Binding studies of antimicrobial peptides to *E.coli* cells

Concetta Avitabile[1], Luca D. D'Andrea[1], Michele Saviano[2], Michele Olivieri[3], Amelia Cimmino[3] and Alessandra Romanelli[1,4*]

[1] Istituto di Biostrutture e Bioimmagini, CNR, via Mezzocannone 16, 80134 Napoli (Italy)

[2] Istituto di Cristallografia, CNR, Via Amendola 122, 70126 Bari (Italy)

[3] Istituto di Genetica e Biofisica "A. Buzzati Traverso", CNR, via Pietro Castellino 111, 80131 Napoli (Italy)

4 Dipartimento di Farmacia, Università di Napoli "Federico II", via Mezzocannone 16, 80134 Napoli (Italy)

Corresponding author e-mail: alessandra.romanelli@unina.it

Abstract

Understanding the mechanism of action of antimicrobial peptides is pivotal to the design of new and more active peptides. In the last few years it has become clear that the behavior of antimicrobial peptides on membrane model systems does not always translate to cells; therefore the need to develop methods aimed at capturing details of the interactions of peptides with bacterial cells is compelling. In this work we analyzed binding of two peptides, namely temporin B and TB_KKG6A, to *E.coli* cells and to *E.coli* LPS. Temporin B is a natural peptide active against Gram positive bacteria but inactive against Gram negative bacteria, TB_KKG6A is an analogue of temporin B showing activity against both gram positive and Gram negative bacteria. We found that binding to cells occurs only for the active peptide TB_KKG6A; stoichiometry and affinity constant of this peptide toward *E.coli* cells were determined.

Keywords: peptide**,** antimicrobial, cell, binding, temporin, dissociation constant

1. Introduction

A deep understanding of the mechanism of action of antimicrobial peptides requires the characterization of the interactions of peptides with bacterial cells. Targets of antimicrobial peptides have been identified in few cases; for example it is known that lantibiotics bind to lipid II and inhibit cell wall biosynthesis [1]. Peptides active against gram negative bacteria seem to interact with the component of the bacterial cell wall, including the lipopolysaccharide (LPS). Antimicrobial cationic peptides massively bind to the bacterial membrane until a critical threshold is reached[2]. Recognition of bacteria by peptides seems to occur through the LPS[3]. The composition of the LPS is critical for a number of processes related to bacterial death such as bacteria agglutination and membrane permeabilization. It is reported that length of the polysaccharide moiety of LPS affects the agglutination of cells mediated by the eosinophil cationic protein (EOP) [4]. When strains with fully truncated LPS are treated with the EOP, agglutination of bacteria does not occur, as demonstrated by scanning electron microscopy experiments. Interestingly, the antimicrobial activity of EOP is related to agglutination of cells[4]. In addition, time-resolved flow cytometry studies reported by Freire et al. demonstrated that the permeabilization kinetics of *E.coli* strains differing for the composition of the LPS by the peptide pepR depend on the composition of the LPS; in particular, membrane permeabilization is blocked when LPS components are removed[5]. The LPS strongly contributes

to the folding of peptides on the cell surface, but is not the only responsible[6,7]. Recent NMR studies reported by our group have demonstrated, in fact, that the three-dimensional structure of the antimicrobial peptide TBKK_G6A obtained in the presence of *E.coli* cells differs from that observed in the presence of *E.coli* LPS, suggesting that all the components of the bacterial outer leaflet do affect the interaction of peptides with bacteria[7,8]. The affinity of antimicrobial peptides for the components of the bacterial outer leaflet such as LPS or lipid mixtures has been widely investigated so far and it has been found that active peptides bind LPS or lipids (tipically binding constants are in the micromolar range). In one case the interaction of the peptide PMAP-23 with *E.coli* bacterial cells and with large unilamellar vescicles formed by lipids extracted by *E.coli* has been investigated: it was shown that the binding isotherms obtained with lipids look similar to those obtained with bacterial cells[9].

A very important question is related to the model system employed to mimick the bacterial outer membrane and to the specificity of binding of peptides towards such model systems as compared to bacterial cells. Interestingly, experiments reported by Bhunia et al [10], demonstrated that the peptide temporin B, which is not active against gram-negative bacteria, shows high binding affinity toward *E.coli* LPS. This was the first indication of the fact that the ability of peptides to bind LPS is not necessarily related to their antimicrobial activity. A very limited number of studies focused on the interaction of antimicrobial peptides with living cells has been reported so far[9,11] and a still unanswered question related to the affinity of binding of active and inactive antimicrobial peptides toward bacterial cells remains. The present work means to fill this gap by analyzing the binding of two different peptides, temporin B (TB) and TB_KKG6A to *E.coli* cells and in parallel to *E.coli* LPS, using fluorescence spectroscopy. Temporin B is a natural peptide active against gram positive bacteria, but inactive against gram negative bacteria[12] while TB_KKG6A is a synthetic analogue of temporin B developed in our lab showing activity against gram positive and gram negative bacteria [8,13].

2. Materials and methods

All peptides investigated in this work were obtained following standard procedures[8,14]. Peptides were purified by RP-HPLC on a Phenomenex Jupiter 10µ Proteo 90 Å (250×10 mm) column and characterized by LC-MS on a Thermo Finnigan instrument equipped with an electrospray source (MSQ) on a Phenomenex Jupiter 5 µ C18 300 Å, (150×4.6 mm) column or on LC-MS Agilent Technologies 6230 ESI-TOF on a Phenomenex Jupiter 3µ C18 (150x2.0 mm) column with a flow rate of 0.2 $mL \cdot min^{-1}$.

Temporin B (TB) sequence: LLPIVGNLLKSLL. Mass calculated (Da): 1390.52 Da; found (Da): 1390.93 . $[M+1H]^{+}$: 1391.93 ; $[M+2H]^{2+}$: 696.48.

Temporin B-NBD (TB-NBD) sequence NBD-Ahx-LLPIVGNLLKSLL. Mass calculated (Da): 1667.95; found (Da): 1667.82. $[M+2H]^{2+}$: 834.96; $[M+3H]^{3+}$: 556.97

TB_KKG6A sequence: KKLLPIVANLLKSLL. Mass calculated (Da): 1661.20 Da; found (Da): 1661.07 Da. $[M+1H]^{+}$: 1662.07; $[M+2H]^{2+}$: 831.54; $[M+3H]^{3+}$: 554.70.

TB_KKG6A –NBD sequence: NBD-Ahx- KKLLPIVANLLKSLL. Mass calculated (Da): 1937.50; found (Da): 1937.82. $[M+2H]^{2+}$: 969.91; $[M+3H]^{3+}$: 646.94.

Escherichia coli BL21 (DE3) cells were grown in LB medium at 37 °C, harvested while in exponential phase (OD_{600} nm: 0.6-0.8), centrifuged (5000 rpm, 10 min, 4ºC) and resuspended in 10 mM phosphate buffer pH

7.0 at the desired concentration. Concentration of peptides was evaluated by UV, reading the absorbance at 205 nm, and using the following ε values: TB_KKG6A (ε_{205}= 39320 M^{-1} cm^{-1}) and TB (ε_{205}= 33760 M^{-1} cm^{-1}). on a Thermo Fisher Scientific Inc (Wilmington, Delaware USA) Nanodrop 2000c spectrophotometer.
Samples for the fluorescence experiments were prepared in a total volume of 50μL, in a Black 384-Well plate (OptiPLATE^{TM}-384F, Perkin Elmer). Fluorescence measurements were carried out on an Espire Multimode Reader (Perkin Elmer). Fluorescence excitation wavelength was set at 468 nm, fluorescence emission at 530 nm. Points were collected every 2.5 minutes for 90 minutes at 25°C. All measurements were carried out in quadruplicate.

2.1 Determination of the peptide/cell binding stoichiometry

Peptides labelled with NBD at 1 μM and 3 μM concentrations were incubated with different amounts of cells in phosphate buffer, 10mM pH 7.0. The concentration of cells ranges from 0 to 2.5 pM (0-1.5 OD_{600}). The fluorescence of cells in buffer was also recorded as a blank.

2.2 Determination of the binding constants of peptides to *E.coli* cells

Peptides at different concentrations were incubated with *E.coli* cells at 0.8 pM concentration (0.5 OD_{600}). The peptide concentration ranges from 0.1 to 6.0 μM for TB_KKG6A and for TB. Peptides in buffer were employed as a blank.

2.3 Microscopy experiments

The peptides TB_KKG6A, TB_KKG6A NBD, TB and TB NBD at 1μM concentration were incubated with *E.coli* cells at 0.5 and 2 pM concentration (0.3 and 1.2 OD_{600}) in phosphate buffer 10mM, pH 7.0 for 30 minutes. Images were taken at the Nikon ECLIPSE Ni-U microscope equipped with a Ds-Riu camera and using a 40× microscope objective. For general fluorescence microscopy purposes, we have used following settings for filters: wavelength excitation 475-495nm, emission 510 nm.

2.4 Determination of the peptide/LPS binding stoichiometry

Peptides TB_KKG6A NBD at 0.5 μM concentration and TB at 3 μM concentration were incubated with different amounts of *E.coli* LPS O111:B4 in phosphate buffer, 10mM pH 7.0. The LPS was treated as described in the literature[8]. The LPS micelle concentration ranges from 0 to 0.08 μM (0-5 μM LPS concentration) for experiments carried out with TB_KKG6A and from 0 to 0.25 μM (0-12 μM LPS concentration) for experiments with TB . The fluorescence of LPS in buffer was also recorded as a blank.

2.5 Determination of the binding constants of peptides to *E.coli* LPS

Peptides at different concentrations were incubated with LPS at 10 μM. The peptide concentration ranges from 0.1 to 1.5 μM for TB_KKG6A and from 0.1 to 12μM for TB. Peptides in buffer were also employed as a blank.

3. Results and discussion

Initial studies were devoted at estimating the stoichiometry of binding of peptides to *E.coli* cells by fluorescence. All peptides were labelled at the N-terminus with the nitrobenzodiazole (NBD) probe, as this probe is sensitive to the environment, showing an intense signal in hydrophobic media. The use of NBD labelled peptides allows us to detect only peptides bound to the bacteria, as the fluorescence signal of free peptides in solution is low as well as the autofluorescence of cells at 530 nm. Experiments were carried out in phosphate buffer at pH7, as these conditions were demonstrated to be compatible with the survival of our cells[6]. Measurements were carried out at 1 and 3 μM concentration of peptides and at cell concentrations ranging from 0 to 2.5 pM. Amount of cells are usually expressed in terms of absorbance at 600nm (OD_{600}). The conversion between OD_{600} and molarity is possible considering the correspondence between the number of cells/mL and OD_{600} ($1x10^9$ cells/mL=1 OD_{600}) ; from the number of cells/mL, using the Avogadro number, the moles of cells/mL and then the concentration of cells can be calculated. The use of molar concentration for peptides and cells allows us to roughly estimate the bound peptide/cells molar ratio. As the fluorescence signal hardly stabilizes after few minutes of incubation of peptides with cells, we measured the fluorescence of different samples obtained mixing a fixed concentration of peptide with increasing concentrations of cells and plotted the intensity of fluorescence at 530nm collected after 60 minutes of incubation (as at this timepoint the fluorescence signal is stable for all mixture) versus cell concentration. The fluorescence signal of the peptide TB_KKG6A without cells is higher than that observed after the addition of a small amount of cells; this data is in agreement with results previously obtained by NMR, demonstrating that the peptide is aggregated in solution and monomeric when bound to *E.coli* cells[7]. At 1 and 3 μM concentration of TB_KKG6A the intensity of fluorescence strongly increases with the cell concentration, reaching an initial plateau when the concentration of cells is about 1.2 pM; a second plateau is reached at about 2pM cell concentration (Figures 1 and S1). For the peptide TB_KKG6A at the two different concentrations the plateau in the fluorescence signal is reached at very high peptide/cell molar ratios. The first plateau is reasonably due to cell surface saturation by peptides, which occurs at a $1x10^6$: 1 molar ratio of peptides/cell. This result supports the finding hypothesized by the group of Castanho that a large excess of peptide saturates the outer membrane of bacterial cell. At higher concentration of bacteria, the increase in the fluorescence intensity might be due to cell aggregation.

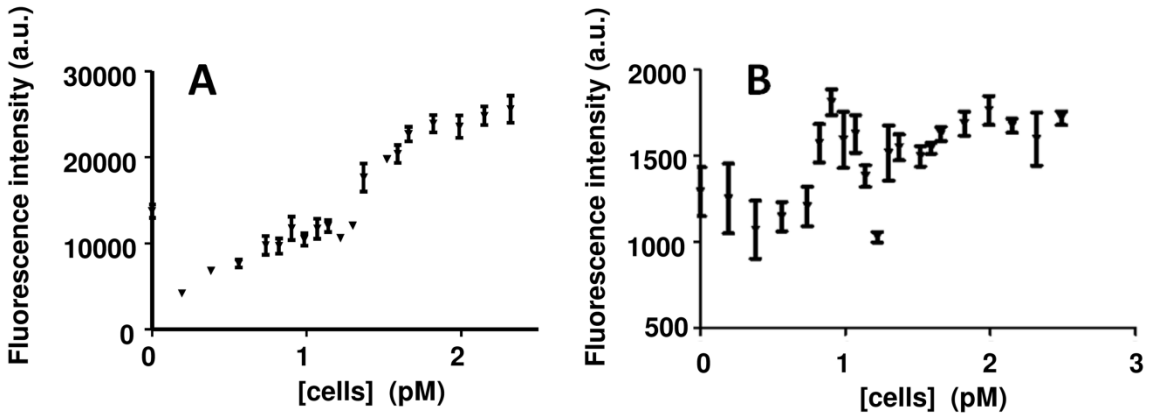

Figure 1. Peptide-cell binding stoichiometry. Plots of fluorescence intensity at 530 nm vs *E.coli* cell concentration recorded after 60 minutes of incubation of the peptides (A: TB_KKG6A, B: TB) with cell mixtures. Peptides' concentration is 3μM.

To verify this hypothesis we performed an analysis by transmission electron microscopy on *E.coli* cells at 0.5 and 2pM concentrations in buffer and incubated with TB_KKG6A and TB_KKG6A NBD at 1μM concentration for 30 minutes (Figures 2, S2 and S3). Images taken for the mixtures TB_KKG6A + cell and TBKK_G6A NBD + cell at the higher cell concentration confirm the formation of aggregates of cells, in some cases branched.

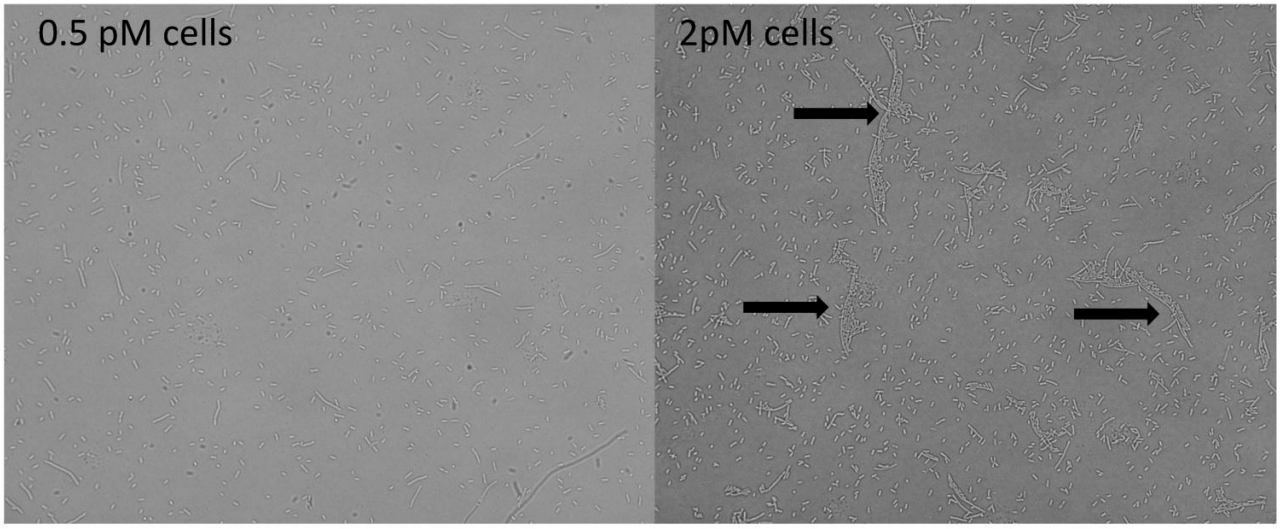

Figure 2. Images of *E.coli* cells + TB_KKG6A 1μM after 30 minutes of incubation at r.t. taken at the transmission electron microscope.

The peptide TBKKG6A may trigger bacterial cell agglutination, a phenomenon observed in few other cases with antimicrobial peptides, which might be mediated by the hydrophobic regions of peptides and results in the inhibition of bacterial growth and consequent death[4,15,16]. Another reasonable hypothesis, supported by the elongated form of the aggregates, is that the peptide inhibits cell division upon membrane disgregation. Bacterial cell division is mediated by the formation of a "Z ring", composed of polymerized FtsZ protein, at the site of division. If the protein FtsZ, is not associated to the membrane, Z rings can form but they are not functional for division and cells give rise to long filaments[17]. It is likely that if the membrane is not intact due to the interaction with the peptide TB_KKG6A, FtsZ cannot associate to it. This mechanism has recently been demonstrated for the cathelin related antimicrobial peptide CRAMP[18].

Fluorescence experiments on mixtures of temporin B and *E.coli* cells do not support a specific interaction of this peptide with *E.coli* cells: large fluctuations of the fluorescence signal were observed (Figures 1 and S1). Transmission electron microscopy experiments also indicate that temporin B does not affect bacterial cells: treated and control cells are identical (Figures S4 and S5).

We measured for 90 minutes the fluorescence of samples obtained incubating cells at 0.5 OD_{600} (0.8 pM) with TB_KKG6A NBD at concentrations ranging from 0 to 6 μM and we found that signals become stable after about 60 minutes (Figure S6). To determine the binding affinity of peptides toward *E.coli* cells, we plotted the fluorescence intensity (measured after 60 minutes of incubation) at 530 nm vs peptide concentration and we calculated the dissociation constant (Kd) of the peptide for *E.coli* cells (Figure 3).

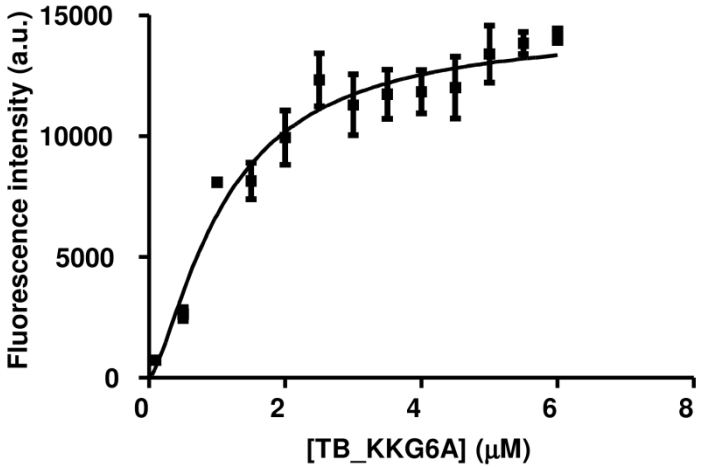

Figure 3. Binding of TB_KKG6A NBD to cells. Plot of the fluorescence intensity at 530 nm vs peptide concentration. The concentration of *E.coli* cells is 0.8 pM (0.5 OD_{600}).

Plots of fluorescence intensity vs peptide concentration approximate hyperbolas for TB_KKG6A. Based on the binding stoichiometry, we considered the following equilibrium reaction:

$$n\ \text{Peptides} + \text{Cell} = (\text{Peptide})_n\text{-Cell}$$

where $n = 1x10^6$. As the peptides are always in large excess as compared to bacterial cells, we can set the nominal concentration of the peptide equal to that of the free peptide. In these conditions it is possible to fit the experimental fluorescence data using the Hill equation, which accounts for binding of n molecules to one target. The Kd value obtained for TB_KKG6A NBD is 1.10±0.19 (μM). We are aware of the fact that the targets of our peptides on a bacterial cell are in principle several, and that the fluorescence signal that we obtain may depend on different binding events. As at the moment we cannot distinguish between different molecular targets on the bacterial cells, we will consider the cell as the "receptor" bearing n identical binding sites for our peptides. In this context the Kd obtained reflects the affinity of binding of this peptide for *E.coli* cells. Identical experiments were carried out also for temporin B; in this case the fluorescence intensity slightly changes with the time (Figure S6). Fitting of the fluorescence vs peptide concentration data was not successful (Figure S7) .

Next we analyzed the binding of peptides to *E.coli* LPS. Experiments aimed at determining the binding stoichiometry were carried out measuring the fluorescence intensity of peptides in presence of LPS micelles, keeping constant the concentration of peptides and increasing micelle concentrations (Figure S8).

Micelle concentration was determined by the equation: [micelle]= ([LPS]-CMC)/N[19]. The [LPS] was determined using an average value of 15000 Da for the molecular weight of LPS; we used the CMC value for *E.coli* LPS O111:B4. of 1.3μM and N, the aggregation number, equal to 43 as reported in the literature[20]. The fluorescence intensity measured for TB_KKG6A at 0.5μM increases at growing micelle concentration, stabilizes in the range of micelle concentration between 0.04 and 0.05 μM reaching a plateau and then keep raising (Figure S8). In the plateau region the molar ratio (TB_KKG6A NBD peptide): (LPS micelle) is about 12. The fluorescence signal measured for TB at 3μM concentration also increases with micelle concentration and stabilizes at 0.13μM LPS micelle (Figure S8). The molar ratio (TB NBD peptide): (LPS micelle) is about 30. These results suggest that an excess of peptides bind to the LPS micelle. In this condition we can assume for the binding of peptides to LPS the same model (n peptides to one LPS micelle)

employed for binding of peptides to cells. To determine the binding affinity of peptides toward LPS we measured the fluorescence signal for mixtures obtained at different peptide concentrations and at LPS micelle concentration of 0.2 μM (Figure 4). In all cases experimental data fit the Hill equation. Fitting of data according to this equation affords the following Kd for temporin B and TB_KKG6A of 1.79±0.38 (μM) and 149±7 (nM), respectively .

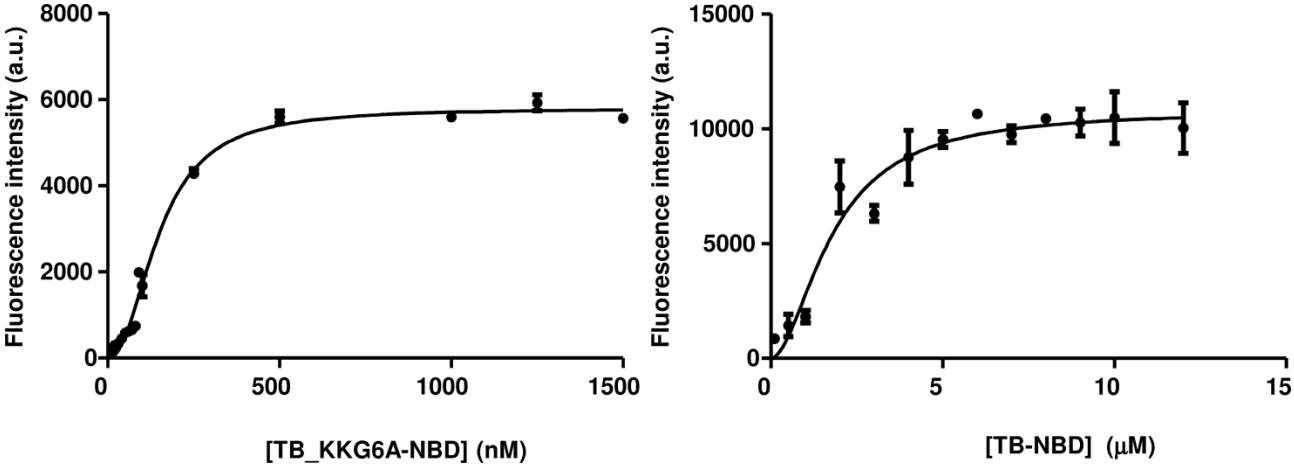

Figure 4. Binding of peptides to LPS micelles. Plots of fluorescence intensity at 530 nm vs peptide concentration at a 0.2μM LPS micelle concentration.

Both peptides bind LPS, although with a different affinity, but only TB_KKG6A binds to cells. These data support the finding that isolated LPS is not a good mimetic of gram negative bacterial cells. We had in fact reported that the three-dimensional structure of the peptide TB_KKG6A in the presence of *E.coli* cells is different as compared to that obtained in the presence of *E.coli* LPS and we have now shown that the binding affinity of peptides to LPS is different as compared to that obtained for *E.coli* cells. In case of TB_KKG6A the difference in the dissociation constant toward cells and LPS might be interpreted in two different ways: or the LPS in the context of a live cell membrane appears different to the peptides as compared to the purified LPS (differences might be due to different aggregation states of LPS for example) or peptides bind to different targets on the cell membrane other than LPS.

In conclusion, we have determined the stoichiometry and the dissociation constant of the antimicrobial peptide TB_KKG6A to *E.coli* cells. The event of bacterial outer membrane saturation by TB_KKG6A is followed by bacteria aggregation. The binding of peptides to *E.coli* LPS is not related to the binding of peptides to bacterial cells.

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