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Cell-Density Dependence of Host-Defense Peptide Activity and Selectivity in the Presence of Host Cells

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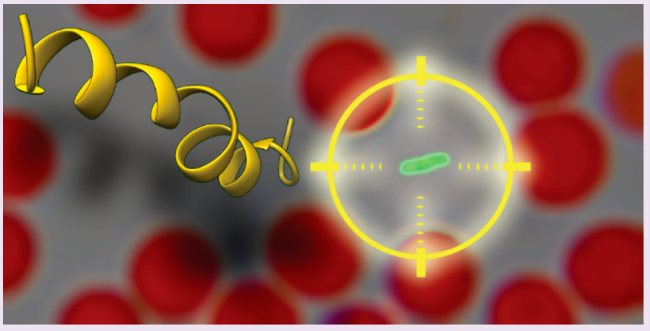
Supporting Information

ABSTRACT: Host-defense peptides (HDPs) are promising compounds against multidrug-resistant microbes. *In vitro*, their bactericidal and toxic concentrations are significantly different, but this might be due to the use of separate assays, with different cell densities. For experiments with a single cell type, the cell-density dependence of the active concentration of the DNS-PMAP23 HDP could be predicted based on the water/cell-membrane partition equilibrium and exhibited a lower bound at low cell counts. On the basis of these data, in the simultaneous presence of both bacteria and an excess of human cells, one would expect no significant toxicity, but also inhibition of the bactericidal activity due to peptide sequestration by host cells. However, this inhibition did not take place in assays with mixed cell populations, showing that for the HDP esculentin-1a(1−21)NH_2, a range of bactericidal, nontoxic concentrations exists and confirming the effective selectivity of HDPs. Mixed-cell assays might be necessary to effectively asses HDP selectivity.



Host-defense peptides (HDPs) are produced by all living organisms as a first defense against pathogens. Multiple immunomodulatory functions of these peptides have been described,[1] but one of their major activities is bactericidal, through the perturbation of the membrane permeability of microbial targets. This mechanism of action makes the development of bacterial resistance particularly unlikely,[2,3] and for this reason these peptides are investigated as lead compounds to fight multidrug-resistant bacteria, a dramatic and increasing worldwide threat.[4] However, several questions still limit their clinical applicability.

A major problem is related to HDP selectivity. *In vitro*, the majority of HDPs are toxic to mammalian cells only at concentrations higher than those needed for bactericidal activity.[5] This behavior is presumably determined by the difference in lipid composition of membranes of the two cell types, as studies on liposomes show a higher affinity for bilayers mimicking bacterial membranes.[6] However, selectivity might be just an experimental artifact resulting from the very different conditions used in assays employed to determine antimicrobial and hemolytic activities, in particular the cell density.[5] Typically, minimal inhibitory concentrations (MICs) are determined with 5×10^5 to 1×10^6 colony-forming units (CFUs) per mL, while minimum hemolytic concentrations (MHCs) are measured in the presence of 5×10^8 cells/mL,[5] and red blood cells (RBCs) have a membrane area approximately 10 times bigger than *E. coli* cells.[7,8] In addition, the use of separate assays is based on the rather drastic assumption that the activity of HDPs against a given cell type is not influenced by the contemporary presence of different cells. However, in principle, when bacteria are in the presence of an excess of human cells, the peptide could be inactivated due to sequestration by host cells. Other important problems relate to the physiological function of HDPs, *i.e.* whether the relatively high peptide concentration needed to kill bacteria can be reached *in vivo* and whether their prevalent activity is bactericidal or immunomodulatory.[9] Clarifying all these issues is essential, since they bear directly on how HDPs are currently screened and optimized.

A recently blooming approach to tackle these questions extends to real cells the quantitative physicochemical experiments normally performed on model membranes.[10−14] Recently, we measured the association to the membranes of *E. coli* cells of a fluorescently labeled analogue of the cathelicidin HDP PMAP-23[15] (DNS-PMAP23, sequence in

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Scheme 1) and determined the threshold value of peptide molecules that must bind to the membranes of a single cell to kill it.[11] On the basis of these results, in the present work we theoretically predicted an unexpected behavior of peptide activity as a function of the density of cells. We also optimized three assay protocols that allowed us to test experimentally this prediction by studying the bactericidal and hemolytic activities in a wide range of cell-density values and to determine both activities under more realistic conditions, *i.e.*, with both cell types present at the same time.

Scheme 1. Sequences of the Peptides Investigated

DNS-PMAP23

Dansyl-RIIDLLWRVRRPQKPKFVTVWVR-NH_2

Esc(1-21)

GIFSKLAGKKIKNLLISGLKG-NH_2

Color code: acid, basic and hydrophobic residues;
Dansyl (DNS) = 5-(dimethylamino)naphthalene-1-sulfonyl.

The so-called "inoculum effect" is a well-known dependence of the MIC of traditional antibiotics on the size of the bacterial inoculum in the growth medium, with the first studies dating back to the 1940s.[16] By contrast, to the best of our knowledge, the cell-density dependence of HDP activity has not been investigated before. In a previous study,[11] we showed that association of at least $T_B = 1.1 \times 10^7$ DNS-PMAP23 molecules to the membranes of each cell is needed to cause the death of 99.9% bacteria, *i.e.*, the % reduction in viable cells normally used to define the minimum bactericidal concentration (MBC).[17] In addition, the measured peptide/cell-membrane association curves (see Supporting Information [SI], Supporting Figure 1) exhibited a behavior that deviated only slightly from an ideal partition,[11,18,19] where the fraction of membrane-associated peptide f_B dependence on bacterial cell-density ([Bacteria]) can be described according to the following equation, derived in the SI:

$$f_B = \frac{[\text{Bacteria}]/K_{app}^B}{1 + [\text{Bacteria}]/K_{app}^B} \quad (1)$$

By fitting the partition curve measured for 10 μM peptide concentration[11] with eq 1, we obtained a value of the apparent partition constant of $K_{app}^B = 1.8 \times 10^8$ bacterial cells/mL (Supporting Figure 1). These data allow a calculation of the fraction of membrane-bound peptide, and thus of the number of membrane-associated peptides per cell, at any value of peptide concentration and cell density. On the basis of these data, we derived an equation (see SI) to predict the minimum total peptide concentration in the sample able to reach the T_B threshold and thus able to kill 99.9% of the bacteria (*i.e.*, the MBC)

$$\text{MBC} = (1 + [\text{Bacteria}]/K_{app}^B)\text{MBC}_{min} \quad (2)$$

with

$$\text{MBC}_{min} = K_{app}^B \frac{10^3}{N_A} T_B \quad (3)$$

Here, N_A is Avogadro's constant, MBC and MBC_{min} are expressed in mol/L, K_{app}^B and the bacterial cell density ([Bacteria]) are reported in cells/mL, and T_B is in molecules per cell.

The MBC values calculated for DNS-PMAP23, according to eq 2, are reported as a continuous line in Figure 1b, on a logarithmic scale. This equation predicts a linear decrease in the MBC with decreasing cell density. However, it also makes a rather surprising prediction, *i.e.*, that even when the cell density becomes extremely low, the MBC does not decrease below a limiting value, equal to MBC_{min} (3.3 μM, in our case). This behavior might seem counterintuitive, because when the cell density decreases, the total amount of cell-bound peptide necessary to cause bacterial death diminishes proportionally, simply because fewer cells need to be killed. Naively, this consideration would lead to the conclusion that, in the limit of [Bacteria] $\rightarrow$ 0, also MBC $\rightarrow$ 0. However, in the low cell-density regime, most of the peptide will stay free in solution. When [Bacteria] $\ll K_{app}^B$, eq 1, like any hyperbolic equation (such as a Langmuir binding isotherm or a Michaelis–Menten enzyme kinetics), can be approximated by linear behavior, and therefore f_B decreases proportionally to [Bacteria]. As a consequence, the total peptide concentration needed in the sample to kill the bacteria remains constant (eq 3).

A similar theoretical prediction (*i.e.*, a linear dependence of MBC, with a nonzero intercept) has been recently reported for the trend of peptide membrane-perturbing activity with the concentration of liposomes.[20] Those calculations were based on

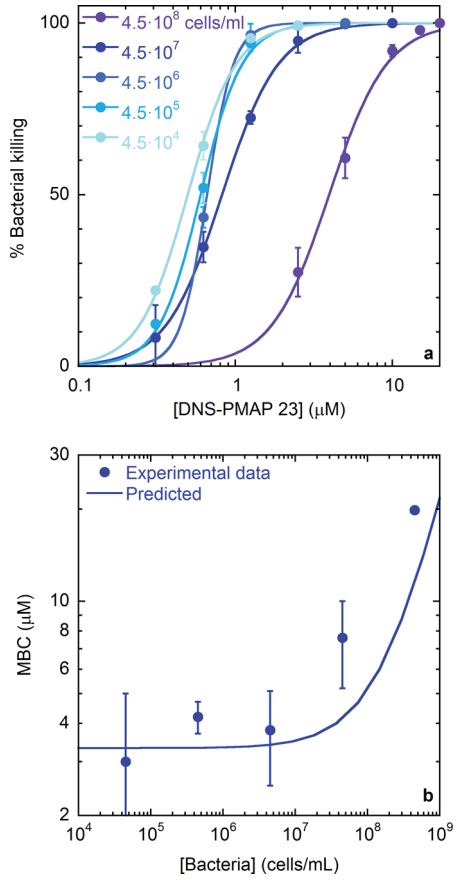

Figure 1. Cell-density dependence of DNS-PMAP23 bactericidal activity. (a) Bactericidal activity in the presence of different *E. coli* cell densities. (b) Predicted (eq 2) and experimental cell-density dependence of the MBC (defined as the concentration that causes the death of 99.9% of bacteria).

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a complex model considering several aspects of the peptide and lipid bilayer behavior at the molecular level. By contrast, the present treatment shows that the predicted trend simply arises from a close to ideal partition equilibrium, without the need of any assumptions on molecular level events. In addition, our previous data on the partition equilibrium and on the value of the threshold T_B[11] allowed us to perform quantitative predictions in cells.

The behavior predicted by eq 2 was verified by experimental measurements of MBCs. Figure 1a reports the killing curves measured in the presence of different cell densities, under the conditions determined previously,[11] where the number of live bacteria in a control sample remains constant during the time of the experiment. The interval of cell densities studied was chosen to cover a realistic range, considering as an upper limit the number of bacteria in an abscess, which may reach up to 10^9 bacterial cells per milliliter of pus.[21] The behavior predicted above was actually observed, with the MBC never decreasing below 3 μM (Figure 1b). The agreement between the experimental data and the predicted curve (which is not a fit) was acceptable even quantitatively.

The simple experiment reported here shows that it is possible to predict the MBC at all cell densities, by determining the threshold of cell-bound peptides necessary to kill a bacterium, and the equilibrium of peptide association to bacterial cells. This possibility was first proposed by Melo et al. in relation to binding studies on model membranes,[22] and it is now demonstrated with studies on real bacteria. In addition, our data show that micromolar total peptide concentrations are necessary to kill the bacteria, even when they are present at low cell counts. Attaining such concentration values in the body by systemic administration of HDPs as drugs might prove problematic. On the other hand, some HDPs can naturally reach concentrations that are even significantly higher, for instance in the granules of leukocytes, in the immediate vicinity of degranulating phagocytes, at the bottom of intestinal crypts,[1] in the hemolymph of insects after a bacterial infection,[23] or on the skin of some frogs.[24] In addition, often multiple HDPs act at the same time and can exhibit synergism.[25] However, for peptides whose physiological concentrations are lower than micromolar, other functions, such as immunomodulation, might be more important than direct bacterial killing.[1]

The considerations that led to the prediction of a lower bound for the active concentration, irrespective of the target cell density, are very general, and therefore a similar behavior could be conceived also for the toxic peptide activity against host cells. To test this hypothesis, we extended our study to erythrocytes, as a convenient model to investigate peptide toxicity. Unfortunately, the high absorbance of the heme group prevented us from determining peptide binding to RBCs with the same spectroscopic approach used for bacteria.[11] However, we developed a protocol to measure hemolysis in the presence of erythrocyte cell densities varying in a range of 4 orders of magnitude. We observed a cell-density dependence of peptide hemolytic activity similar to our findings for bacteria, with a limiting value at low cell counts (Figure 2). From these data, through a fitting with eq 2, an order of magnitude estimate can be obtained for the partition constant and for the threshold of bound peptides relative to the lysis of erythrocytes: $K^E_{app} \sim 10^7$ RBCs/mL and $T_E \sim 10^7$ molecules/RBC (the E super/subscript indicates that these values refer to erythrocytes, rather than to bacteria). Even if this determination of the parameters is rather indirect, the estimate for the order of magnitude of

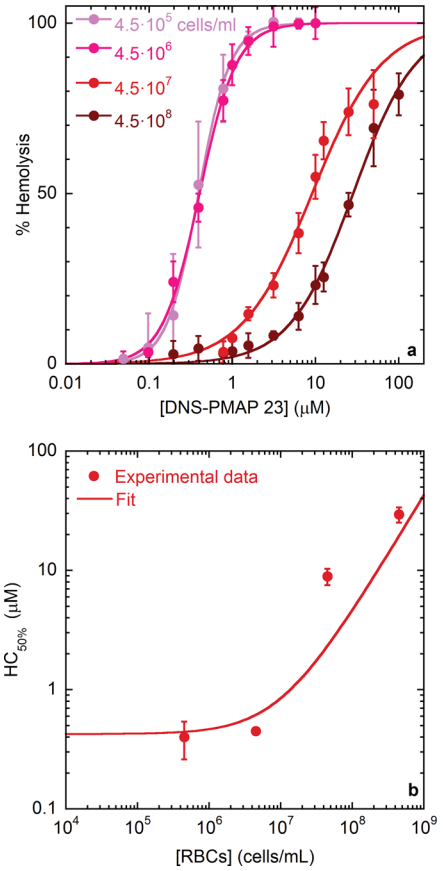

Figure 2. Cell-density dependence of DNS-PMAP23 hemolytic activity. (a) Curves of hemolytic activity determined in the presence of different RBC densities. (b) Cell-density dependence of the peptide concentration needed to cause 50% hemolysis ($HC_{50\%}$). The continuous line is a fit with eq 2.

K^E_{app} can be considered reliable, as it corresponds to the cell density for which the minimum active concentration doubles with respect to the limiting value at low cell counts (eq 2), and the data of Figure 2 definitely indicate that this happens between 4.5×10^6 and 4.5×10^7 cells/mL. In discussing these findings, in comparison with the corresponding values for bacteria, it should be considered that the membrane area of RBCs is about 10 times that of *E. coli* cells but also that different criteria were used to determine the bactericidal and hemolytic concentrations (99.9% killing for MBC and 50% hemolysis for $HC_{50\%}$). In addition, DNS-PMAP23 is a particularly challenging system for testing HDP selectivity, since this property was reduced with respect to the parent peptide by the introduction of the fluorescent label.[11,26] From the standard activity assays commonly performed to determine peptide activity and selectivity (see SI), we obtained for DNS-PMAP23 a MIC of 16 μM and a concentration causing 50% hemolysis ($HC_{50\%}$) of 29 μM, with a therapeutic index (*i.e.*, the ratio between the two values) of only 1.8. The K_{app} values obtained for the two cell types are compatible with this limited selectivity. However, selectivity under realistic conditions would depend on the cell densities of both cell types. To place these data in context, it should be considered that the RBC density in whole blood is on the order of 5×10^9 cells/mL.[27] Therefore, by comparing the data reported in Figures 1 and 2, and extrapolating to the RBC density in blood, it would appear that peptide concentration values might exist for which even the

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marginally selective DNS-PMAP23 could be active, without being toxic.

This conclusion rests on the assumption that separate assays with one cell type only allow the prediction of the behavior in a mixture of bacteria and host cells. However, the bactericidal activity of the peptide might be inhibited due to sequestration by the predominant host cells. A quantitative discussion of this hypothesis, based on equilibrium equations similar to eq 2, is reported in the SI. Indeed, the partition constants reported above lead to the prediction that, when the RBC density exceeds K^{E}_{app}, significant inhibition of the bactericidal activity should be observed. To test for this possibility, we developed a novel assay to measure bacterial killing and RBC lysis in the presence of both cell types (Figure 3). The conditions used

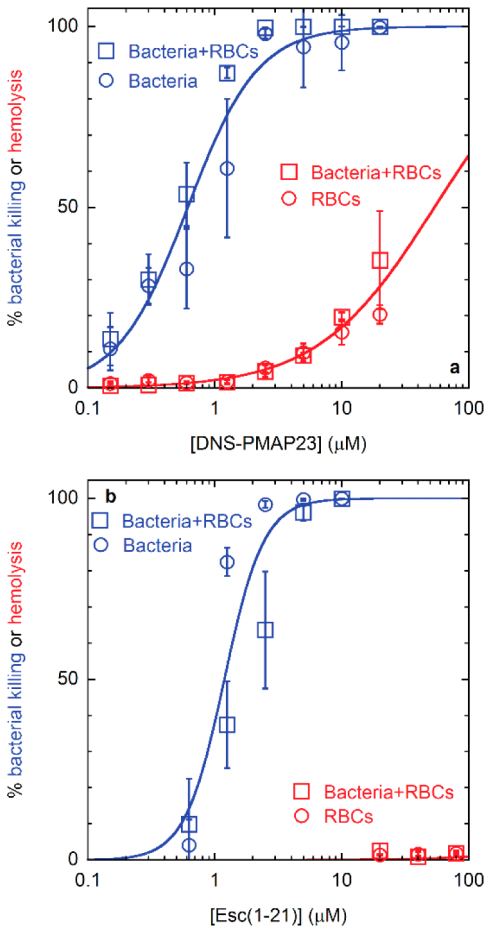

Figure 3. Bactericidal and hemolytic activities of DNS-PMAP23 (a) and Esc(1−21) (b) in the presence of both bacteria and erythrocytes or of one cell type only. 4.5×10^7 *E. coli* cells/mL, 4.5×10^8 RBCs/mL.

were of 4.5×10^7 *E. coli* cells/mL and 4.5×10^8 RBCs/mL. Considering also the different size of the two cell types, these values represent a large excess of eukaryotic versus bacterial cell membranes. Rather surprisingly, we observed that the antimicrobial activity was not affected by the presence of RBCs (Figure 3a). Peptide toxicity, too, was the same in the absence or in the presence of the bacteria.

The lack of alteration of the hemolytic activity in the presence of bacteria is compatible with an equilibrium partition treatment: due to the excess of RBCs, the fraction of peptide bound to bacteria is negligible (see SI). By contrast, the peptide capability to maintain its bactericidal activity also in the presence of a large excess of erythrocytes contrasts with the prediction based on partition equilibria between water and the two cell types, since a large fraction of peptide molecules should be sequestered by RBCs. Therefore, our finding indicates that an equilibrium treatment is inadequate in the presence of both host and bacterial cells, and that other effects (such as a faster peptide binding to bacteria than to RBCs) might be important. Of course, further experiments are needed to clarify this point.

It is reasonable to assume that if the bactericidal activity of a peptide with low selectivity like DNS-PMAP23 was unaffected by the presence of erythrocytes, a more selective HDP would also be insensitive to the simultaneous presence of both cell types. To test this hypothesis, and to verify if our findings can be generalized to other peptides, the killing and hemolysis curves were measured for separated and mixed cell populations also for esculentin-1a(1−21)NH_2, abbreviated as Esc(1−21). This peptide (sequence in Scheme 1) is a derivative of esculentin-1a, a HDP isolated from the skin of the *Pelophylax lessonae/ridibundus* frog, which has been demonstrated to be highly selective, with a therapeutic index of 77 for *E. coli*.[28,29] Also in this case, only a minor inhibition of the antibacterial activity was caused by the presence of a large excess of RBCs (Figure 3b). In addition, in contrast to what has been observed for DNS-PMAP23, Esc(1−21) was highly active against bacteria, without being significantly toxic in a large range of concentrations. These results provide support for a possible direct bactericidal function of HDPs under realistic conditions. They also bode well for the possible therapeutic application of HDPs, at least for topical treatments, while systemic administration might be faced with other hurdles (sequestration by serum components, proteolytic degradation, rapid clearance, etc.). However, it should be noted that several reports exist regarding the efficacy of HDPs *in vivo*[30] and their ability to concentrate at sites of infection.[31]

In conclusion, our findings can be summarized as follows: (i) In the presence of one cell type only, the cell-density dependence of the active peptide concentration is determined by the peptide/cell binding equilibrium and can be predicted, based on the determination of the apparent water/cell-membrane partition constant and of the threshold of membrane-bound peptides per cell needed to cause death. (ii) At low cell densities, the MBC does not decrease below a minimum limit, which in our case was in the micromolar range. This finding begs the question whether these relatively high concentrations can be reached *in vivo*. (iii) Since both the peptide activity and toxicity depend on cell density, the effective selectivity of HDPs depends on the amounts of host and target cells present. (iv) At the same time, the antibacterial activity was not affected by the contemporary presence of a large excess of host cells, as could have been expected based on water/cell-membrane partition equilibria. This finding indicates that a reconsideration of the protocols and assays currently used in the evaluation of HDP selectivity might be necessary. Extension of the present results to other peptides and cell types is essential, but due to the similarity of the properties of many HDPs, our conclusions might apply also to other systems.

Note: While the present work was under revision, an article by Wimley and co-workers was published online,[32] reporting the inhibition of the bactericidal activity of some HDPs by an excess of RBCs. However, this effect was observed only when the peptide was preincubated with RBCs before adding the

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ACS Chem. Biol. 2017, 12, 52−56

peptide/erythrocyte solution to bacteria. In agreement with our findings, no significant inhibition took place when the peptide was added directly to a bacteria/RBCs mixture. The fact that bacterial killing depends on the order of addition of the various components in the assay strongly supports the hypothesis that kinetic effects might be important when different cell populations are present at the same time.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00910.

Model for the interaction of HDPs with different cell populations and for the prediction of peptide activity, with derivation of eqs 1 and 2 and Methods (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Hancock, R. E., Haney, E. F., and Gill, E. E. (2016) The immunology of host defence peptides: beyond antimicrobial activity. *Nat. Rev. Immunol. 16*, 321−334.

(2) Wimley, W. C. (2010) Describing the mechanism of antimicrobial peptide action with the interfacial activity model. *ACS Chem. Biol. 5*, 905−917.

(3) Fox, J. C. (2013) Antimicrobial peptides stage a comeback. *Nat. Biotechnol. 31*, 379−382.

(4) Ventola, C. L. (2015) The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *Pharm. Ther. 40*, 277−283.

(5) Matsuzaki, K. (2009) Control of cell selectivity of antimicrobial peptides. *Biochim. Biophys. Acta, Biomembr. 1788*, 1687−1692.

(6) Bobone, S., Bocchinfuso, G., Park, Y., Palleschi, A., Hahm, K. S., and Stella, L. (2013) The importance of being kinked: role of Pro residues in the selectivity of the helical antimicrobial peptide P5. *J. Pept. Sci. 19*, 758−769.

(7) Yawata, Y. (2003) *Cell Membrane: the Red Blood Cell As a Model*, Wiley-VCH, Weinheim.

(8) Schulz, H. N., and Jorgensen, B. B. (2001) Big bacteria. *Annu. Rev. Microbiol. 55*, 105−137.

(9) Hilchie, A. L., Wuerth, K., and Hancock, R. E. W. (2013) Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat. Chem. Biol. 9*, 761−768.

(10) Melo, N. M., Ferre, R., and Castanho, M. A. R. B. (2009) Antimicrobial peptides: linking partition, activity and high membrane bound concentrations. *Nat. Rev. Microbiol. 7*, 245−250.

(11) Roversi, D., Luca, V., Aureli, S., Park, Y., Mangoni, M. L., and Stella, L. (2014) How many antimicrobial peptide molecules kill a bacterium? The case of PMAP-23. *ACS Chem. Biol. 9*, 2003−2007.

(12) Freire, J. M., Gaspar, D., Veiga, A. S., and Castanho, M. A. (2015) Shifting gear in antimicrobial and anticancer peptides biophysical studies: from vesicles to cells. *J. Pept. Sci. 21*, 178−185.

(13) Malgieri, G., Avitabile, C., Palmieri, M., D'Andrea, L. D., Isernia, C., Romanelli, A., and Fattorusso, R. (2015) Structural basis of a temporin 1b analogue antimicrobial activity against Gram negative bacteria determined by CD and NMR Techniques in cellular environment. *ACS Chem. Biol. 10*, 965−969.

(14) Choi, H., Rangarajan, N., and Weisshaar, J. C. (2016) Lights, camera, action! antimicrobial peptide mechanisms imaged in space and time. *Trends Microbiol. 24*, 111−122.

(15) Orioni, B., Bocchinfuso, G., Kim, J. Y., Palleschi, A., Grande, G., Bobone, S., Park, Y., Kim, J., Hahm, K. S., and Stella, L. (2009) Membrane perturbation by the antimicrobial peptide PMAP-23: A fluorescence and molecular dynamics study. *Biochim. Biophys. Acta, Biomembr. 1788*, 1523−1533.

(16) Brook, I. (1989) Inoculum effect. *Clin. Infect. Dis. 11*, 361−368.

(17) Schwalbe, R., Steele-Moore, L., and Goodwin, A. C. (2007). *Antimicrobial Susceptibility Testing Protocols*, CRC Press, Boca Raton.

(18) Stella, L., Mazzuca, C., Venanzi, M., Palleschi, A., Didonè, M., Formaggio, F., Toniolo, C., and Pispisa, B. (2004) Aggregation and water-membrane partition as major determinants of the activity of the antibiotic peptide trichogin GA IV. *Biophys. J. 86*, 936−945.

(19) White, S. H., Wimley, W. C., Ladokhin, A. J., and Hristova, K. (1998) Protein folding in membranes: determining energetics of peptide-bilayer interactions. *Methods Enzymol. 295*, 62−87.

(20) Bagheri, A., Taheri-Araghi, S., and Ha, B. Y. (2015) How cell concentrations are implicated in cell selectivity of antimicrobial peptides. *Langmuir 31*, 8052−8062.

(21) König, C., Simmen, H. P., and Blaser, J. (1998) Bacterial concentrations in pus and infected peritoneal fluid−implications for bactericidal activity of antibiotics. *J. Antimicrob. Chemother. 42*, 227−232.

(22) Melo, N. M., Ferre, R., Feliu, L., Bardaji, E., Planas, M., and Castanho, M. A. R. B. (2011) Prediction of antibacterial activity from physicochemical properties of antimicrobial peptides. *PLoS One 6*, e28549.

(23) Lemaitre, B., and Hoffmann, J. (2007) The Host Defense of Drosophila melanogaster. *Annu. Rev. Immunol. 25*, 697−743.

(24) Mangoni, M. L., Di Grazia, A., Cappiello, F., Casciaro, B., and Luca, V. (2016) Naturally occurring peptides from Rana temporaria: antimicrobial properties and more. *Curr. Top. Med. Chem. 16*, 54−64.

(25) Mangoni, M. L., and Shai, Y. (2009) Temporins and their synergism against Gram-negative bacteria and in lipopolysaccharide detoxification. *Biochim. Biophys. Acta, Biomembr. 1788*, 1610−1619.

(26) Kim, J. Y., Park, S. C., Yoon, M. Y., Hahm, K. S., and Park, Y. (2011) C-terminal amidation of PMAP-23: translocation to the inner membrane of Gram-negative bacteria. *Amino Acids 40*, 183−195.

(27) Sender, R., Fuchs, S., and Milo, R. (2016) Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. *Cell 164*, 337−340.

(28) Islas-Rodrìguez, A. E., Marcellini, L., Orioni, B., Barra, D., Stella, L., and Mangoni, M. L. (2009) Esculentin 1−21: a linear antimicrobial peptide from frog skin with inhibitory effect on bovine mastitis-causing bacteria. *J. Pept. Sci. 15*, 607−614.

(29) Luca, V., Stringaro, A., Colone, M., Pini, A., and Mangoni, M. L. (2013) Esculentin(1−21), an amphibian skin membrane-active peptide with potent activity on both planktonic and biofilm cells of the bacterial pathogen Pseudomonas aeruginosa. *Cell. Mol. Life Sci. 70*, 2773−2786.

(30) Zhang, L., and Falla, T. J. (2006) Antimicrobial peptides: therapeutic potential. *Expert Opin. Pharmacother. 7*, 653−663.

(31) Bunschoten, A., Welling, M. M., Termaat, M. F., Sathekge, M., and Van leeuwen, F. W. (2013) Development and prospects of dedicated tracers for the molecular imaging of bacterial infections. *Bioconjugate Chem. 24*, 1971−1989.

(32) Starr, C. G., He, J., and Wimley, W. C. (2016) Host cell interactions are a significant barrier to the clinical utility of peptide antibiotics. *ACS Chem. Biol.*, DOI: 10.1021/acschembio.6b00843.

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ACS Chem. Biol. 2017, 12, 52−56