectively disrupt the outer membrane of Gram-negative bacteria.

In **Chapter 1** an overview is presented of the current literature on outer membrane disrupting synergistic compounds. This overview includes linear and cyclic cationic peptides, positively charged peptide-mimics, small molecules, either chelating, lipophilic, or positively charged, cationic steroids, and derivatives of aminoglycoside antibiotics. Synergy is defined by a fractional inhibitory concentration index (FICI) of ≤0.5 (Equation 1). The FICI functions as a numerical representation of potentiation: the lower the number, the stronger the synergistic combination. However, the drawback of focusing on FICI is that one loses sight of the concentration at which the synergy occurs. Selectivity for the outer membrane over membranes of human cells is vital and often overlooked in literature reports. A red blood cell based hemolysis assay can provide insight into this selectivity. This review aims to provide an assessment of the body of literature which facilitates the comparison of published synergistic agents and therefore aid the optimization and development of synergists.

$$\mathrm{FICI} = \frac{\mathrm{MSC_{ant}}}{\mathrm{MIC_{ant}}} + \frac{\mathrm{MSC_{syn}}}{\mathrm{MIC_{syn}}} \quad (1)$$

Equation 1. Calculation of FICI. MSC_{ant} = MIC of antibiotic in combination with synergist; MIC_{ant} = MIC of antibiotic alone; MSC_{syn} = MIC of synergist in combination with antibiotic; MIC_{syn} = MIC of synergist alone.

In **Chapter 2** the synergistic potential of bis-amidines was explored. Pentamidine, an anti-parasitic drug, has been reported to potentiate Gram-positive specific antibiotics against Gram-negative bacteria and a limited structure-activity relationship (SAR) study was described using a commercially available set of pentamidine analogues. In **Chapter 2**, this SAR was expanded with synthesized bis-amidines allowing for specific modifications to be compared. A screening using checkerboard assays revealed hits with improved FICI values. However, a hemolysis assay revealed that the most potent hits resulted in high percentages of hemolysis. Our focus shifted towards bis-amidines **21** and **22** containing a xylene linker which showed no hemolytic activity (Figure 1). A wide range of potentiation was confirmed by screening of compounds **21** and **22** in combination with several antibiotics: erythromycin, rifampicin, vancomycin, and novobiocin. Synergy was first assessed in *E. coli* and subsequently established for *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and resistant *E. coli* strains.

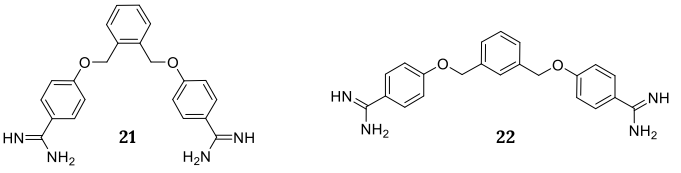


Figure 1. Molecular structures of the most potent, non-hemolytic bis-amidines **21** and **22**

Thrombin-derived peptides have been reported to bind to lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria. We hypothesized that LPS-binding results in outer membrane disruption by these peptides as seen for other synergists. In **Chapter 3** screening of four thrombin-derived peptides confirmed this hypothesis through potentiation of erythromycin and rifampicin against *E. coli*. Optimization of the lead peptide was achieved through amidation of the C-terminus followed by an alanine scan. Of the 12 alanine scan peptides, the peptides with the highest synergy and lowest hemolytic activity are presented in Table 1. The range of potentiation was investigated as reported for the bis-amidines in **Chapter 2**. Again, the synergistic activity for the optimized peptides extended to multiple antibiotics, resistant *E. coli* strains and other Gram-negative bacteria.

Table 1. Overview of the thrombin-derived peptides sequence, synergistic and hemolytic activity.

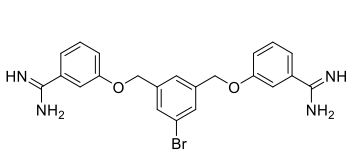
Peptide	Peptide sequence	MIC[a]	$MSC_{peptide}$[b]	FICI[c]	Hemolysis[d]
6	H_2N-VFRLKKWIQKVI-NH_2	12.5	3.13	0.313	4%
14	H_2N-VFRLKK**A**IQKVI-NH_2	>200	6.25	0.078	1%
19	H_2N-VFRLKKWIQKV**A**-NH_2	100	3.13	0.094	1%

[a]Minimum inhibitory concentration (MIC); [b]Minimum synergistic concentration (MSC); [c]Fractional inhibitory concentration index (FICI); [d]Hemolysis after 20 hours of incubation of the compounds (200 μg/ml) with defibrinated sheep blood.

Recently, human serum was reported to potentiate both vancomycin and nisin against Gram-negative bacteria. The membrane-attack complex (MAC) present in human serum was found to be responsible for this potentiation through pore formation in bacterial membranes. This finding was further supported by the loss of synergy upon inhibition of MAC. The potentiation of other Gram-positive specific antibiotics by human serum was explored in **Chapter 4**. The inner membrane permeability assay produced nisin and vancomycin as hits against *E. coli*, while only modest activity was observed for daptomycin and the other glycopeptide antibiotics tested; telavancin, oritavancin, and dalbavancin. The bacterial viability assay functioned as a validation of these hits: the viability of *E. coli* was significantly reduced by nisin, vancomycin, and dalbavancin in the presence of serum. The bacterial viability assay also revealed other antibiotics with a significant effect on bacterial viability on *E. coli* in combination with serum: erythromycin, quinupristin & dalfopristin, and rifampicin. Unfortunately, the serum concentrations employed resulted in a reduction of viability for *K. pneumoniae* and *P. aeruginosa*, which overshadowed the results of the antibiotics. Prolonged exposure or

high concentration of human serum leads to inner membrane permeabilization and subsequent bactericidal activity by serum alone. Therefore, further optimization of the serum concentration would be recommended. Still, the current data clearly indicates that nisin, rifampicin, and vancomycin are potentiated against *K. pneumoniae* and nisin, quinupristin & dalfopristin, rifampicin, and vancomycin synergize with serum against *P. aeruginosa.*

Pentamidine has been reported as moderately active against Gram-positive bacteria. Therefore, the bis-amidines described in **Chapter 2** were screened for their antibacterial activity against Gram-positive bacteria in **Chapter 5**. The lowest minimum inhibitory concentration (MIC) observed was 0.25 μg/mL. However, like in **Chapter 2**, the most potent bis-amidines were found to be hemolytic. The range of hemolytic activity was quite broad even though several bis-amidines had similar lipophilic structures. To investigate whether the orientation of the linker compared to the amidine-position could be responsible for the difference in hemolysis, a set of bis-amidines was synthesized with altered amidine positions. Of the four novel bis-amidines synthesized, bis-amidine **6b** was found to be non-hemolytic whilst retaining activity (Figure 2). Together with the slightly hemolytic bis-amidine **7**, the antibacterial activity was evaluated against multiple Gram-positive strains in different growth media. Overall, bis-amidine **7** was found to be more potent than compound **6b**. A therapeutic window has been proposed based on the large difference between the concentration resulting in hemolysis (128 μg/mL) and the MIC values (0.25-4 μg/mL) for bis-amidine **7**.



6b

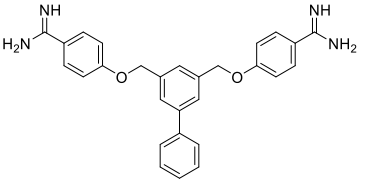


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Figure 2. Chemical structures of the most potent bis-amidines **6b** and **7**

2. Future outlook

1.1. Bis-amidines

Both SARs performed on the bis-amidines have revealed clear and important trends; their hemolytic activity is driven by the compounds' lipophilicity as well as the orientation of the linker and positioning of the amidine moiety. Using CLogP as a measure of lipophilicity, the CLogP values of the bis-amidines were plotted against both the FICI values and the percentage of hemolysis (Figure 3).

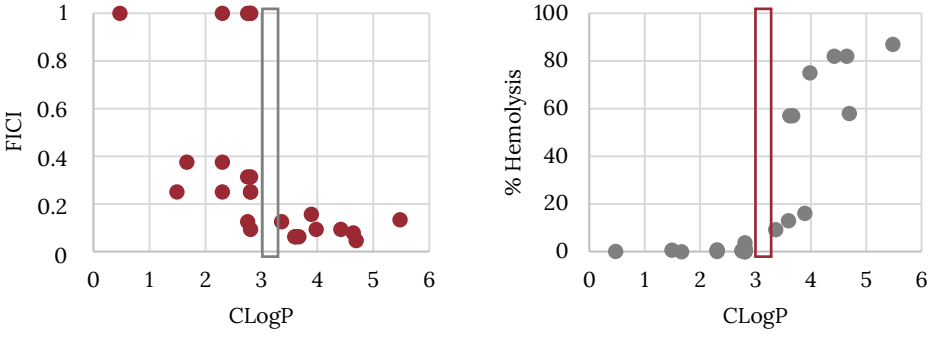

Figure 3. CLogP values of screened bis-amidines plotted vs. the FICI values or hemolysis percentages

The graphs indicate that a CLogP of 3.0 to 3.3 could be the ideal lipophilicity for minimal hemolysis and potent synergy. The lipophilicity of lead compound **22** (CLogP 2.809, Figure 1) could be slightly increased by the introduction of a methyl group (Figure 4A). The ideal position of the methyl group can be investigated through the synthesis of three analogues and, if successful, the same approach could be applied to the other two lead compounds: bis-*para*-amidine **21** (Figure 1) and bis-*meta*-amidine **23b** (**Chapter 2**).

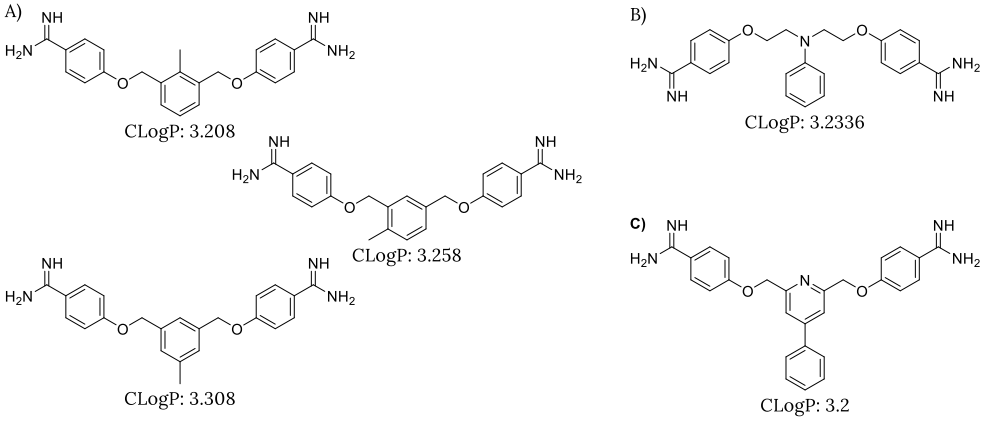

Figure 4. Structures of proposed bis-amidines and their ClogP values calculated by Chemdraw. A) Proposed methylation of compound **22** (Figure 1) to increase its lipophilicity; B) Optimized lead by the Brown group; C) Introduction of the nitrogen group in compound **7** (Figure 3) to reduce its lipophilicity.

A recent paper by the Brown group, which initially reported the synergistic potential of pentamidine, revealed a new lead compound based off of their original SAR study (Figure 4B).[1,2] The introduction of a nitrogen in the lead compound reduces its lipophilicity. In our own SAR study we had reported the parent compound of this analogue to be hemolytic (**Chapter 2**), while the new analogue was reported to be non-hemolytic (after 45 minutes of incubation).[1] The CLogP value calculated for this compound is 3.2, well within the proposed optimal range proposed and the nitrogen could also be implemented in our most potent, but hemolytic, bis-amidine **7** (Figure 2 and 4C).

While bis-amidines represent interesting leads as antibiotic synergists, the clinical history of pentamidine offers important insights into the side-effects, nephrotoxicity, hypotension, and hypoglycaemia associated with such compounds.[3-5] In addition to standard cytotoxicity screens (i.e. with HEK293 cells), the ADME and PK profile of the new analogues will certainly also need to be determined. Recent work from the Brown group provides not only a convenient ADME screening roadmap, but also assays tailored for the specific side-effects of pentamidine (QT prolongation and cell cycle arrest with HepG2 cells).[1] The PK profile of the new bis-amidines is also highly relevant since the clinical efficacy is dependent on how well this profile matches that of the antibiotics it potentiates. In literature *in vivo* potentiation of novobiocin with both pentamidine and the new lead compound has been established in mouse models.[1,2] It could be predicted that other structurally similar bis-amidines are likely to exhibit similar PK profiles.

1.2. Synergistic peptides

The original paper describing thrombin-derived peptides as LPS-binding is part of the research field focusing on sepsis and endotoxin-neutralization.[6] LPS is an endotoxin, binding LPS can lead to endotoxin-neutralization, and sepsis research could therefore provide a wealth of new leads for outer membrane disrupting synergists. Vice-versa, this field could also benefit from the LPS-binding outer membrane synergists reported.

The thrombin-derived peptides reported in **Chapter 3** can be further optimized by investigating truncations of the C- and N-termini and preparation of the mirror-image enantiomers using D-amino acids as D-peptides are more resistant to proteolysis.

Another experimental approach could be a systematic modification of the peptide sequence: to evaluate which set of amino acids needs to be kept intact as a group (for example, from VF**RLKKW**IQKVI to IVF**RLKKW**IQKV). A strongly synergistic peptide with a similar sequence was reported by the Kuipers group[7] in which the RLKKW sequence shifted towards the C-terminal end of the peptide (Table 2).

Table 2. Overview of the thrombin-and cathelicidin-derived peptides sequences.

	Peptide sequence
6	H_2N-VF**RLKKW**IQKVI-NH_2
L-11	H_2N-RIVQ**RIKKW**LR-NH_2

While peptide based synergists have in some cases shown potent *in vitro* activity, their *in vivo* efficacy is hampered by degradation and rapid clearance. In this regard, serum/plasma stability assays can provide insight into the stability and the sites of proteolytic degration. Given the clinical potential of peptide-based therapeutics, a variety of modification have been investigated as a means of improving their stability. These strategies include replacing specific L-amino acids with D-amino acids, capping the termini, as well as completely transforming the peptide to its mirror image as was reported for **L-11**.[7-14] The plasma stability of this peptide was greatly improved upon transforming this peptide to its mirror image **D-11**.[7] Notably, while the mirror image form of our most potent peptide synergist **6** was not synergistic (see **Chapter 3**), the enantiomeric peptide did display antimicrobial activity suggesting the possibility to further optimize its inherent and/or synergistic activity.

1.3. Synergy with human serum

As described in both the summary and in **Chapter 4**, the optimal serum concentration for *K. pneumoniae* and *P. aeruginosa* needs further investigation. Subsequently, the screening of antibiotics and synergists can be repeated with these two strains. In addition, preliminary data was obtained for *A. baumannii* indicating that 25% of serum could potentiate nisin, although the data was hard to reproduce and the serum concentration needs further optimization.

With an optimal serum concentration established, the screening could be expanded to include more Gram-positive specific antibiotics. For example, from the macrolides only erythromycin was screened, while reports of potentiation of clarithromycin by chemical outer membrane disruptors has been reported.[15] This could result in new hits, as the potentiation of antibiotics by human serum is not equal for every member of an antibiotic class. This is evident from the data obtained for several glycopeptides: while dalbavancin and vancomycin resulted in a significant reduction in viability for *E. coli* in the presence of serum, no effect was observed for telavancin and oritavancin.

After screening with a fixed concentration of antibiotic, the antibiotic concentration required for potentiation can be investigated and optimized. The screenings could also be followed-up with time-kill assays. This would provide insight into the bacteriostatic or bactericidal activity of the serum and antibiotic combination. Resistance assay are also recommended as the resistance against the Gram-positive antibiotics is only known for Gram-positive species.

Traditional antibiotic susceptibility assays have generally neglected to consider the potential for serum proteins, including those of the innate immune system, to enhance the activity of antibiotics. While the *in vitro* research in **Chapter 4** points toward this intriguing possibilty, it remains to be seen if *in vivo* assays would corroborate this synergistic effect, since the synergy is dependent on a window of time and serum concentration. Furthermore, it should be noted that the synergistic effect is limited to bacterial cells that are in contact with serum. With this in mind, the formation of biofilms, known to reduce the efficacy of antibiotics, could also limit the synergistic activity of serum. Further studies are needed to more fully elucidate the extent to which serum proteins can effectively potentiate antibiotics.

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