AMPHIPHILIC MEMBRANE-ACTIVE PEPTIDES: BROAD-SPECTRUM ANTIBACTERIAL ACTIVITY ALONE AND IN COMBINATION WITH ANTIBIOTICS AND STRUCTURAL INSIGHTS

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Way2Drug PREDICTIVE SERVICES
Understanding Chemical-Biological Interactions

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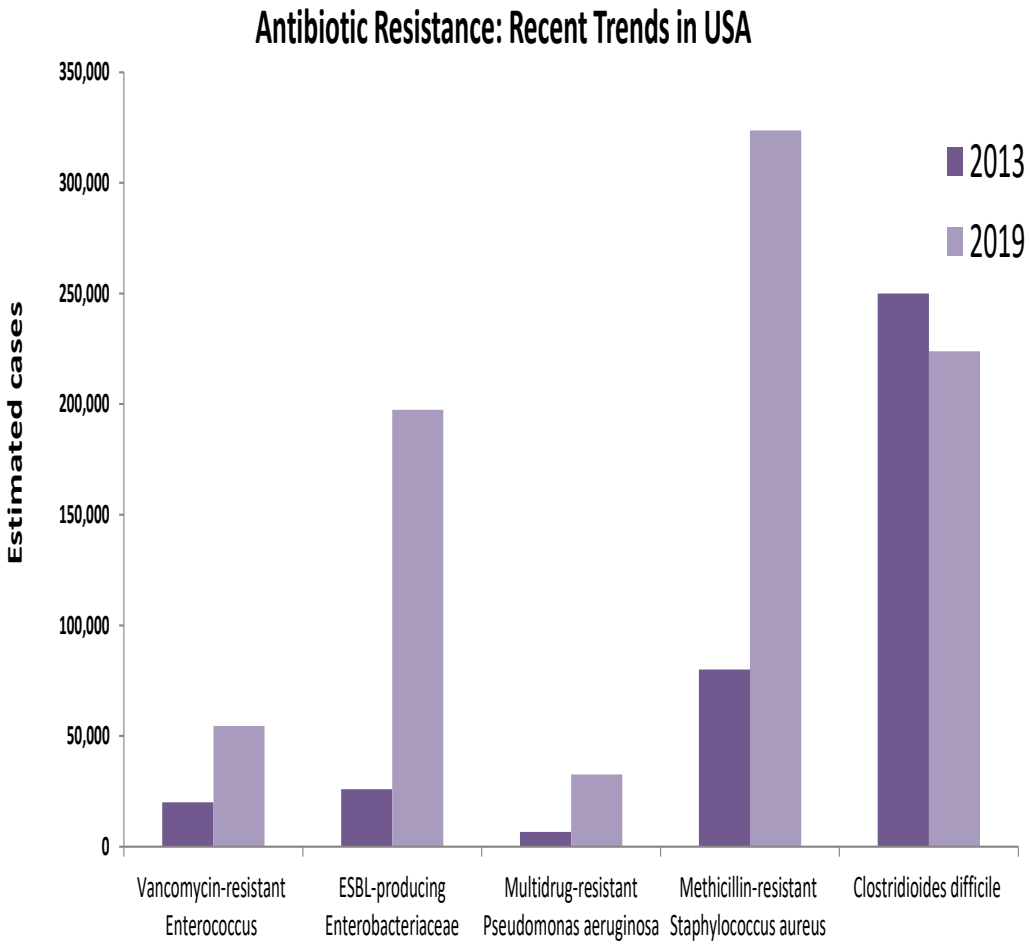
Antibiotic resistance remains a threat to global health

- Infections with drug-resistant bacteria lead to longer and more costly hospital care.
- >2 million antimicrobial-resistant infections and over 35,000 deaths in the US
- >700,000 antimicrobial-resistant infections worldwide
- **Silent Pandemic:** Estimated the death of 10 million people a year by 2050.

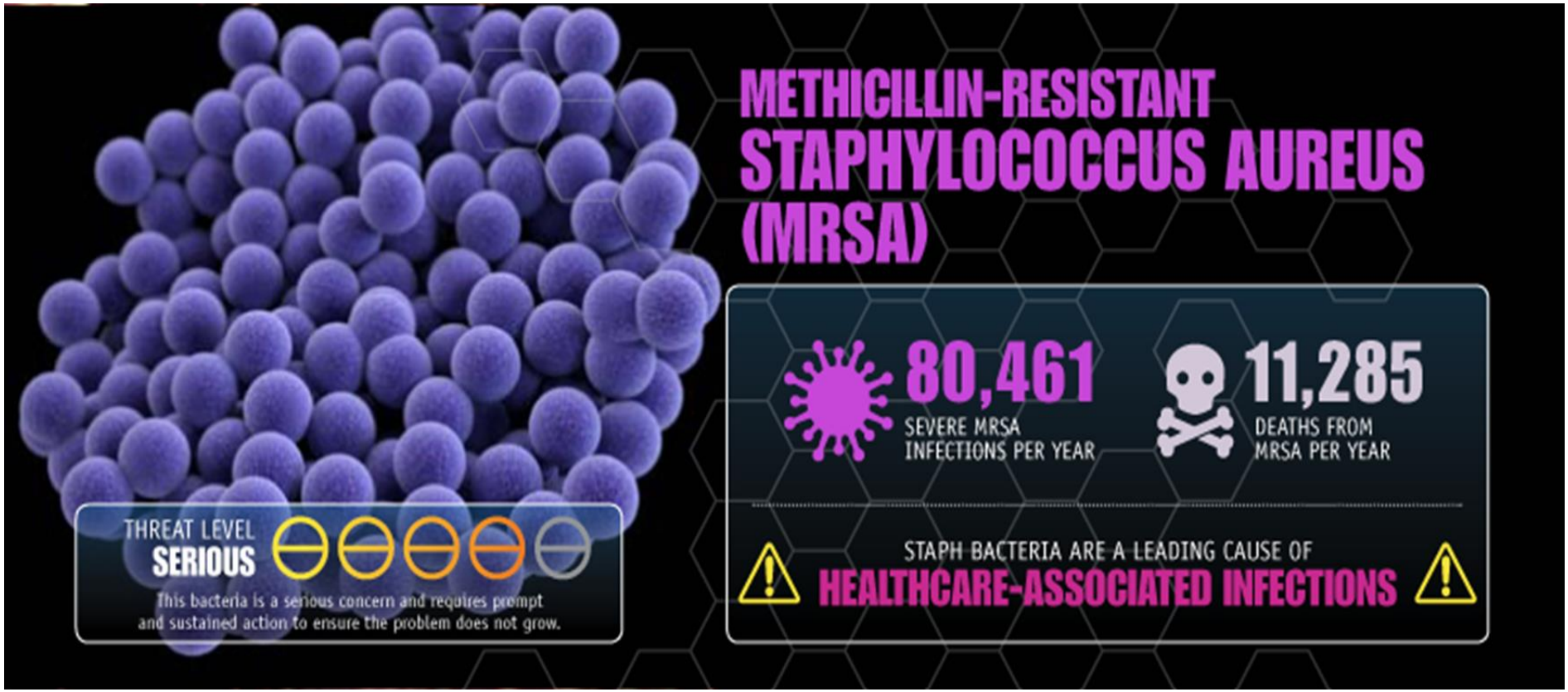


Global action plan on antimicrobial agent WHO publication 2015

Source: CDC. Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019

Antimicrobial Drug-Resistance in "ESKAPE" Pathogens

Enterococcus faecium
Methicillin- resistant Staphylococcus aureus (MRSA)
Acinetobacter baumannii
Klebsiella pneumoniae
Pseudomonas aeruginosa
Escherichia coli

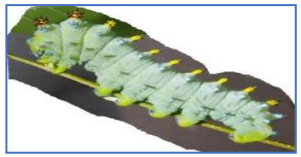


Worldwide hospital anti-MRSA antibiotic market estimated to be worth $2.6B-Source IQVIA Dec2020

Antimicrobial Peptides (AMPs)

Native source

- More than 2,000 AMPs have been isolated from various forms of life



Insects (Cecropin, 1981)

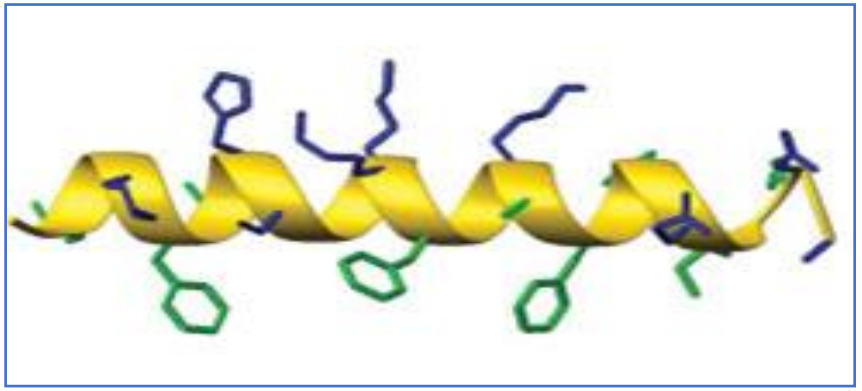
Frogs (Maganin, 1987)

Human (LL-37, 1991)

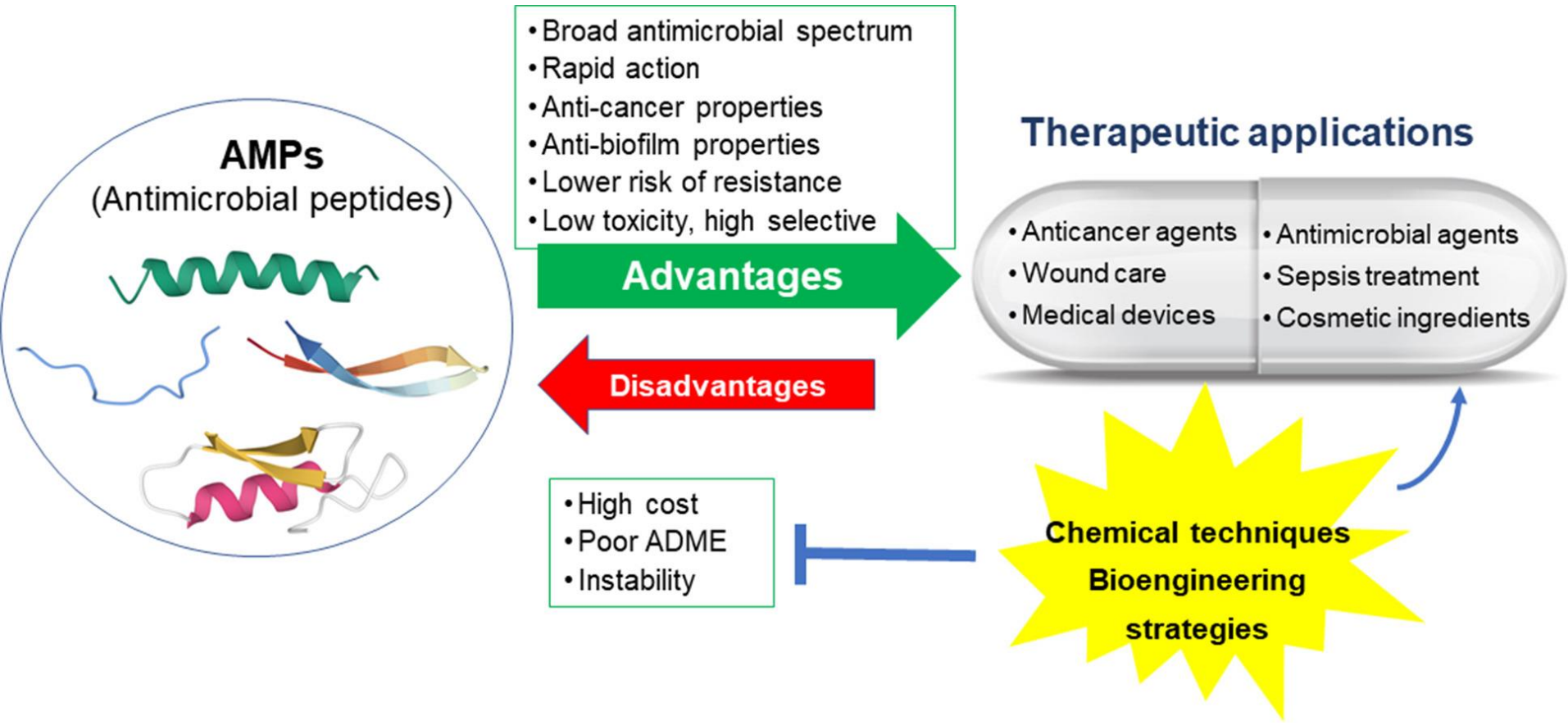
Plants

Structural features

- Relatively large (12 to 50 amino acids)
- Cationic charge (+2 to +9)
- Hydrophobicity (generally >50%).
- Amphipathic conformation

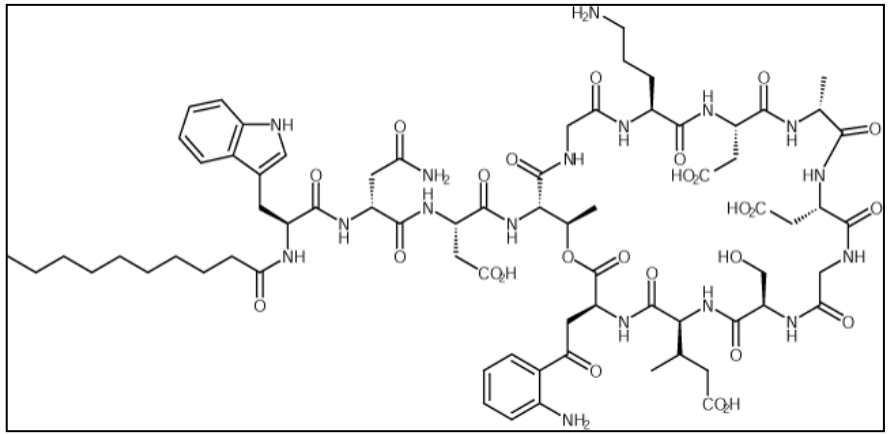


Pros and Cons of AMPs



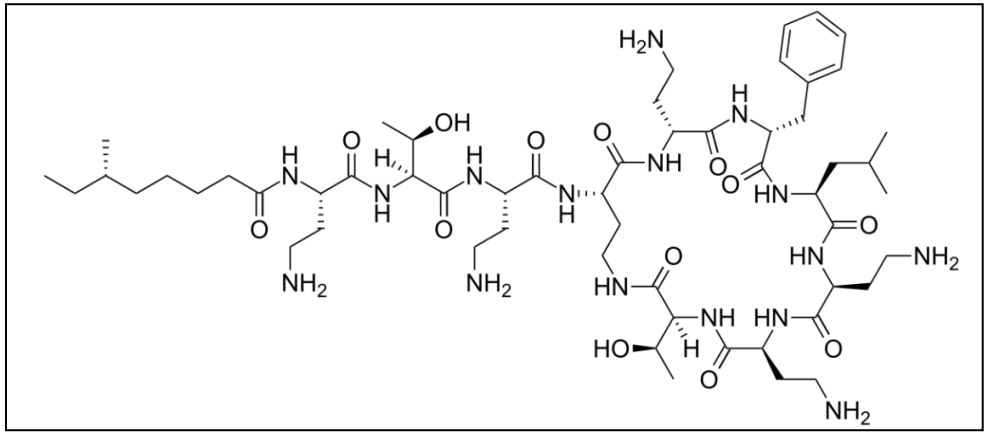
Luong et al., Life Sciences *260* **(2020)**, 118407.

AMPs in the Clinical Use



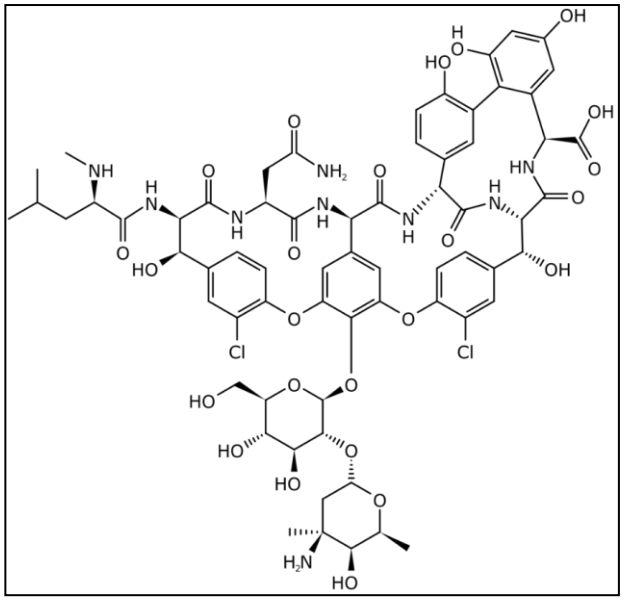
Daptomycin

Cubicin® is a lipopolypeptide obtained from *Streptomyces roseosporus*



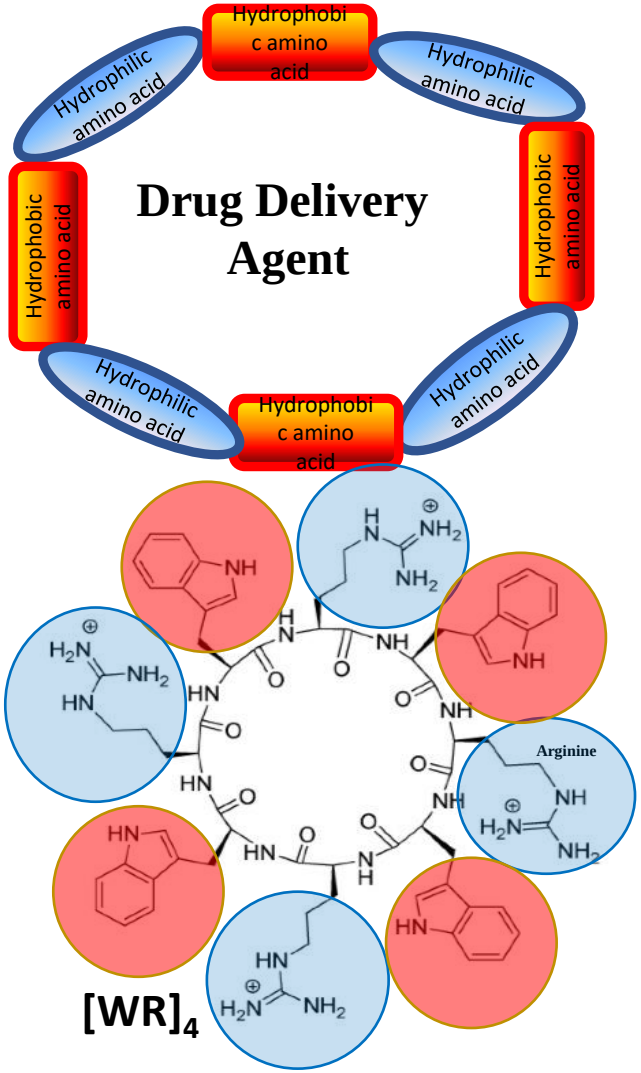
Polymyxin B

It is derived from the bacterium *Paenibacillus polymyxa*

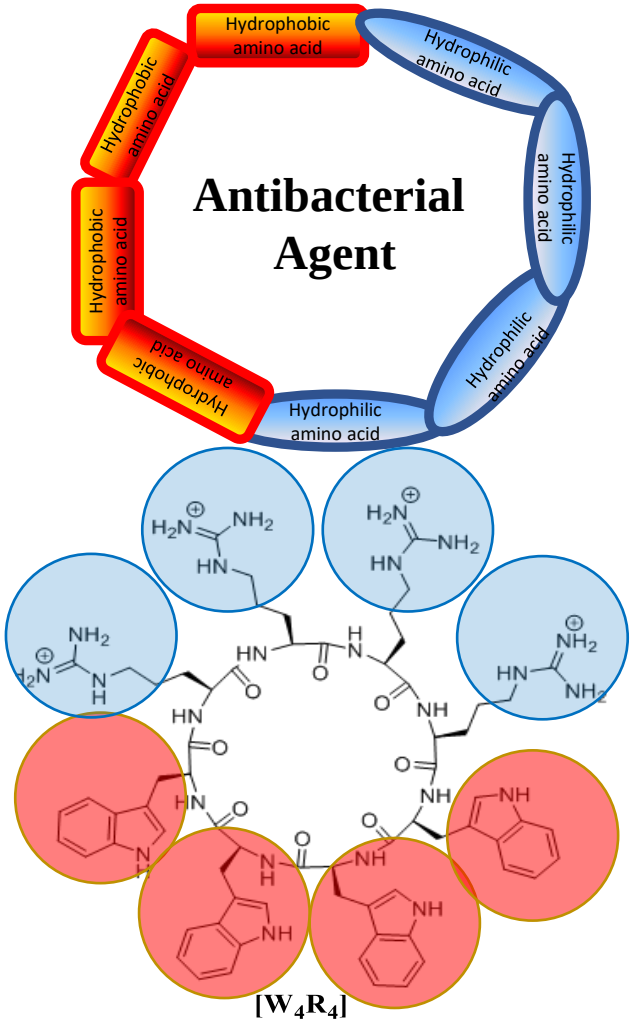


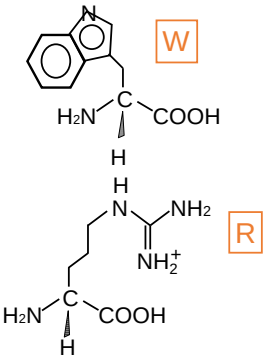
Vancomycin

is made by the soil bacterium *Amycolatopsis orientali*



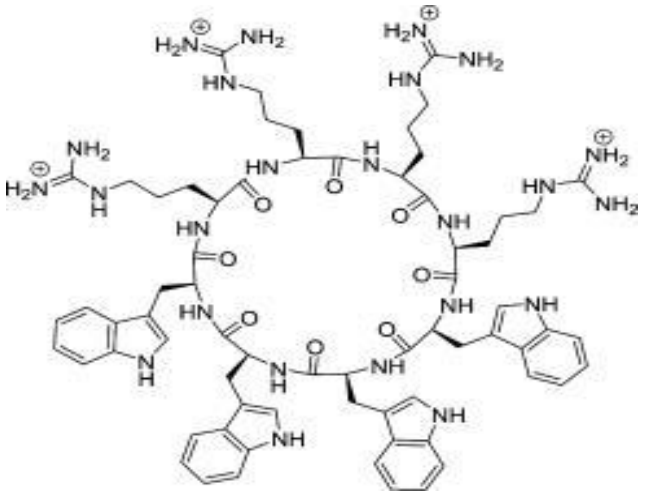
Angewandte Chemie, **2011**, 50(41), 9633-9637; *ACS Omega*, **(2018)**, *3*, 16281-16291.





J. Med. Chem. **2022**, *65*, 665-687; *Mol. Pharmaceutics* **(2014)** *11*, 3528-3536.

Amphipathic Cyclic Antibacterial Peptide [R_4W_4]

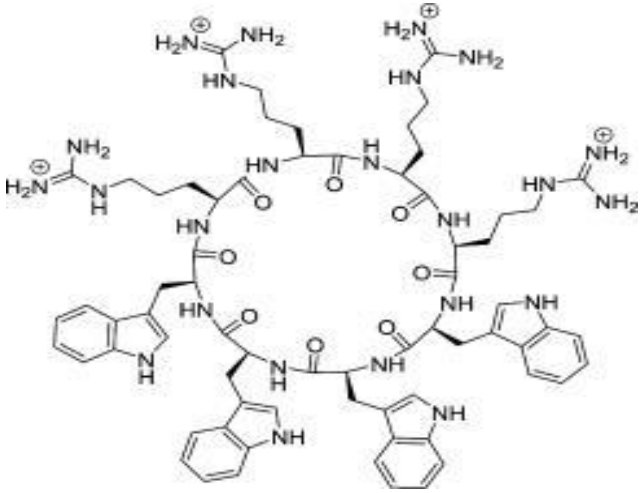


[R_4W_4]

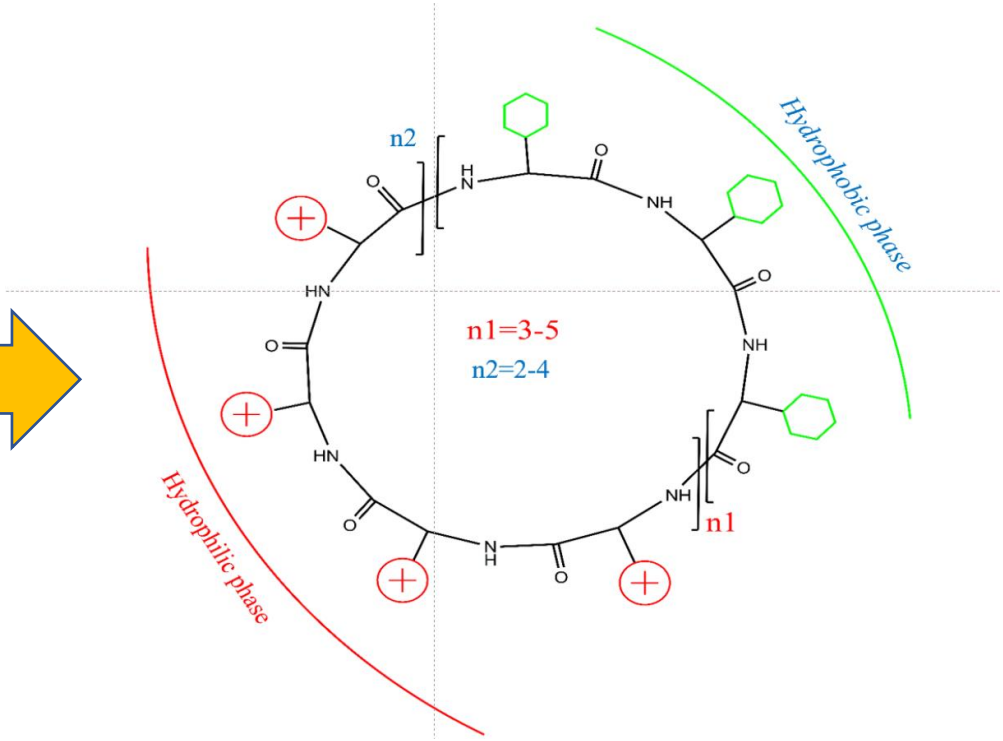
Mol. Pharmaceutics **(2014)** *11*, 3528-3536.

- [R_4W_4] is a cyclic antibacterial peptide containing W and R.
- **Minimum Inhibitory Concentration (MIC):** 2.67 μg/mL against MRSA and 42 μg/mL against *Pseudomonas aeruginosa (PSA)*.
- **Mechanism of action:** Disruption of bacterial cell membrane functions, interfering with biological structure integrity, leakages by creating pores on the bacterial cell membrane.

Hypothesis: Peptides containing appropriate hydrophobic and positively charged residues can have potential antibacterial and/or synergistic activity with other antibiotics.



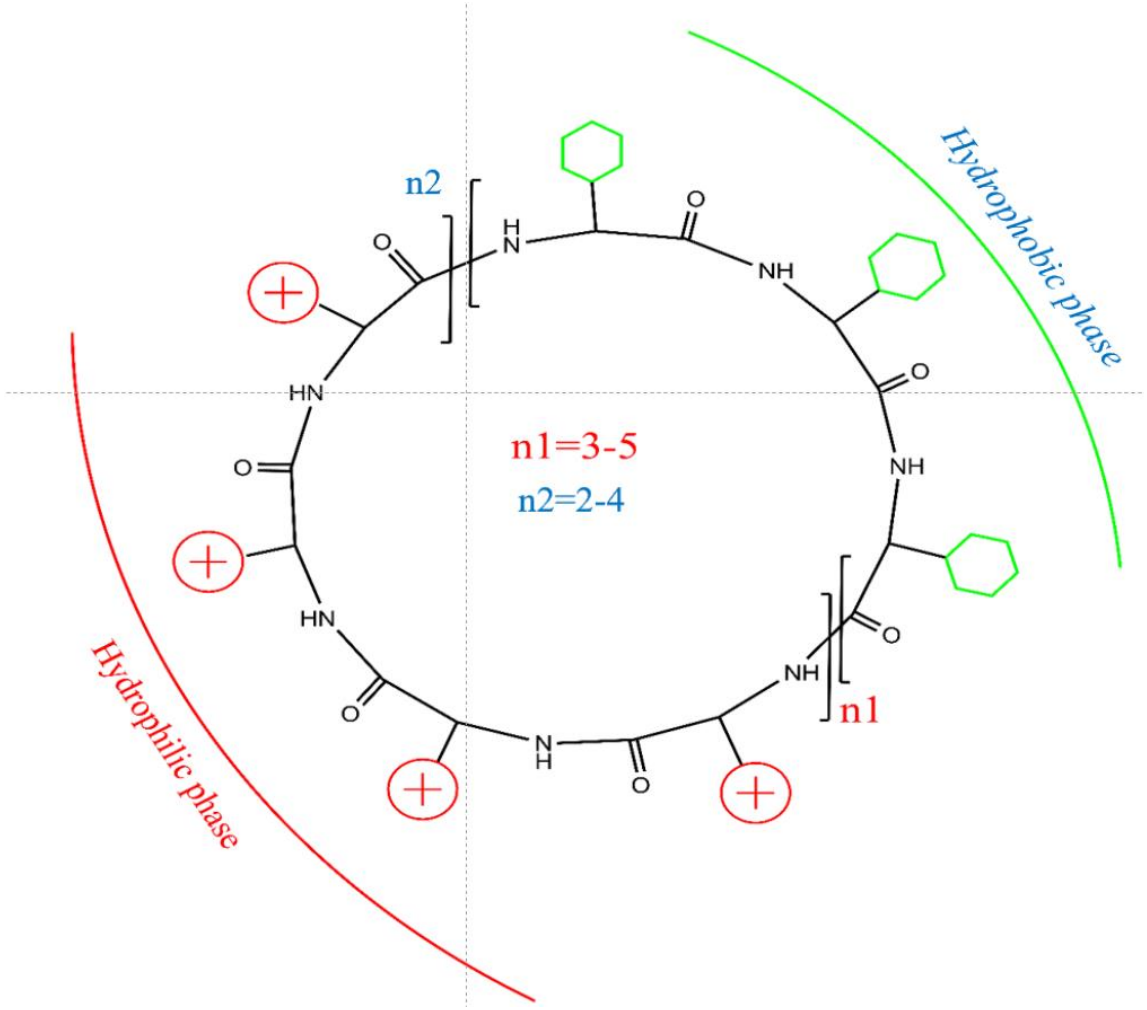
$[R_4W_4]$

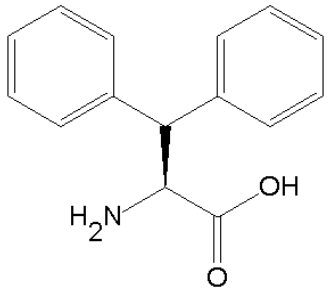


Molecules **2017**, *22*(6), 957
Molecules **2018**, *23*(10), 2722
Antibiotics **2022**, *11*(3), 416

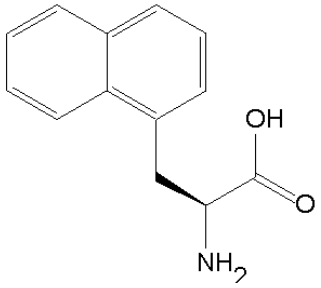
J. Med. Chem. **2023**, *66(1)*, 855-874
J. Med. Chem. **2022**, *65(23)*,15819-15839
J. Med. Chem. **2022**, *65(1)*:665-687
Eu. J. Med. Chem, **2022**, *235*, 114278

Strategy 1. Using Non-genetically coded amino acid residues

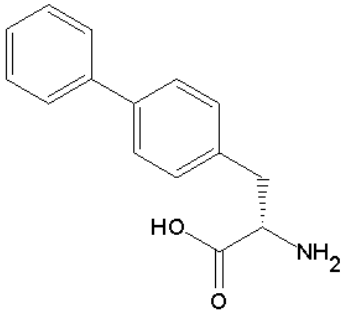




Dip



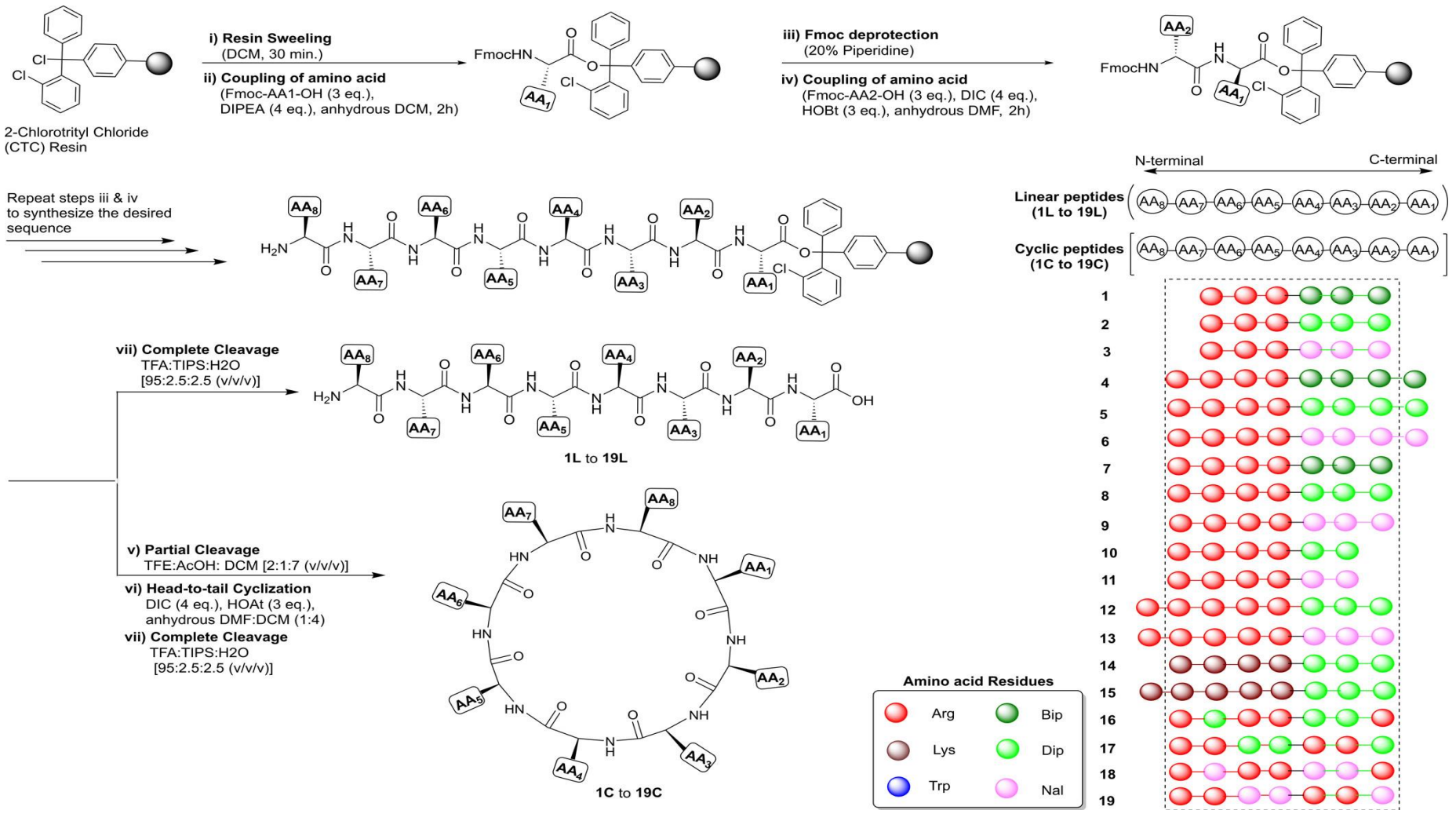
Nal



Bip

J. Med. Chem. **2022,** *65(1)*, 665-687.

Cationic Macrocyclic peptides (CMPs) (n >250)



Overview of the Different Steps Involved in the Synthesis of Linear (**1L** to **19L**) and Cyclic (**1C** to **19C**) Peptides

J. Med. Chem. **2022,** *65(1)*, 665-687.

Antibacterial and Hemolytic Activity Results of Linear Peptides

code	peptide sequence[b]	MIC[c] (μg/mL) MRSA (ATCC BAA-1556)	*S. aureus* (ATCC 29213)	*P. aeruginosa* (ATCC 27883)	*E. coli* (ATCC 25922)	HC_{50}[d]
1L	NH_2-Arg-Arg-Arg-Bip-Bip-Bip-OH	50	50	>100	>100	ND[e]
2L	NH_2-Arg-Arg-Arg-Dip-Dip-Dip-OH	6.2	6.2	25	6.2	120
3L	NH_2-Arg-Arg-Arg-Nal-Nal-Nal-OH	6.2	6.2	25	12.5	105
4L	NH_2-Arg-Arg-Arg-Arg-Bip-Bip-Bip-Bip-OH	>100	>100	>100	>100	ND[e]
5L	NH_2-Arg-Arg-Arg-Arg-Dip-Dip-Dip-Dip-OH	12.5	6.2	50	50	75
6L	NH_2-Arg-Arg-Arg-Arg-Nal-Nal-Nal-Nal-OH	12.5	12.5	>100	50	70
7L	NH_2-Arg-Arg-Arg-Arg-Bip-Bip-Bip-OH	12.5	6.2	25	>100	ND[e]
8L	NH_2-Arg-Arg-Arg-Arg-Dip-Dip-Dip-OH	6.2	6.2	12.5	12.5	145
9L	NH_2-Arg-Arg-Arg-Arg-Nal-Nal-Nal-OH	6.2	6.2	25	12.5	130
10L	NH_2-Arg-Arg-Arg-Arg-Dip-Dip-OH	12.5	12.5	50	50	185
11L	NH_2-Arg-Arg-Arg-Arg-Nal-Nal-OH	6.2	6.2	>100	50	180
12L	NH_2-Arg-Arg-Arg-Arg-Arg-Dip-Dip-Dip-OH	12.5	12.5	50	25	165
13L	NH_2-Arg-Arg-Arg-Arg-Arg-Nal-Nal-Nal-OH	6.2	6.2	25	25	190
14L	NH_2-Lys-Lys-Lys-Lys-Dip-Dip-Dip-OH	25	25	>100	>100	255
15L	NH_2-Lys-Lys-Lys-Lys-Lys-Dip-Dip-Dip-OH	50	50	>100	>100	280
16L	NH_2-Arg-Dip-Arg-Arg-Dip-Dip-Arg-OH	25	12.5	>100	25	220
17L	NH_2-Arg-Arg-Dip-Dip-Arg-Arg-Dip-OH	12.5	12.5	25	25	240
18L	NH_2-Arg-Nal-Arg-Arg-Nal-Nal-Arg-OH	25	12.5	50	25	205
19L	NH_2-Arg-Arg-Nal-Nal-Arg-Arg-Nal-OH	25	12.5	25	25	195
	daptomycin	1.5	0.7	ND[e]	ND[e]	ND[e]
	polymyxin B	ND[e]	0.7	0.7	0.7	ND[e]
	ciprofloxacin	3.1	1.5	0.7	0.7	ND[e]

[a]Results of three independent experiments performed in triplicate. [b]All amino acid residues are represented in three-letter notation. Bip, 4,4′-biphenyl-L-alanine; Dip, 3,3-diphenyl-L-alanine; Nal, 3-(2-naphthyl)-L-alanine. [c]Minimum inhibitory concentrations (MICs) were determined as the lowest concentration of the peptides that inhibited bacterial growth. [d]HC_{50} is the concentration in μg/mL of peptides at which 50% hemolysis is observed. [e]ND = not determined.

Antibacterial and Hemolytic Activity Results of Cyclic Peptides

code	peptide sequence[b]	MIC[c] (μg/mL) MRSA (ATCC BAA-1556)	*S. aureus* (ATCC 29213)	*P. aeruginosa* (ATCC 27883)	*E. coli* (ATCC 25922)	HC_{50}[d]
1C	c[Arg-Arg-Arg-Bip-Bip-Bip]	50	50	>100	>100	ND[e]
2C	c[Arg-Arg-Arg-Dip-Dip-Dip]	3.1	3.1	12.5	12.5	45
3C	c[Arg-Arg-Arg-Nal-Nal-Nal]	6.2	3.1	50	25	50
4C	c[Arg-Arg-Arg-Arg-Bip-Bip-Bip-Bip]	>100	50	>100	>100	ND[e]
5C	c[Arg-Arg-Arg-Arg-Dip-Dip-Dip-Dip]	6.2	3.1	25	25	40
6C	c[Arg-Arg-Arg-Arg-Nal-Nal-Nal-Nal]	6.2	3.1	50	25	30
7C	c[Arg-Arg-Arg-Arg-Bip-Bip-Bip]	6.2	3.1	25	>100	ND[e]
8C	c[Arg-Arg-Arg-Arg-Dip-Dip-Dip]	3.1	3.1	12.5	12.5	70
9C	c[Arg-Arg-Arg-Arg-Nal-Nal-Nal]	3.1	3.1	12.5	25	80
10C	c[Arg-Arg-Arg-Arg-Dip-Dip]	6.2	6.2	25	50	110
11C	c[Arg-Arg-Arg-Arg-Nal-Nal]	6.2	6.2	50	50	125
12C	c[Arg-Arg-Arg-Arg-Arg-Dip-Dip-Dip]	6.2	6.2	25	25	100
13C	c[Arg-Arg-Arg-Arg-Arg-Nal-Nal-Nal]	6.2	3.1	25	50	90
14C	c[Lys-Lys-Lys-Lys-Dip-Dip-Dip]	12.5	12.5	>100	>100	165
15C	c[Lys-Lys-Lys-Lys-Lys-Dip-Dip-Dip]	12.5	6.2	>100	50	180
16C	c[Arg-Dip-Arg-Arg-Dip-Dip-Arg]	6.2	6.2	50	>100	130
17C	c[Arg-Arg-Dip-Dip-Arg-Arg-Dip]	12.5	6.2	>100	>100	145
18C	c[Arg-Nal-Arg-Arg-Nal-Nal-Arg]	6.2	6.2	>100	25	105
19C	c[Arg-Arg-Nal-Nal-Arg-Arg-Nal]	6.2	6.2	50	50	110

Antibacterial Activity of Selected Peptides against Drug-Resistant Gram-Positive and Gram-Negative Bacterial Strains

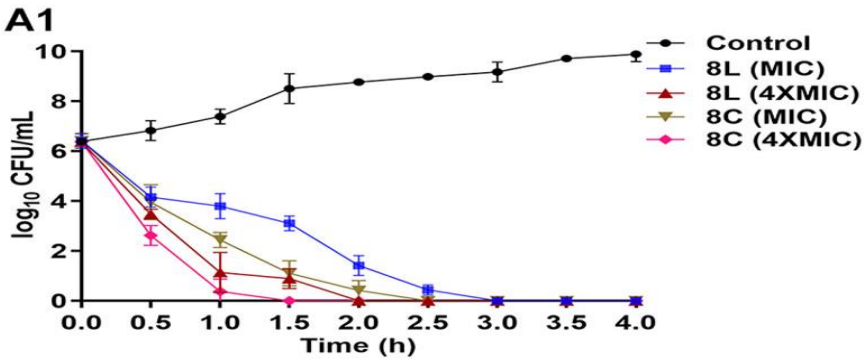
bacterial strain	MIC[h] (μg/mL)						
	2C	8C	9C	daptomycin	vancomycin	ciprofloxacin	polymyxin B
Gram-Positive							
Enterococcus faecium (ATCC 27270)	3.1	3.1	3.1	1.5	1.5	ND[i]	ND[i]
Enterococcus faecium[b] (ATCC 700221)	6.2	3.1	3.1	6.25	>50	ND[i]	ND[i]
Enterococcus faecalis (ATCC 29212)	12.5	3.1	3.1	6.25	0.7	ND[i]	ND[i]
Enterococcus faecalis[b] (ATCC 51575)	12.5	6.2	3.1	12.5	>50	ND[i]	ND[i]
Staphylococcus pneumoniae (ATCC 49619)	50	25	25	12.5	3.1	ND[i]	ND[i]
Staphylococcus pneumoniae[c] (ATCC 700677)	25	25	12.5	12.5	1.5	ND[i]	ND[i]
Bacillus subtilis (ATCC 6633)	3.1	1.5	1.5	0.7	0.7	ND[i]	ND[i]
Bacillus cereus (ATCC 13061)	6.2	3.1	3.1	1.5	0.7	ND[i]	ND[i]
Gram-Negative							
Escherichia coli[d] (ATCC BAA-2452)	25	12.5	12.5	ND[i]	ND[i]	0.7	0.7
Klebsiella pneumonia (ATCC 13883)	>50	50	50	ND[i]	ND[i]	1.5	6.2
Klebsiella pneumonia[e] (ATCC BAA-2470)	12.5	12.5	12.5	ND[i]	ND[i]	0.7	1.5
Acinetobacter baumannii[f] (ATCC BAA1605)	25	12.5	12.5	ND[i]	ND[i]	0.7	0.7
Pseudomonas aeruginosa (ATCC 10145)	50	25	25	ND[i]	ND[i]	0.7	0.7
Pseudomonas aeruginosa[g] (ATCC BAA-1744)	25	12.5	12.5	ND[i]	ND[i]	0.7	0.7

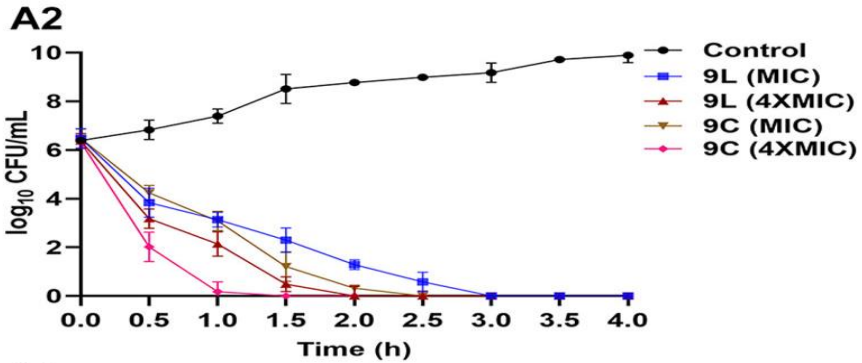
[a]Results of three independent experiments performed in triplicate. [b]Vancomycin-resistant bacterial strains. [c]Multidrug-resistant (penicillin, tetracycline, and erythromycin) bacterial strains. [d]NDM-1-resistant bacterial strains. [e]Carbapenem-resistant bacterial strains. [f]Ciprofloxacin-resistant bacterial strains. [g]Imipenem-resistant bacterial strains. [h]Minimum inhibitory concentrations (MICs) were determined as the lowest concentration of the peptides that inhibited bacterial growth. [i]ND = not determined.

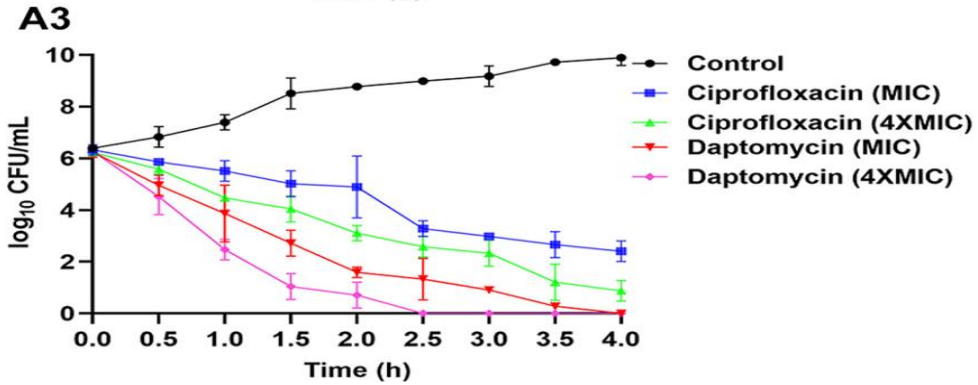
2C	c[Arg-Arg-Arg-Dip-Dip-Dip]
8C	c[Arg-Arg-Arg-Arg-Dip-Dip-Dip]
9C	c[Arg-Arg-Arg-Arg-Nal-Nal-Nal]

Bactericidal kinetics of test peptides and standard antibiotics at the MIC and 4× the MIC

MRSA

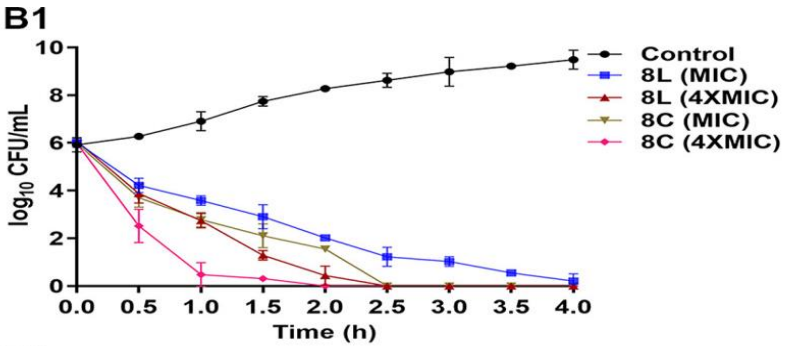


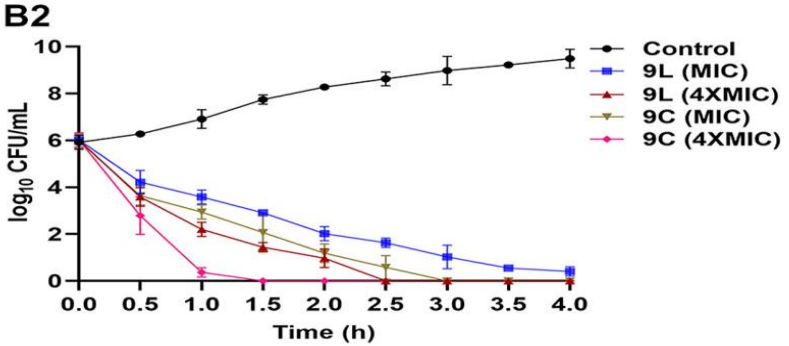


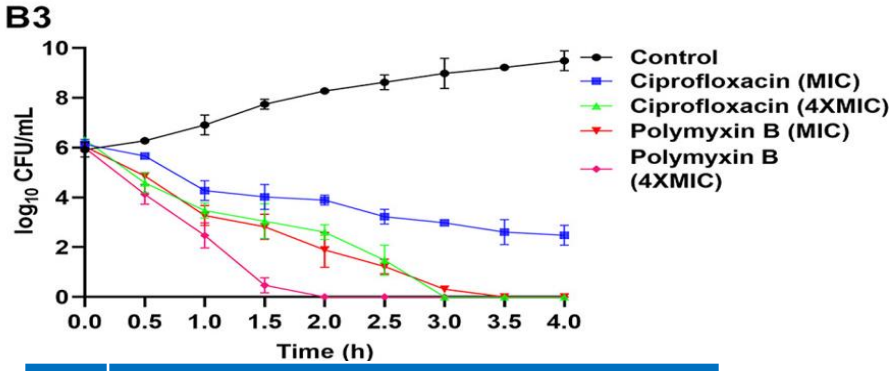


8C	c[Arg-Arg-Arg-Arg-Dip-Dip-Dip]
9C	c[Arg-Arg-Arg-Arg-Nal-Nal-Nal]

E.coli

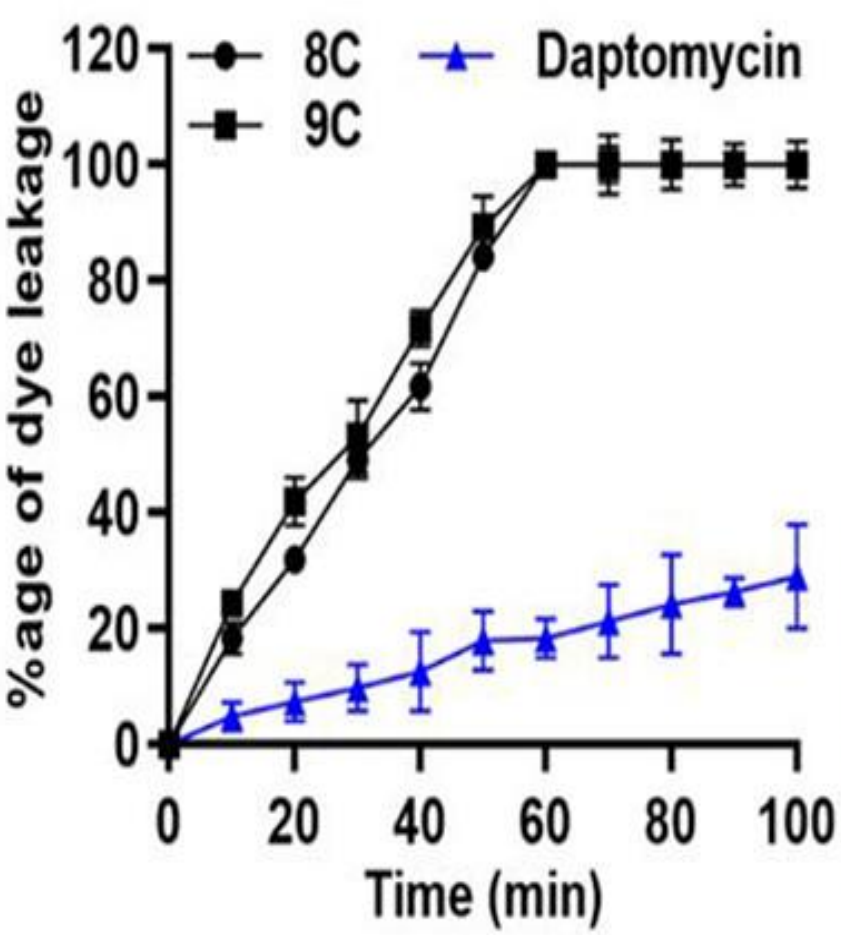


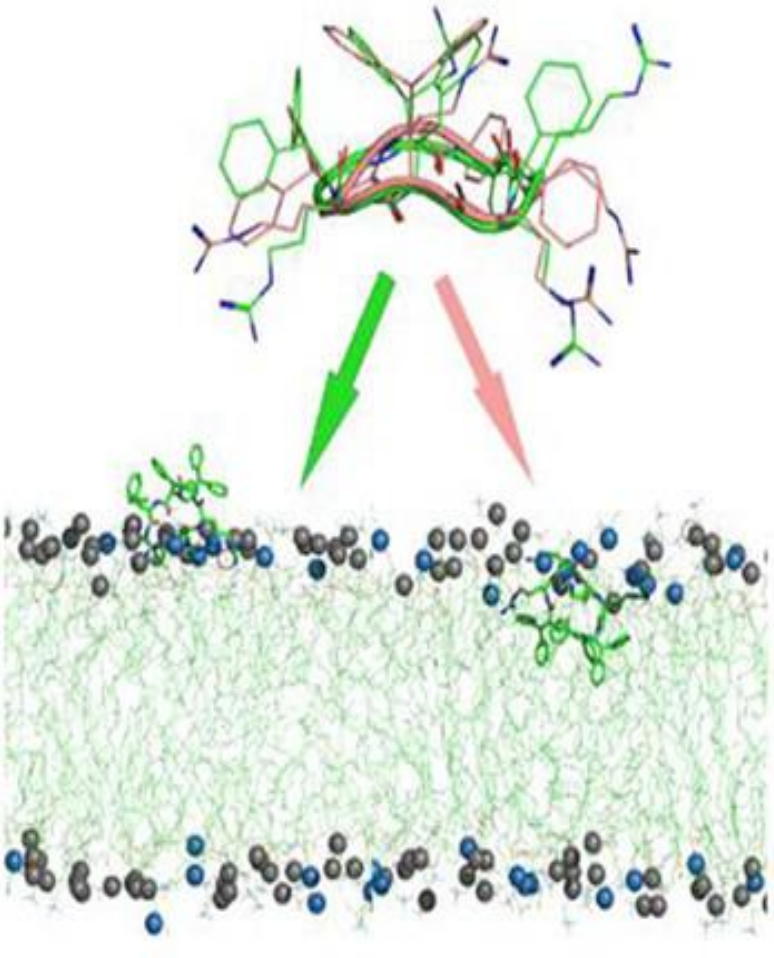




8L	NH_2-Arg-Arg-Arg-Arg-Dip-Dip-Dip-OH
9L	NH_2-Arg-Arg-Arg-Arg-Nal-Nal-Nal-OH

Calcein Dye Leakage Studies





8C	c[Arg-Arg-Arg-Arg-Dip-Dip-Dip]
9C	c[Arg-Arg-Arg-Arg-Nal-Nal-Nal]

Bacterial Membrane Mimicking Liposomes

Mammalian Membrane Mimicking Liposomes

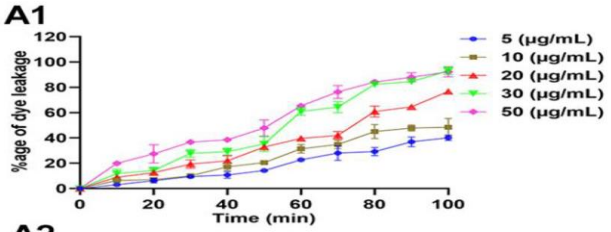
8L

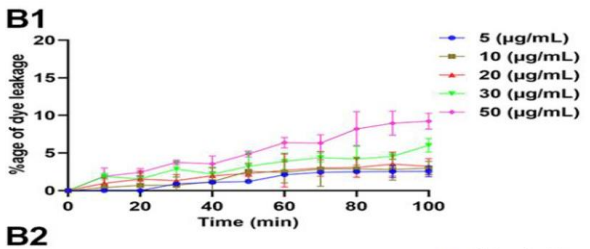
9L

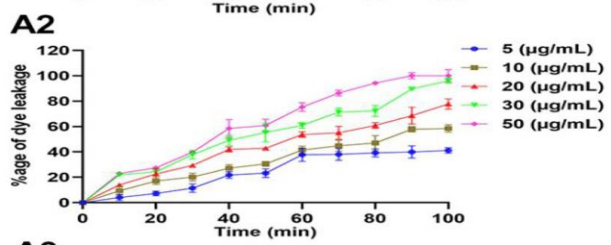
8C

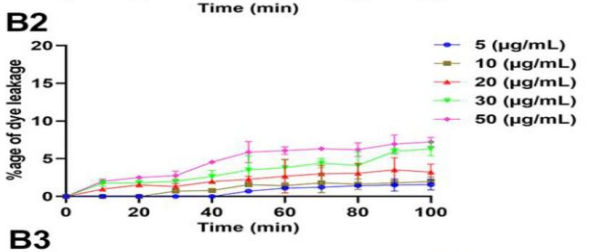
9C

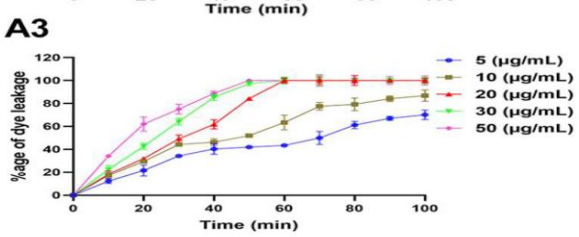
Daptomycin

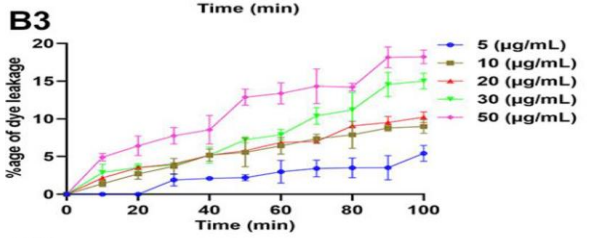


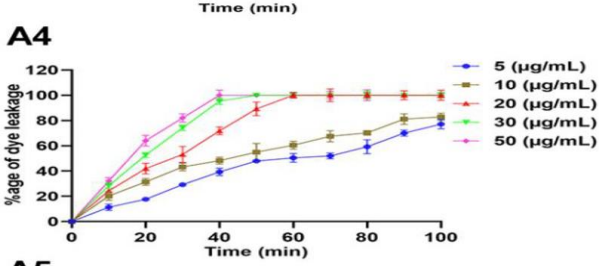


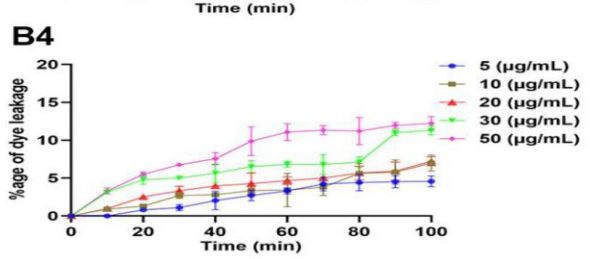


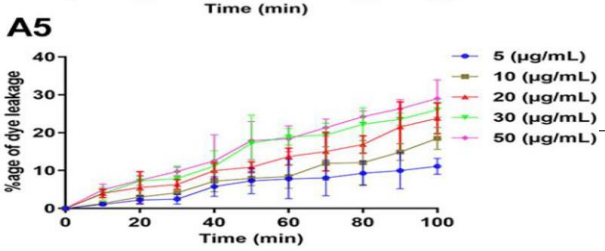


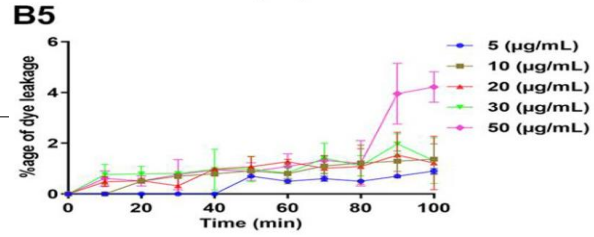






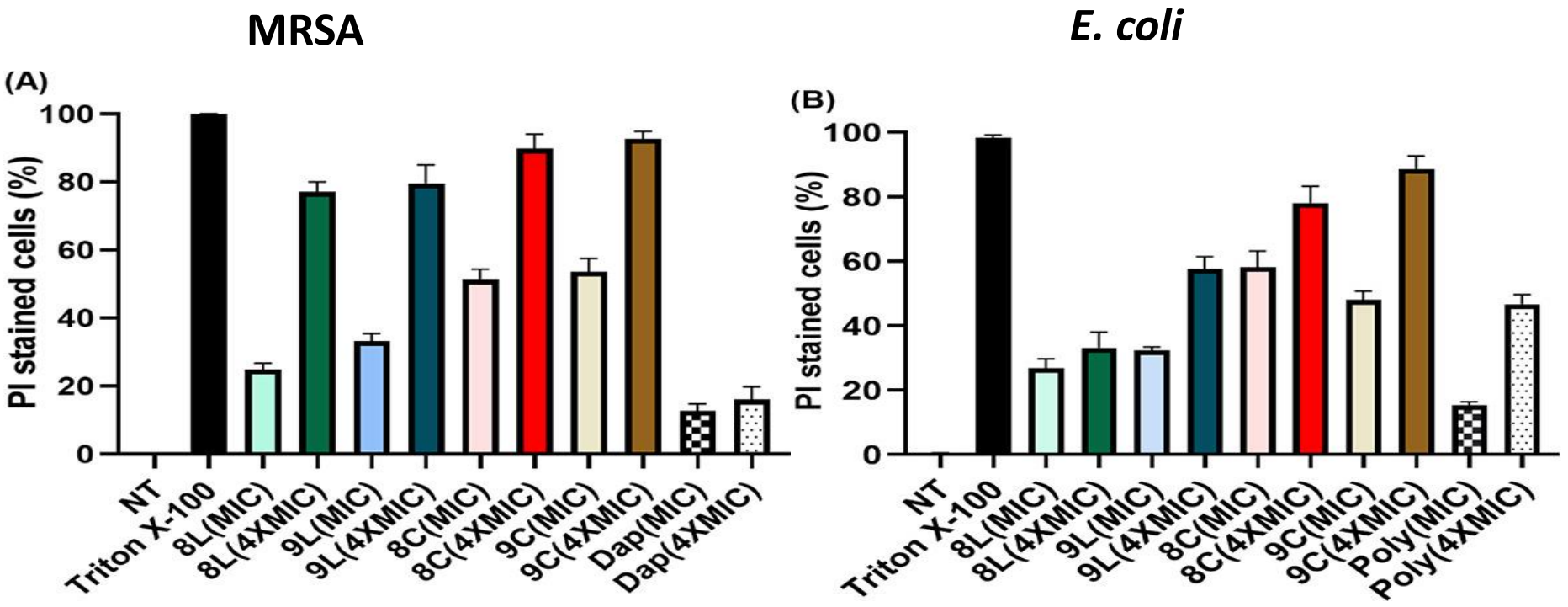






Concentration-dependent leakage of calcein dye

Flow cytometric analysis of bacterial cells treated with test peptides (8L, 8C, 9L, and 9C) and standard antibiotics (daptomycin (Dap) and polymyxin B (Poly)) at the MIC and 4× the MIC.

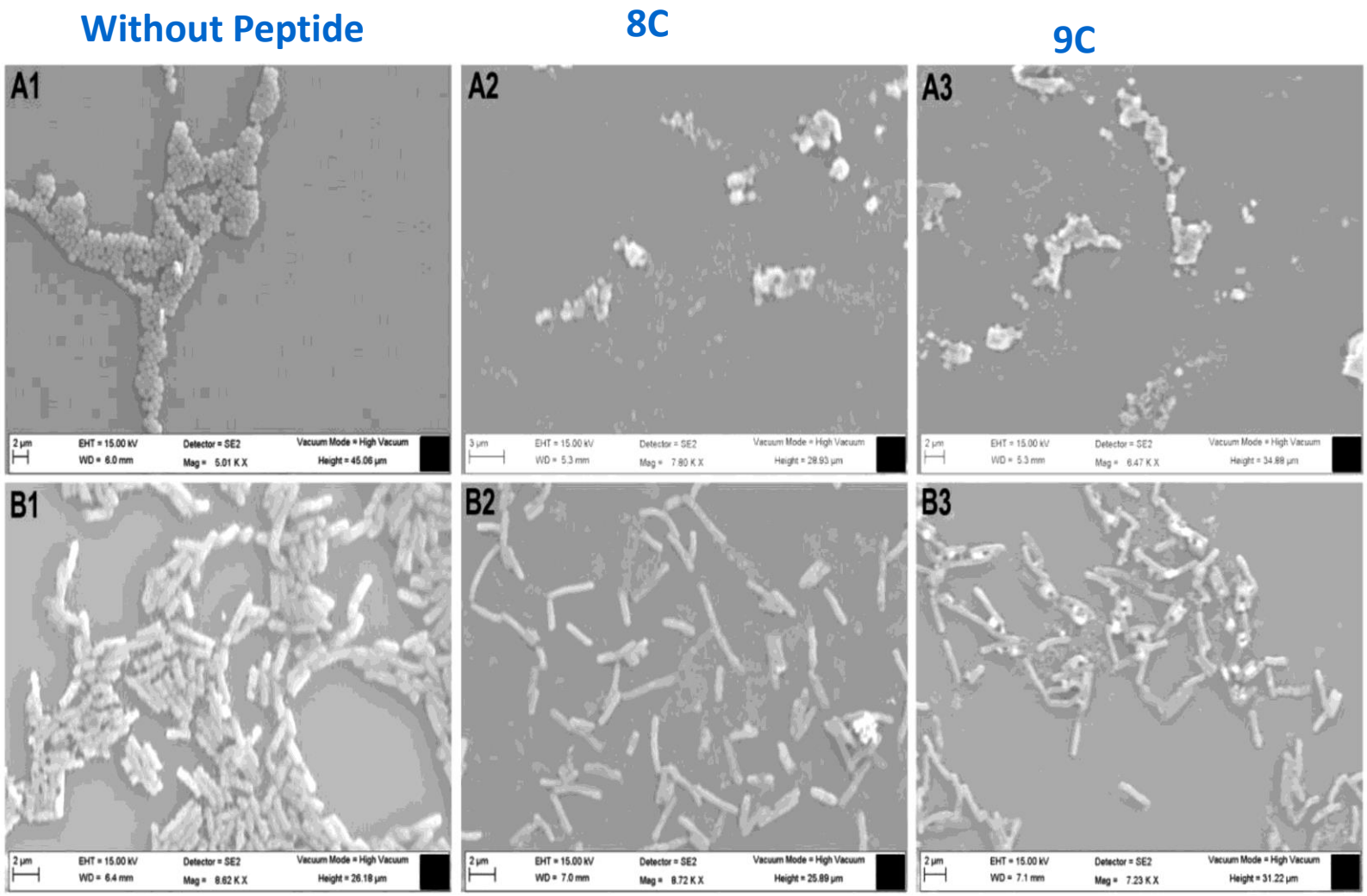


The data represent the increase in propidium iodide-stained cells (%) upon treatment with test peptides and standard antibiotics.

8C	c[Arg-Arg-Arg-Arg-Dip-Dip-Dip]
9C	c[Arg-Arg-Arg-Arg-Nal-Nal-Nal]

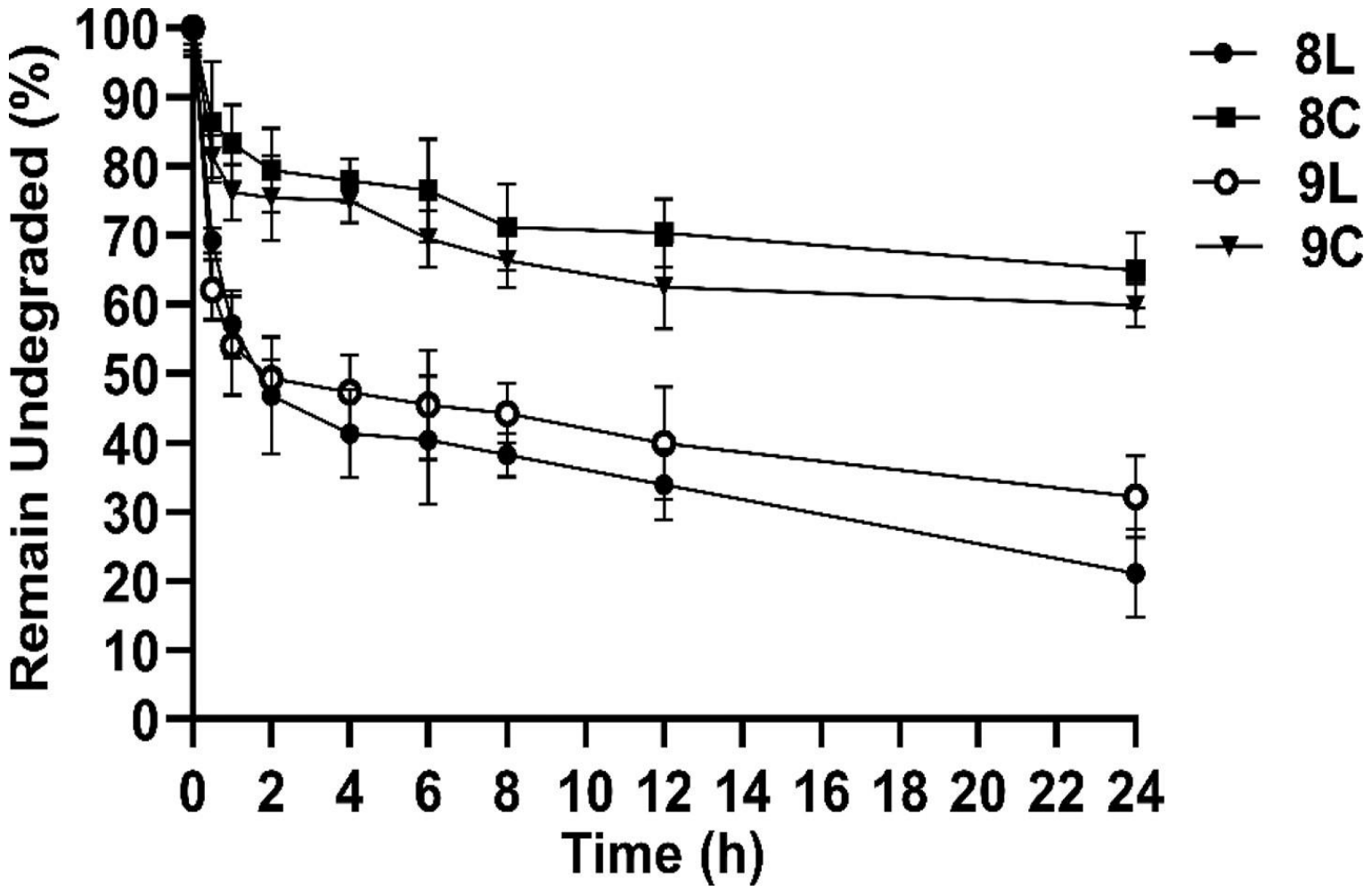
8L	NH_2-Arg-Arg-Arg-Arg-Dip-Dip-Dip-OH
9L	NH_2-Arg-Arg-Arg-Arg-Nal-Nal-Nal-OH

SEM images Mid logarithmic-phase bacterial cells were incubated with **8C** (A2 and B2) and **9C** (A3 and B3) at a final concentration of 4× the MIC for 1 h.



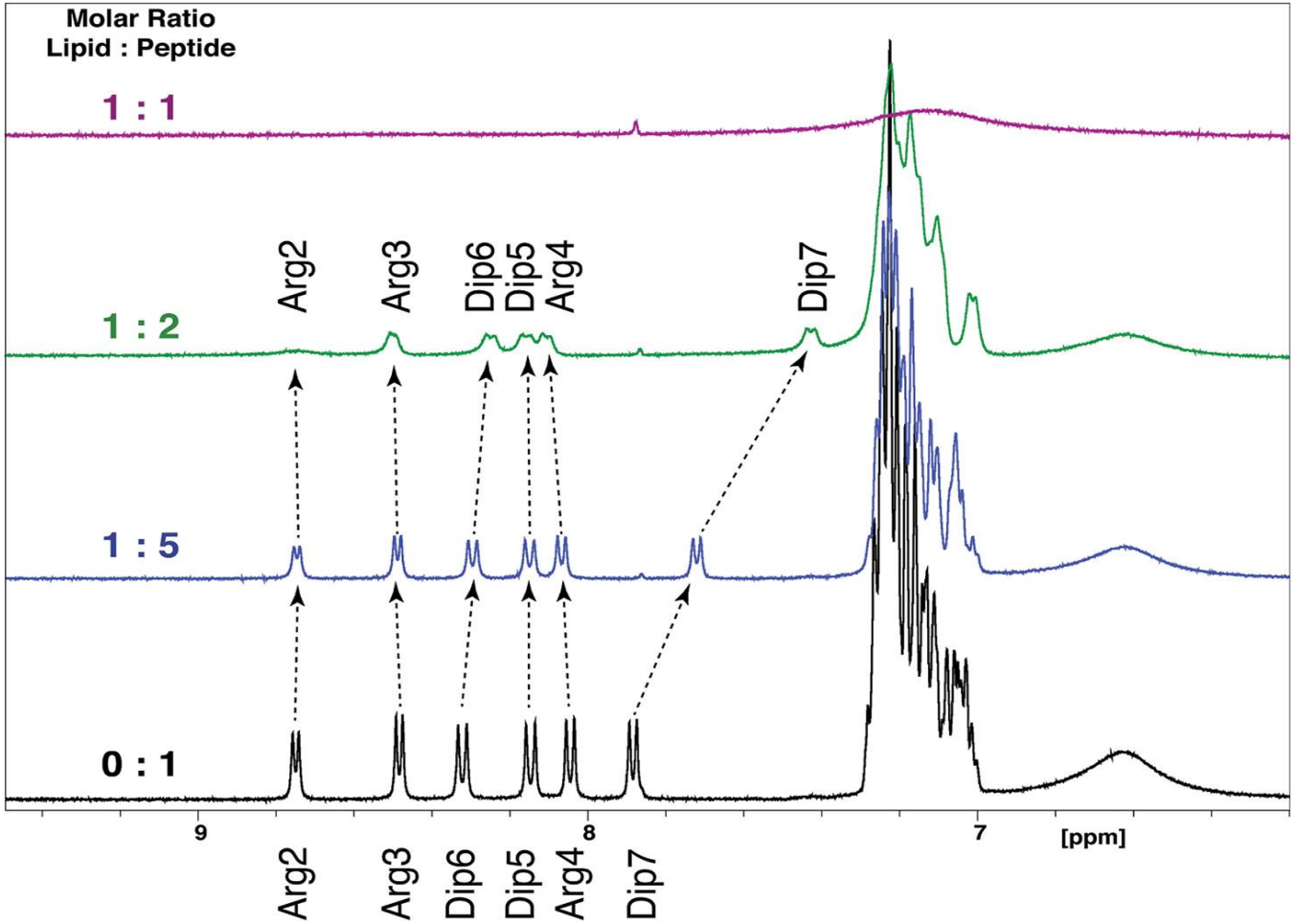
8C	c[Arg-Arg-Arg-Arg-Dip-Dip-Dip]
9C	c[Arg-Arg-Arg-Arg-Nal-Nal-Nal]

***In vitro* enzymatic stability assay** of the lead cyclic peptides **8C** and **9C** and their linear analogs **8L** and **9L** in human plasma.



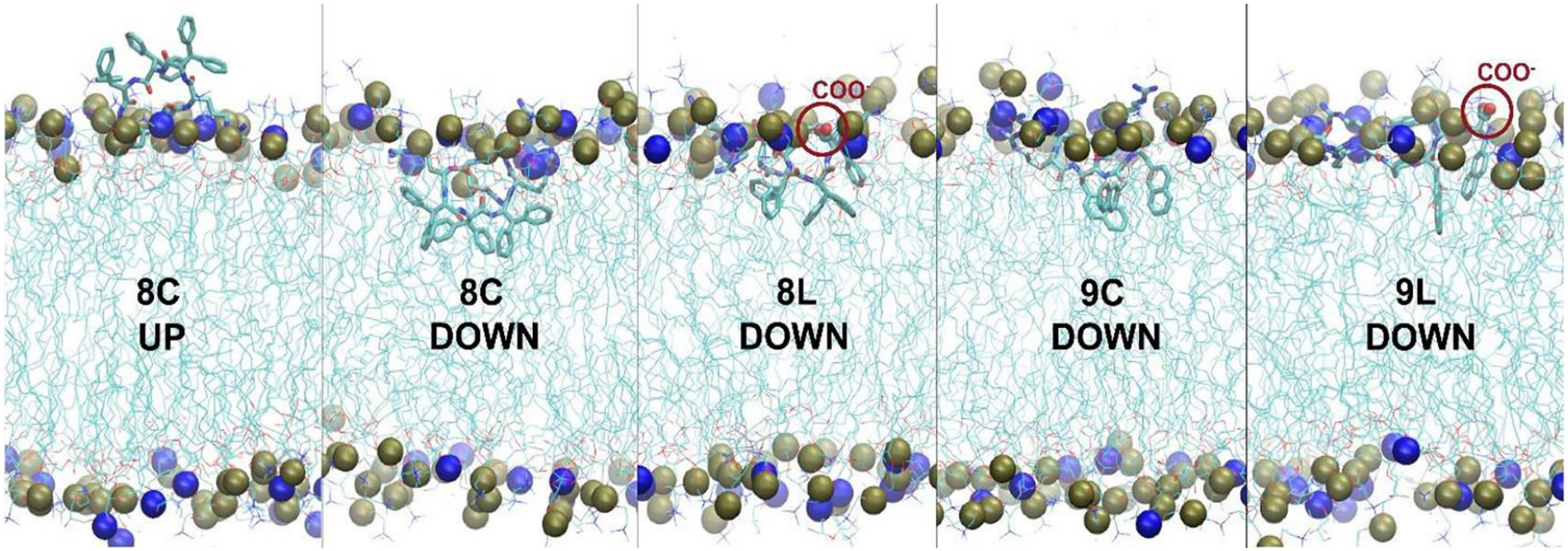
8C	c[Arg-Arg-Arg-Arg-Dip-Dip-Dip]
9C	c[Arg-Arg-Arg-Arg-Nal-Nal-Nal]

8L	NH_2-Arg-Arg-Arg-Arg-Dip-Dip-Dip-OH
9L	NH_2-Arg-Arg-Arg-Arg-Nal-Nal-Nal-OH



Changes in 1H NMR spectra of peptide 8L upon addition of the liposomes. The lipid–peptide molar ratios are shown on the left starting from 0:1 (no lipids, bottom spectrum). The assignment of the amide hydrogens for residues 2–7 is shown for the peptide alone (black spectrum) and for the lipid–peptide molar ratio of 1:2 (green spectrum).

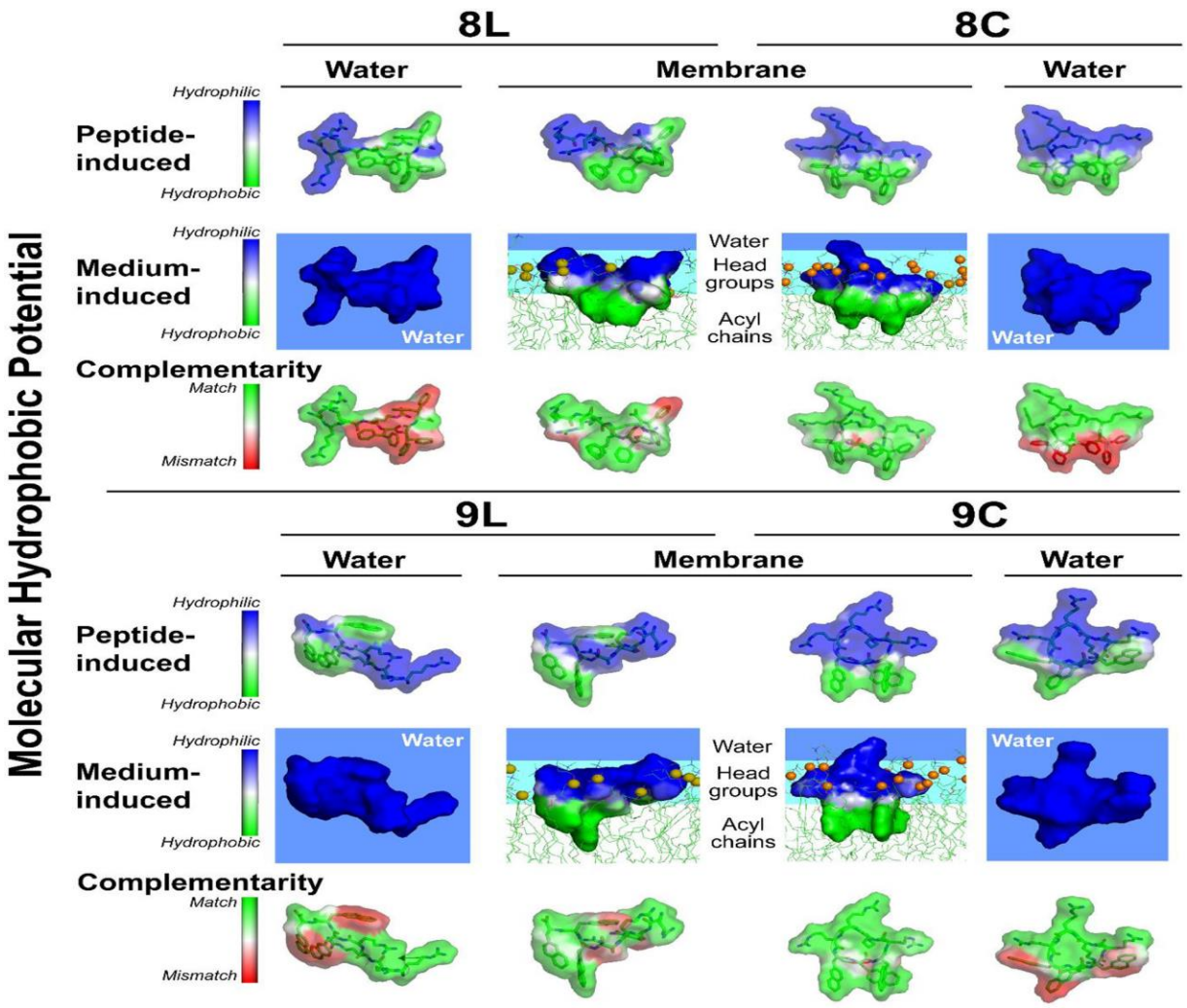
Orientation of the Lead Peptides in Proximity of the Membrane



Snapshots of the binding modes of linear (**8L** and **9L**) and cyclic (**8C** and **9C**) peptides obtained via MD simulations in the lipid bilayer. The peptide binding modes “UP” and “DOWN” are indicated. Phosphorus atoms of lipid head groups are given with the spheres (blue for DOPG, golden for DOPC).

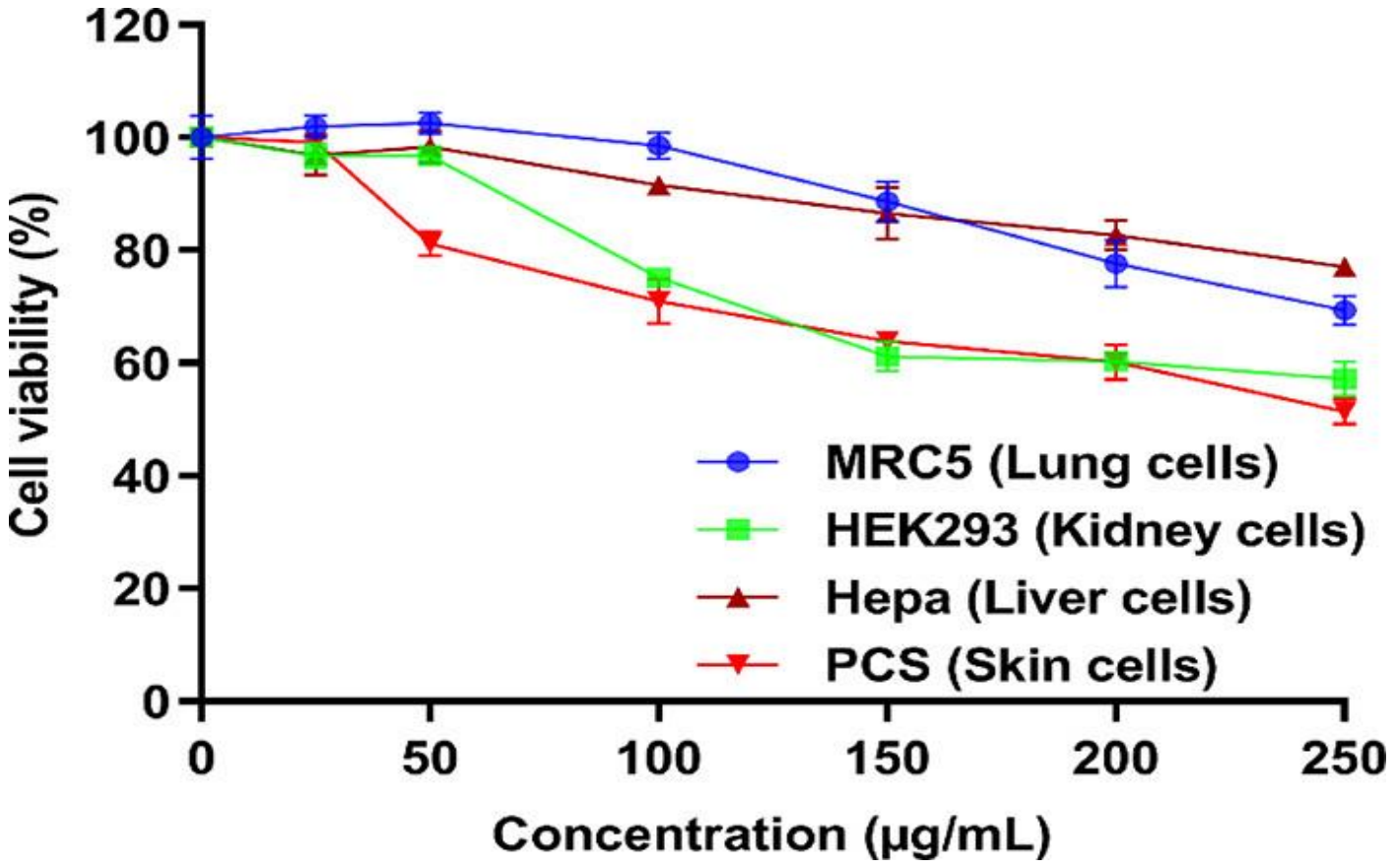
8C	c[Arg-Arg-Arg-Arg-Dip-Dip-Dip]
9C	c[Arg-Arg-Arg-Arg-Nal-Nal-Nal]

8L	NH_2-Arg-Arg-Arg-Arg-Dip-Dip-Dip-OH
9L	NH_2-Arg-Arg-Arg-Arg-Nal-Nal-Nal-OH

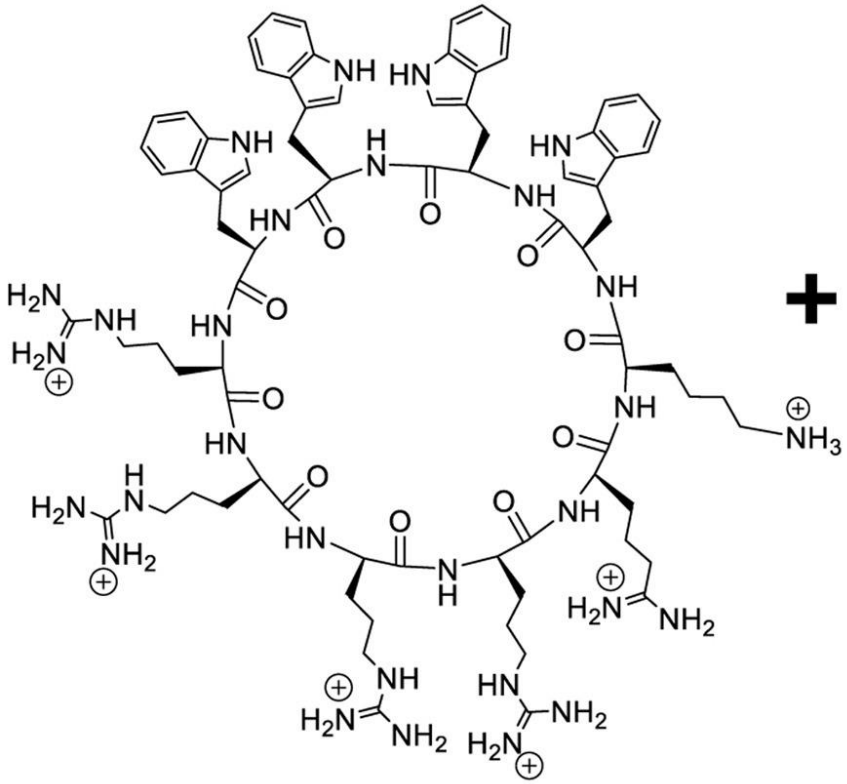


Complementarity of hydrophobic/hydrophilic surface properties for the cyclic peptides **8C**, **8L**, **9C**, and **9L**. The molecular hydrophobicity potential (MHP) values (blue, hydrophilic; green, hydrophobic) in the surface points of the peptide molecule calculated for the peptide atoms (peptide-induced rows) or the peptide's environment (medium-induced rows; water or water-bilayer). Complementarity rows demonstrate the color representation of the MHP complementarity (match (green) or mismatch (red) of the MHP induced by the peptide and the environment) on the peptide surface.

Cytotoxicity of Lead Cyclic Peptide [R_5W_4]



AMPs and Antibiotics in combination therapy?



$[W_4KR_5]$

\+

Antibiotics

[Meropenum]
[Tetracycline]
[Tobramycin]
[Levofloxacin]
[Ciprofloxacin]
[Clindamycin]
[Daptomycin]
[Polymyxin B]
[Kanamycin]
[Vancomycin]

Synergism

Gram-Positive Bacteria

Gram-Negative Bacteria

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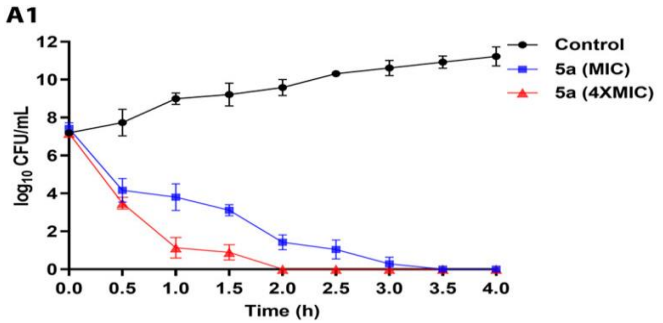
The Synergistic Effect of the Peptide 5a/Antibiotic Combination

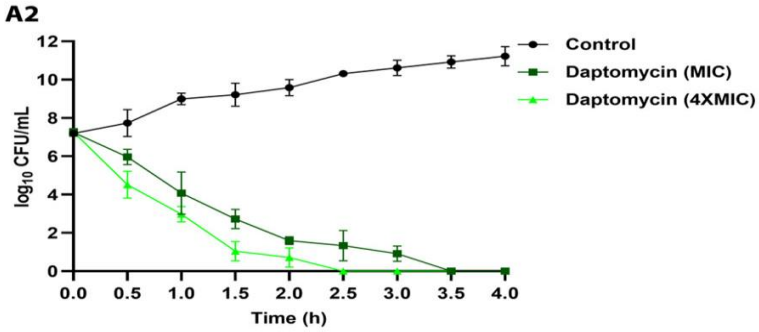
combination	bacterial strain	MIC (μg/mL)		FIC antibiotic	FICI[b]	integrative category
		antibiotic in combination	antibiotic alone			
$[R_5W_4]$ + tetracycline	*S. aureus* (ATCC BAA-1556)	0.0625	0.250	0.25	0.500	synergy
	P. aeruginosa (ATCC 27883)	4.00	32.0	0.125	0.375	synergy
	E. coli (ATCC 25922)	1.00	8.00	0.125	0.375	synergy
	K. pneumoniae (ATCC BAA-1705)	2.00	16.0	0.125	0.375	synergy
$[R_5W_4]$ + tobramycin	*S. aureus* (ATCC BAA-1556)	0.125	0.500	0.250	0.500	synergy
	P. aeruginosa (ATCC 27883)	0.125	0.500	0.250	0.500	synergy
	E. coli (ATCC 25922)	2.00	8.00	0.250	0.500	synergy
	K. pneumoniae (ATCC BAA-1705)	2.00	16.0	0.125	0.375	synergy
$[R_5W_4]$ + clindamycin	*S. aureus* (ATCC BAA-1556)	0.0312	0.125	0.249	0.499	synergy
	P. aeruginosa (ATCC 27883)	8.00	512	0.016	0.266	synergy
	E. coli (ATCC 25922)	4.00	64.0	0.063	0.313	synergy
	K. pneumoniae (ATCC BAA-1705)	2.00	512	0.0039	0.254	synergy
$[R_5W_4]$ + kanamycin	*S. aureus* (ATCC BAA-1556)	ND[a]	256	ND[a]	ND[a]	ND[a]
	P. aeruginosa (ATCC 27883)	8.00	256	0.0313	0.281	synergy
	E. coli (ATCC 25922)	8.00	32.0	0.250	0.500	synergy
	K. pneumoniae (ATCC BAA-1705)	8.00	64.0	0.125	0.375	synergy
$[R_5W_4]$ + levofloxacin	*S. aureus* (ATCC BAA-1556)	1.00	4.00	0.25	0.500	synergy
	P. aeruginosa (ATCC 27883)	0.125	1.00	0.125	0.375	synergy
	E. coli (ATCC 25922)	8.00	64.0	0.125	0.375	synergy
	K. pneumoniae (ATCC BAA-1705)	8.00	64.0	0.125	0.375	synergy

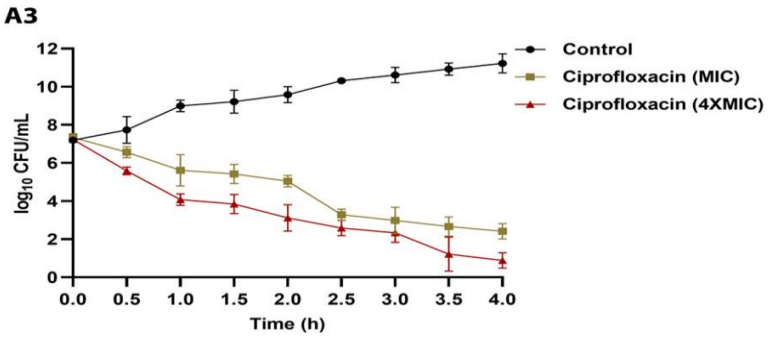
[a]ND, not determined. [b]FIC of the peptide in combination is 0.25 (peptide concentration equivalent to one-fourth of its MIC). All experiments were performed in triplicate.

Bactericidal kinetics of lead cyclic peptide [R_5W_4] (5a) and standard antibiotics

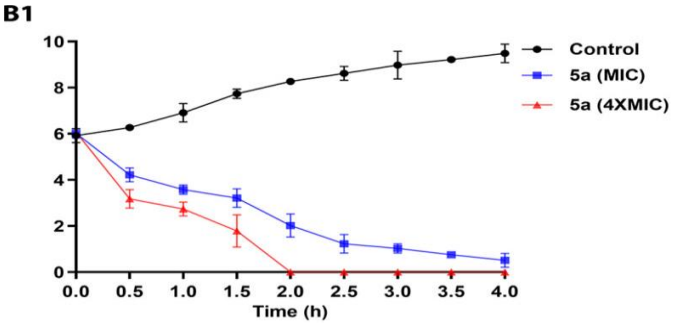
MRSA

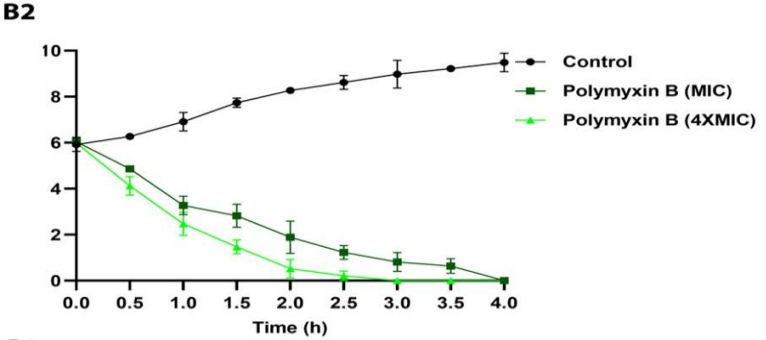


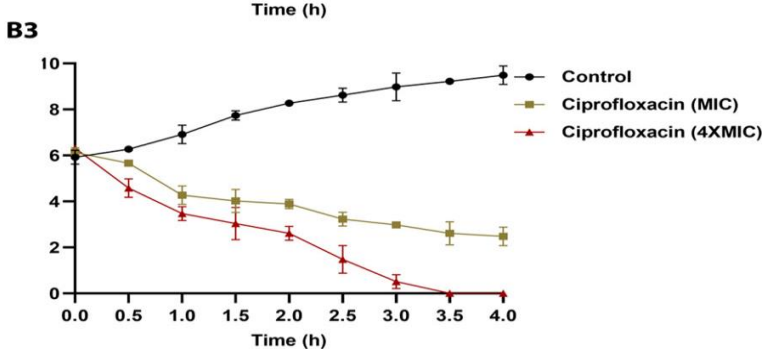




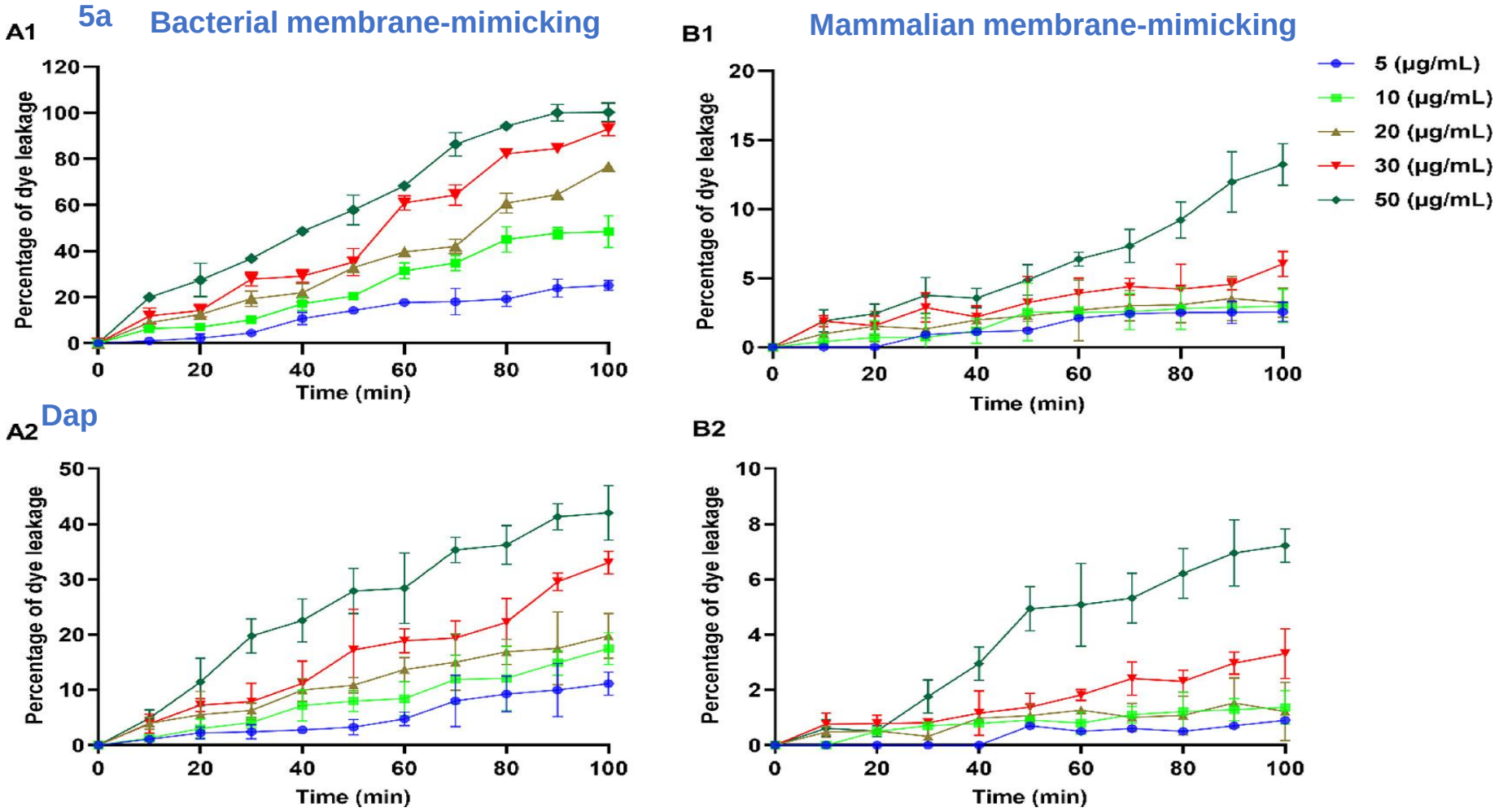
E. Coli







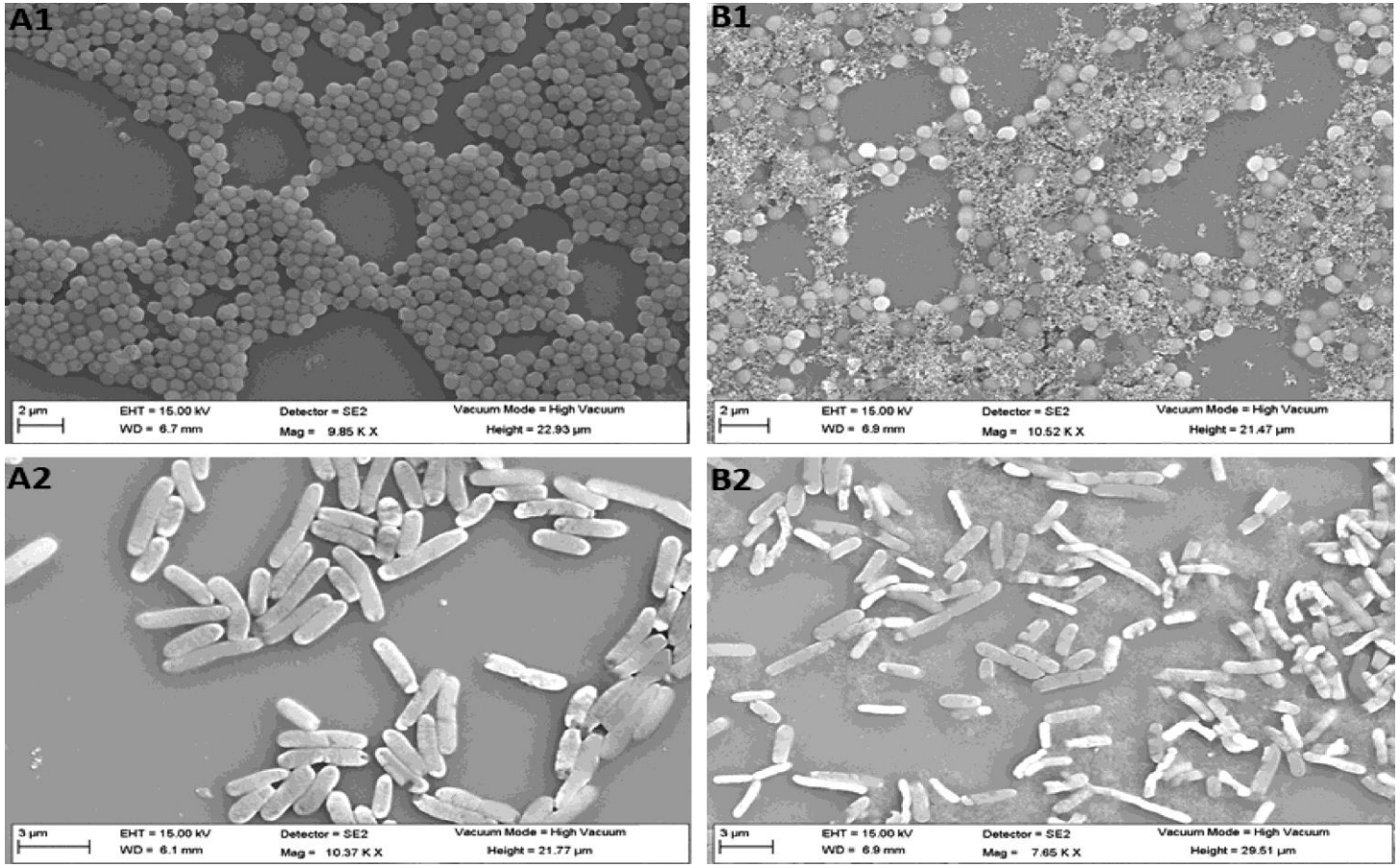
Selectivity of $[R_5W_4]$ (5a)
Bacterial versus Mammalian membrane-mimicking liposome



SEM micrograph of bacteria after treatment with $[R_5W_4]$

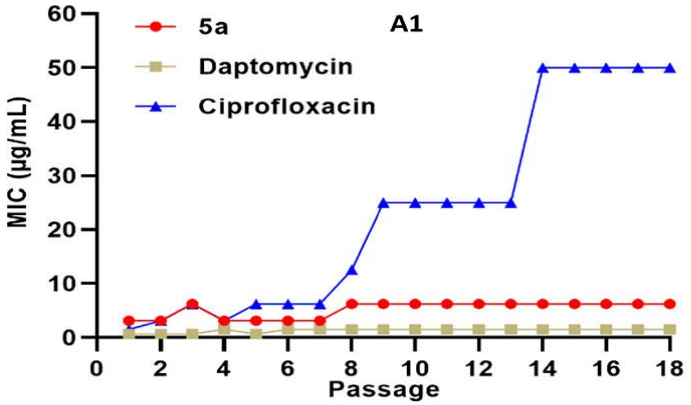
MRSA

E. coli

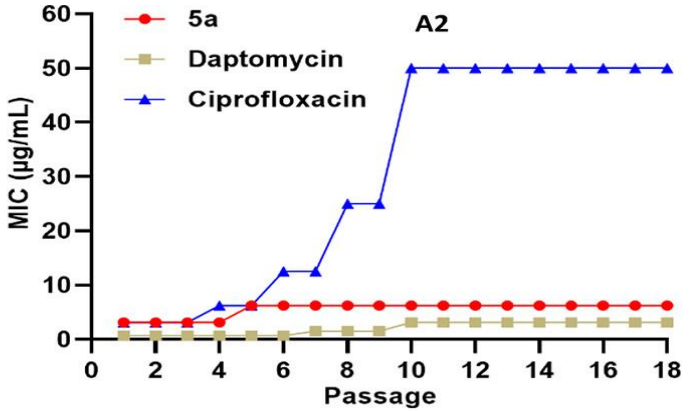


Resistance induction after 18 repeated times of exposure of peptide [R_5W_4] (5a)

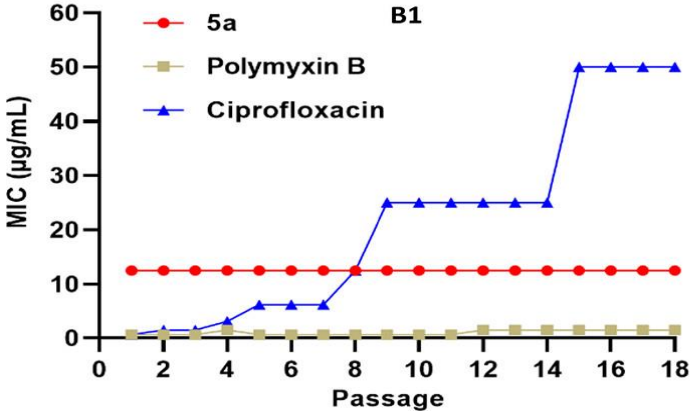
S. aureus (ATCC 29213)



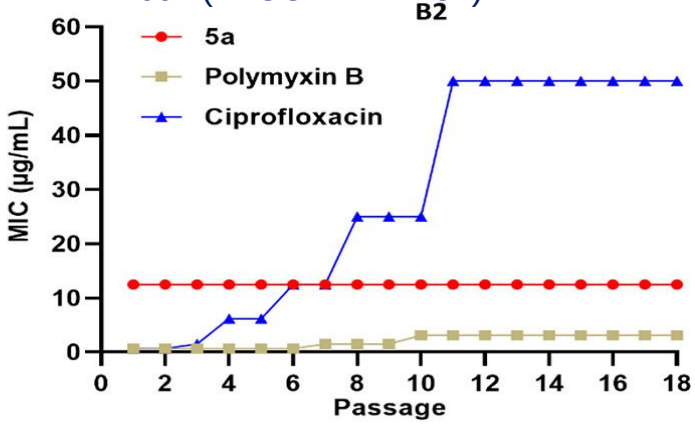
MRSA (ATCC BAA-1556)



E. coli (ATCC 25922)



E. coli (ATCC BAA-2452)



In-Vivo Toxicity Study (preliminary studies)

Animal: CD-1 Mice
Route of Administration: IP
Dose: Once daily for 6 days

Group (Dose level)	No. of Animals	Observation [R_4W_5]
Vehicle	6 (3M/3F)	Non-toxic
5 mg/kg	6 (3M/3F)	Non-toxic
10 mg/kg	6 (3M/3F)	Non-toxic
25 mg/kg	6 (3M/3F)	Non-toxic
50 mg/kg	6 (3M/3F)	Toxic (mortality)

Confidential

Confidential

Efficacy of IV administered IFX-301 [R_5W_4] against a lethal dose of MRSA Infection 2×10^7 CFU/neutropenic mouse (50% of mice survived through 6 days, 13% with PBS) Vancomycin (10 mg/kg)

Confidential

Body Weight after IV administration of IFX-301 [R_5W_4] against a lethal dose of MRSA (NRS-71) Infection 2×10^7 CFU/neutropenic mouse (50% of mice survived through 6 days). Vancomycin (10 mg/kg)

Conclusions

- Broad-spectrum activity of lead peptides against Gram-Positive and Gram-Negative Bacterial Pathogens
- Activity against multi-drug resistant bacteria
- NMR and Molecular Dynamic Simulations confirmed the peptide interactions with the lipids mimicking bacterial membrane.
- Significant synergistic activity with other antibiotics
- Low cytotoxicity
- Efficacy *in vivo*

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- Dr. Rakesh Tiwari
- Dr. Jason Yamaki

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- Dr. Dindyal Mandal
- Dr Naglaa Salem El-Sayed

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- Ajayi Akinwale David
- Eman Mohammed

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CHAPMAN UNIVERSITY

AJK BIOPHARMACEUTICAL

NIH National Institute of Allergy and Infectious Diseases