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A short non-cytotoxic antimicrobial peptide designed from Aβ29-40 adopts nanostructure and shows *in vivo* anti-endotoxin activity†

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Aβ29-40 residues with tryptophan in place of the lone methionine residue and three arginine residues added to its C-terminus exhibited augmented antibacterial activity and protected mice against a lethal dose of LPS. The results show the conversion of Aβ29-40 segment into a cell-selective antimicrobial/anti-endotoxin peptide having nanostructure and cation-π interaction.

Treatment against microbial infection faces considerable challenges due to the development of resistance in microorganisms against many of the conventional antibiotics.[1, 2]. Besides, there is no remedy against sepsis which results from the uncontrolled production of pro-inflammatory responses by the bacterial membrane components mostly when dead/live bacteria come in contact with the blood in the host. Cationic AMPs lyse the membrane of microorganisms with drastic alteration in morphology within a short time and hence the development of resistance against these molecules will not be easy[3, 4]. Thus the molecules that selectively target the membrane of microorganisms are considered as appropriate candidates for future antimicrobial drug development. Self-assembling property, particularly the ability of the molecules to adopt nanostructures has found advantages for their use as biomaterials in different biomedical applications[5-8]. Even antimicrobial molecules with nanostructures seem to have more positive points than their counterpart without nanostructures[9]. Antimicrobial peptide, protegrin-1 adopts nanofibrillar structure like Aβ peptide[10]. Interestingly, broad-spectrum antimicrobial activities have been reported for $A\beta_{40}$ and $A\beta_{42}$ that self-assemble into nanofibrillar structures[11, 12]. Further, nanobiomaterials have been prepared with $A\beta_{16-22}$ segment[13]. Thus we hypothesize that Aβ peptide could be a suitable parent molecule for designing short AMPs with nanostructure.

Amino acid region, 16-22, employed previously for the design nanobiomaterial, contains a FF stretch. In search of new sequences in Aβ peptide with the ability to form nanostructures, we have identified the longest hydrophobic segment of Aβ peptide from G_{29} to V_{40} (12-residues) which is present in both $A\beta_{40}$ and $A\beta_{42}$ and contains II, GG and VV stretches that frequently occur in other amyloid-forming peptides[14, 15]. $A\beta_{29-40}$ is not soluble in water, acetonitrile or in the mixture of both in different ratios. In order to solubilize as well as to introduce cationicity in this Aβ stretch for enabling it to interact with the negatively charged membrane of bacteria, varying number of cationic arginine residues were introduced at its C-terminus. We observed that the variant of $A\beta_{29-40}$ containing three arginine residues ($A\beta_{29-40}$-V1) possesses moderate antimicrobial activity. To introduce an intrinsic fluorescent reporter in the molecule, a variant of $A\beta_{29-40}$-V1 was designed ($A\beta_{29-40}$-V2) by replacing the methionine residue at the 7th position with a tryptophan residue. Further, to investigate any specific effect of the tryptophan residue, a variant ($A\beta_{29-40}$-V3) was designed by replacing the methionine residue of $A\beta_{29-40}$-V1 with another aromatic residue namely, phenylalanine. To distinguish between arginine and lysine residues and explore if tryptophan-containing peptide has any preference for arginine residues, three arginine residues of $A\beta_{29-40}$-V2 were replaced with three lysine residues ($A\beta_{29-40}$-V4). The designed variants resemble lipid-like peptides with a polar head (arginine/lysine residues) and a non-polar tail (12 a.a. stretch) that are known to undergo self-assembly to form stable and well-ordered nanostructures[16].

Table 1 Sequences and molecular weights of $A\beta_{29-40}$ and its variants*

$A\beta_{29-40}$ Variants	Sequence	Calculated Mass	Observed Mass	RP-HPLC retention time (min)
$A\beta_{29-40}$	GAIIGLMVGGVV	1084.38	ND	ND
$A\beta_{29-40}$-V1	GAIIGLMVGGVVRRR	1552.94	1552.9	12.05
$A\beta_{29-40}$-V2	GAIIGLWVGGVVRRR	1607.95	1607.9	12.66
$A\beta_{29-40}$-V3	GAIIGLFVGGVVRRR	1568.91	1568.8	12.91
$A\beta_{29-40}$-V4	GAIIGLWVGGVVKKK	1523.91	1523.9	12.53

*HPLC chromatograms and mass spectra are shown in Fig. S1-S3 (ESI†).

The designed Aβ-variants did not show any significant hemolytic activity against human red blood cells (hRBCs) even at 100 μM concentration (Fig. 1a). The variants also did not cause any significant loss of viability of 3T3 cells (Fig. 1b) as determined by MTT assay at the same concentration. Further,

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all the mice survived and remained healthy after administering 25 mg/kg dose of $A\beta_{29-40}$-variants in seven days experiment ruling out any *in vivo* cytotoxic effect of these peptides (Fig. 1c).

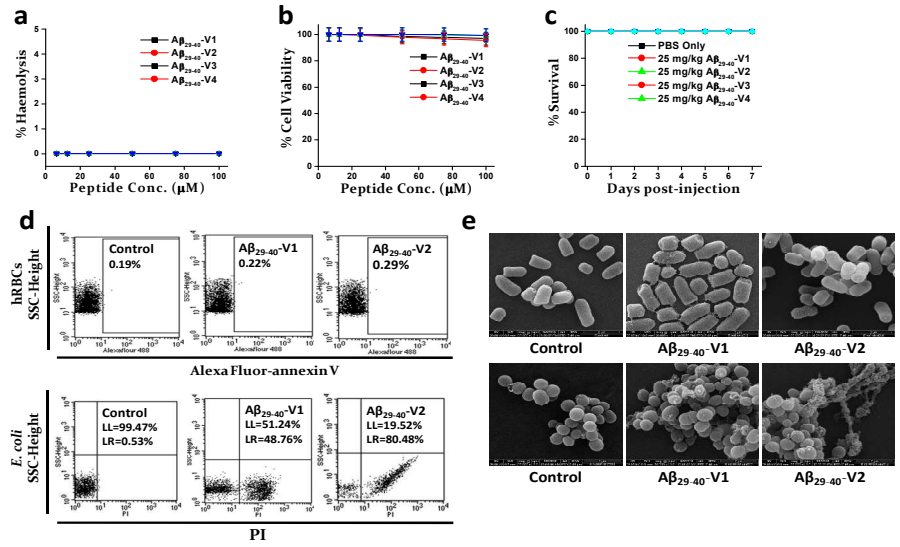

Fig. 1 (a) Hemolytic activity of $A\beta_{29-40}$-variants against hRBCs. (b) Viability of murine 3T3 cells in the presence of $A\beta_{29-40}$-variants. (c) *In vivo* toxicity of the variants, was determined using 4-6 week old male BALB/c mice (n=5). The mice were injected intraperitoneally (i.p.) with $A\beta_{29-40}$-variants (25 mg/kg of body weight). All mice were alive after 7 days. (d) Detection of peptide-induced Alexa Fluor-annexin V staining of hRBCs (upper panel) and PI staining of *E. coli* ATCC 25922 (lower panel). Left quadrants of upper and lower panels show the staining of control hRBCs and *E. coli* without any peptide treatment. $A\beta_{29-40}$-V3 and $A\beta_{29-40}$-V4 are neither hemolytic nor possess any significant antibacterial activity and did not induce any staining of either hRBCs or *E. coli* by these probes and hence data are not shown. Concentrations of each of the peptides for hRBCs and bacteria were 50 and 15 μM, respectively. (e) SEM images of *E. coli* and *S. aureus* in the presence of peptides

As mentioned already that $A\beta_{29-40}$-V1 exhibited moderate antimicrobial activity against the selected Gram-negative and Gram-positive bacteria (Table 2). However, $A\beta_{29-40}$-V2 showed significantly augmented antibacterial activity against the same bacteria when methionine residue of $A\beta_{29-40}$-V1 was replaced with a tryptophan residue (Table 2). Interestingly, $A\beta_{29-40}$-V3 with tryptophan residue of $A\beta_{29-40}$-V2 replaced with phenylalanine residue or $A\beta_{29-40}$-V4 with three arginine residues in $A\beta_{29-40}$-V2 replaced with three lysine residues did not show any appreciable antibacterial activity against tested microorganism. Antibacterial activity of the active peptides, $A\beta_{29-40}$-V1 and $A\beta_{29-40}$-V2, was also examined in the presence of serum and Na^+/K^+ salt at physiological concentrations

Table 2 Antibacterial activity of $A\beta_{29-40}$ variants

$A\beta_{29-40}$ variant	Minimum inhibitory concentration (MIC) in μM										
	Normal					10% Serum		Salts			
	EC	*PA*	*KP*	*SA*	*BS*	*EC*	*SA*	*EC*		*SA*	
								NaCl	KCl	NaCl	KCl
$A\beta_{29-40}$-V1	17.0	45.0	45.0	17.0	17.0	58.5	>100	20.5	18.5	48.8	45.5
$A\beta_{29-40}$-V2	4.0	6.5	4.0	4.0	4.0	14.0	32.5	6.5	6.0	5.0	4.5
$A\beta_{29-40}$-V3	100	100	100	100	100	>100	>100	>100	>100	>100	>100
$A\beta_{29-40}$-V4	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

Minimum inhibitory concentrations (MICs) were determined as the lowest concentration of peptide that caused 100% inhibition of microbial growth. The bacteria are EC, *E. coli* ATCC 25922; PA, *P. aeruginosa* ATCC BAA-427; KP, *K. pneumoniae* ATCC 27736; SA, *S. aureus* ATCC 25923; BS, *B. subtilis* ATCC 6633

(Table 2). Though there were losses in activity, $A\beta_{29-40}$-V2 still showed appreciable antibacterial activity in the presence of serum whereas and it almost retained its activity both in the presence of NaCl (150 mM) and KCl (4.5 mM) against *E. coli* and *S. aureus*.

Tryptophan substituted variant, $A\beta_{29-40}$-V2 showed faster bactericidal kinetics than that of methionine containing variant and killed bacteria within 2 h (Fig. S4, ESI†). Further, $A\beta_{29-40}$-V1 and $A\beta_{29-40}$-V2 induced significant propidium iodide (PI) staining of *E. coli* suggesting their ability to damage the membrane organization of bacteria (Fig. 1d, lower panel). FACS data of Alexa Fluor-annexin V staining of hRBCs suggested that both the variants didn't cause any damage to the membrane organization of hRBCs (Fig. 1d, upper panel). Both *E. coli* and *S. aureus* treated with either $A\beta_{29-40}$-V1 or $A\beta_{29-40}$-V2 showed appreciable changes in their cellular morphology, including wrinkling, surface roughening, membrane blebbing and leakage of bacterial contents (Fig. 1e). The results indicated that

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bacterial membrane could be the possible targets of these peptides.

To get more insight on the mode of action of these peptides, Peptides-induced depolarization of hRBCs, live bacteria and mammalian (PC/Chol) and bacterial membrane mimetic (PC/PG) lipid vesicles was evaluated by employing a potential-sensitive dye diSC$_3$-5. These Aβ$_{29-40}$-variants failed to induce depolarization in hRBC membrane, as well as in zwitterionic, PC/Chol lipid vesicles as indicated by the plot of percentage of fluorescence recovery versus peptide concentration (Fig. S5a and Fig. S6a, ESI†). However, both Aβ$_{29-40}$-V1 and Aβ$_{29-40}$-V2 depolarized either *E. coli* or *S. aureus* and negatively charged, PC/PG vesicles appreciably (Fig. S5b, c and Fig. S6b, ESI†).

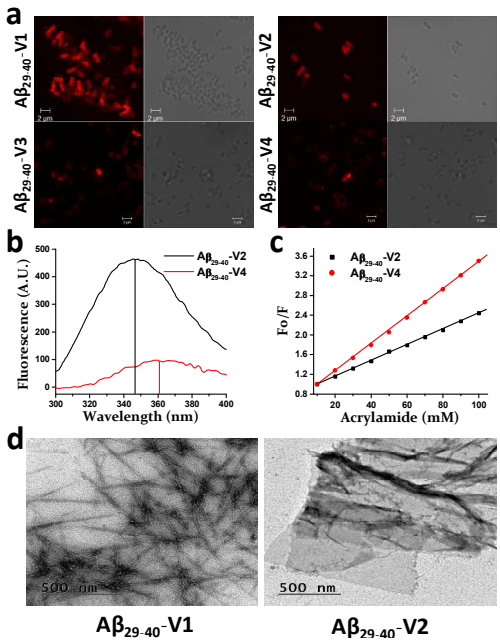

Fig. 2 (a) Confocal microscopy for the detection of localization of Rho-labeled Aβ$_{29-40}$-variants onto *E. coli*. For each variant treatment (concentration, 15 μM), fluorescence and DIC images of *E. coli* are shown. (b) Fluorescence spectra of Aβ$_{29-40}$-V2 and Aβ$_{29-40}$-V4 in the presence of PC/PG lipid vesicles (200 μM) in PBS. (c) Stern-Volmer plots for acrylamide quenching of tryptophan fluorescence of Aβ$_{29-40}$-V2 and Aβ$_{29-40}$-V4 in PC/PG lipid vesicles. (d) TEM images of nanostructure forming property of Aβ$_{29-40}$-V1 and Aβ$_{29-40}$-V2.

Confocal microscopic images indicated that Rho-labeled Aβ$_{29-40}$-V1 and Aβ$_{29-40}$-V2 significantly bound to bacteria whereas binding of Rho-labeled Aβ$_{29-40}$-V3 and Aβ$_{29-40}$-V4 was significantly lower (Fig. 2a). The Images further suggest that Aβ$_{29-40}$-V1 and Aβ$_{29-40}$-V2 localized throughout the cells indicating their ability to penetrate *E. coli* membrane. Since fluorescence of tryptophan residue is sensitive to its environment, fluorescence spectra of Aβ$_{29-40}$-V2 and Aβ$_{29-40}$-V4 that contain single tryptophan residue were recorded. Following the addition of bacterial membrane mimetic PC/PG lipid vesicles, a significant enhancement in fluorescence along with blue-shift of tryptophan emission maximum was observed for Aβ$_{29-40}$-V2. However, Aβ$_{29-40}$-V4 neither showed appreciable enhancement in fluorescence nor shift of its emission maximum towards the shorter wavelength (Fig. 2b). The result suggested that Aβ$_{29-40}$-V2 interacted appreciably with PC/PG vesicles resulting in the localization of its tryptophan residue in the hydrophobic milieu of this kind of membrane, whereas, tryptophan residue of Aβ$_{29-40}$-V4 was located in the polar environment of PC/PG lipid vesicles suggesting its weaker interaction with bacterial membrane mimetic lipid vesicles. Further, Aβ$_{29-40}$-V2 exhibited lesser acrylamide quenching of tryptophan fluorescence than that of Aβ$_{29-40}$-V4 (Fig. 2c) in PC/PG vesicles indicating lower accessibility of its tryptophan residue to the quencher which is supportive of the blue-shift of its tryptophan emission maximum in these lipid vesicles. Self-assembly of Aβ$_{29-40}$-V1 and Aβ$_{29-40}$-V2 was studied by TEM. TEM images indicated that Aβ$_{29-40}$-V1 formed filamentous structure and Aβ$_{29-40}$-V2 formed sheet-like assemblies (Fig. 2d). FTIR spectra of these two Aβ$_{29-40}$-variants Aβ$_{29-40}$-V1 and Aβ$_{29-40}$-V2 also showed a prominent peak (low frequency) at around 1620 cm^{-1} and 1623 cm^{-1}, respectively, corresponding to amide I region (1600-1700 cm^{-1}) which indicates parallel β-sheet (1615-1637 cm^{-1})[17] structure of the peptide (Fig. S7, ESI†). CD spectra of these Aβ$_{29-40}$-variants also showed characteristic β-sheet structure (Fig. S8, ESI†).

THP-1 monocytes, bound to Fluorescein isothiocyanate-labeled lipopolysaccharide (FITC-LPS), showed MFI of 28.19. However, THP-1 cells showed MFI of 6.04 when they were treated with FITC-LPS which was pre-treated with Aβ$_{29-40}$-V2 suggesting that Aβ$_{29-40}$-V2 significantly inhibited the binding of FITC-LPS to THP-1 cells (Fig. 3a). Aβ$_{29-40}$-V1 too inhibited binding of FITC-LPS to THP-1 cells but to a lesser extent than Aβ$_{29-40}$-V2. Aβ$_{29-40}$-V2 inhibited LPS-induced production of TNF-α, IL-1β in THP-1 cells more efficiently than that of Aβ$_{29-40}$-V1 (Fig. 3b, c).

After examining *in vitro* anti-endotoxin activities of Aβ$_{29-40}$-V1 and Aβ$_{29-40}$-V2, we further investigated their efficacy in protecting BALC/c mice against LPS-challenge. The mice group challenged with LPS alone died within 24 h of LPS administration (Fig. 3d). However, Aβ$_{29-40}$-V2 at doses of 5 and 10 mg/Kg demonstrated 60 and 100 % survival of mice challenged with lethal dose of LPS in seven days experiment (Fig. 3d). When Aβ$_{29-40}$-V2 was replaced with Aβ$_{29-40}$-V1 in the same experiment 40 and 80% survival of mice were observed. Aβ$_{29-40}$-V1 and Aβ$_{29-40}$-V2 inhibited LPS-induced production of TNF-α and IL-6 in mice which could be the basis of survival of mice against LPS-challenge in presence of these peptides (Fig. S9, ESI†).

The most remarkable observation in this study is the significant augmentation (~4 times) of antibacterial activity of Aβ$_{29-40}$-variant, Aβ$_{29-40}$-V1 after replacing its lone methionine residue with tryptophan residue. Moreover, Aβ$_{29-40}$-V2 showed faster kinetics to kill bacteria than Aβ$_{29-40}$-V1. Remarkably, despite having very similar amino acid composition and cationic charge both Aβ$_{29-40}$-V3 and Aβ$_{29-40}$-V4 failed to exhibit any appreciable antibacterial activity. Moreover, Aβ$_{29-40}$-V3 and Aβ$_{29-40}$-V4 bound to *E. coli* much weakly than Aβ$_{29-40}$-V1 and Aβ$_{29-40}$-V2 (Fig. 2a). Tryptophan fluorescence studies indicated that unlike Aβ$_{29-40}$-V2, Aβ$_{29-40}$-V4 bound poorly to PC/PG vesicles.

We propose that this discriminatory ability of these Aβ$_{29-40}$-variants to participate in membrane-interaction and show antibacterial activity is due to the strong cation-π interaction between the tryptophan and arginine residues which could be the driving force for the interaction of positively charged arginine residues of Aβ$_{29-40}$-V2 with either negatively charged lipid vesicles or negatively charged bacterial membrane.

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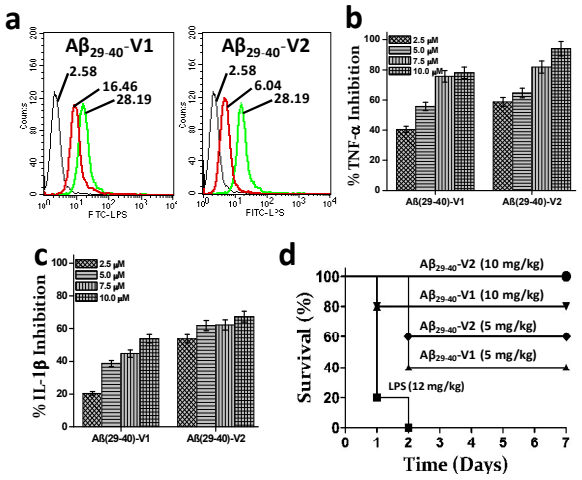

Fig. 3 (a) Effects of $A\beta_{29-40}$-V1 and $A\beta_{29-40}$-V2 on binding of FITC-LPS to THP-1 cells. (b) and (c), Dose-dependent percentage inhibition of LPS-induced secretion of TNF-α and IL-1β respectively in THP-1 cells. (d), Survival of BALB/c mice against intraperitoneally (i.p.) injected *E. coli* LPS (12 mg/kg) followed by administration of single dose of $A\beta_{29-40}$-V1 or $A\beta_{29-40}$-V2 at two different concentrations (5 and 10 mg/kg) after 15 min. Only LPS treated mice died within 24 h, mice treated with LPS and peptides were observed for seven days.

Cation-π interaction between arginine and tryptophan residues could facilitate interaction between $A\beta_{29-40}$-V2 and negatively charged bacterial membrane[18-20] resulting in the augmentation of its antibacterial activity. When arginine and tryptophan residues are engaged in cation-π interaction, arginine residues are still capable of participating in hydrogen bonding with the water molecules in the surrounding membrane environments[21-23]. However, when lysine residues are involved in cation-π interaction with tryptophan residues, these cationic residues are no-longer capable of forming hydrogen bonds with the surrounding water molecules in the membrane environments[23]. Probably thus $A\beta_{29-40}$-V4 failed to show any significant binding to either bacterial membrane or bacterial membrane mimetic negatively charged lipid vesicles and exhibit any appreciable antibacterial activity. Inactivity of $A\beta_{29-40}$-V3 suggested that aromatic phenylalanine residue cannot compensate the tryptophan residue in cation-π interaction as also indicated in the literature that arginine-tryptophan is the best combination for cation-π interaction[18, 20]. Being an aliphatic amino acid, methionine is not involved in cation-π interaction; therefore, no augmentation or attenuation of interaction between the cationic arginine residues of $A\beta_{29-40}$-V1 with negatively charged bacterial membrane occurs. Thus $A\beta_{29-40}$-V1 exhibits moderate antibacterial activity.

In conclusion, the results show the engineering of a short stretch of Aβ peptide into non-cytotoxic, potent antibacterial and anti-endotoxin peptide with nanostructures for the first time to our knowledge. The results further strengthen the notion that Aβ sequence could be used for the design of novel nanobiomaterials including short antimicrobial agents. The results also demonstrate the manifestation of cation-π interaction between arginine and tryptophan residues in augmenting antimicrobial activity of a peptide. Further, $A\beta_{29-40}$ derived peptides seem to be nice models to show the implication of cation-π interaction in antimicrobial activity of short peptides.

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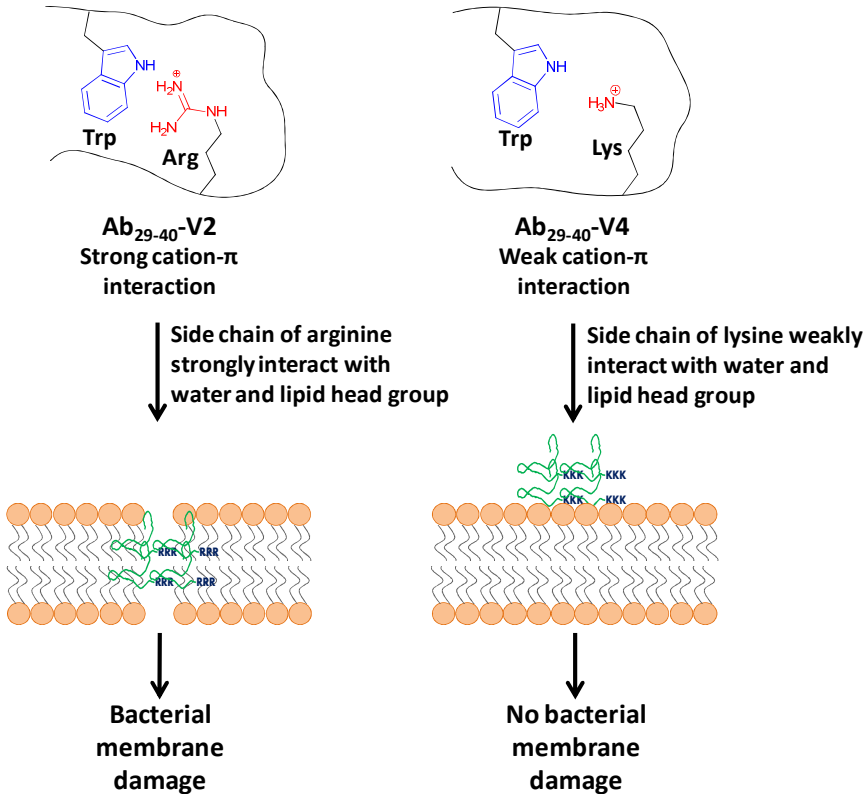

A schematic presentation showing the plausible mechanism of antimicrobial activity of $A\beta_{29\text{-}40}$-V2 and $A\beta_{29\text{-}40}$-V4.