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Article

Cooperativity in Bacterial Membrane Association Controls the Synergistic Activities of Antimicrobial Peptides

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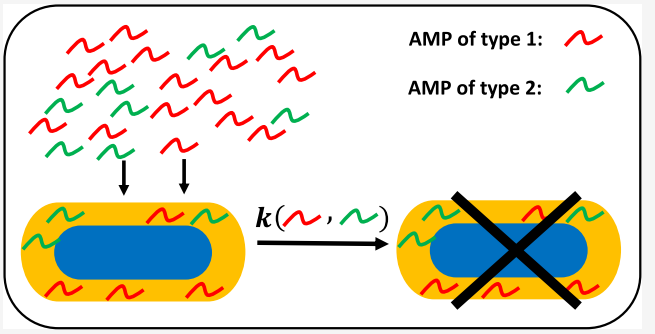
ABSTRACT: Antimicrobial peptides (AMPs), or defence peptides, are compounds naturally produced during immune responses of living organisms against bacterial infections that are currently actively considered as promising alternatives to antibiotics. Recent experimental studies uncovered that in many situations, combinations of different AMPs are much more successful in eliminating the bacterial pathogens than single peptide species. However, the microscopic origin of such synergistic activities remains not fully understood. We present and investigate a possible mechanism of synergy between AMPs. It is based on the idea that due to inter-molecular interactions, the presence of an AMP of one type stimulates the association of an AMP of another type, and this accelerates the overall association to the membrane, eventually killing the bacteria. This approach allows us to fully quantify the synergistic activities of AMPs, and it is successfully applied for several experimental systems. It is found that strong cooperativity can be achieved for relatively weak inter-molecular interactions, suggesting that the application of combinations of AMPs can be further rationally optimized to make it a powerful antibacterial treatment.



INTRODUCTION

Essentially all multi-cellular organisms can produce specific compounds, known as antimicrobial peptides (AMPs), that are critically important in their immune responses to external infections.[1−7] AMPs are relatively short peptide chain molecules with a large fraction of positively charged groups that exhibit a broad spectrum of activities against bacteria, fungi, parasites, cancer, and viruses.[2,8−15] These unique properties stimulated significant research efforts to explore AMPs as potential alternatives to conventional antibiotics.[7] This is especially important in light of growing resistance to antibiotics from various bacterial strains, which is one of the greatest threats to modern medicine, while the resistance is much weaker against AMPs.[1,6,7,16,17] Multiple applications of AMPs in other industries, such as food production, cosmetics, and agriculture, have been also actively discussed.[7,18]

Although the molecular details of how AMPs function are still not fully understood, significant progress has been achieved in clarifying the biological pathways that help them to remove bacterial pathogens.[10,19,20] One of the major pathways explores the association of AMPs to bacterial cellular membranes that happens mostly due to electrostatic interactions between positively charged groups on AMPs and negatively charged species in membranes. This association eventually leads to the formation of the pores in membranes, effectively killing the bacteria via the leakage of its cellular material and disruption of all cellular activities.[5,19] The advantage of this pathway is that it is much less probable for the bacteria to acquire a resistance to AMPs because of very rare instances of mutations in cell membranes.[21−23] In addition, this pathway allows AMPs to be selective in their toxicity. The AMPs are less likely to destroy their own host cells, while the bacteria are efficiently killed. This is mainly due to the difference in chemical composition and charge density of cellular membranes in bacterial and eukaryotic cells. In particular, the presence of the acidic phospholipids in the outer layer of prokaryotic cell membranes as well as the abundance of cholesterol in eukaryotic cell membranes have been suggested as explanations for the AMPs' preferences to attack only the bacterial cells.[24] It should be noted, however, that the pore formation is not the only mechanism by which AMPs can eliminate the bacterial pathogens.[20]

One of the most important factors in the development of new medical treatments is the requirement to minimize possible side effects. This is also crucial for future applications of AMPs as substitutes of antibiotics. Although the appearance

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of resistance against AMPs is rare, the rapid evolution of bacteria still remains a threat to the efficacy of potential AMP treatments. In addition, despite being relatively safe to the host, long-term usage of AMPs can induce drastic toxic side effects such as hemolytic activities,[3] affecting the treatment outcomes. To minimize such effects, the strategy of using combinations of drugs has been explored also for AMPs.[16,25,26] The main idea of this approach is that the total amount of required AMPs in such a combination will be less than using each AMP separately to achieve the same result, thus reducing the possibility of negative side effects and the development of resistance. In addition, a combination of AMPs might lead to new, more efficient mechanisms of killing the bacteria that each of the AMP separately does not exhibit.

An illustrative example of synergistic activities for combinations of AMPs is a system of temporin A and temporin B molecules that are polypeptides secreted by the granular glands of the European red frog.[25] The experiments show that, when used individually, temporin A molecules are more active against Gram-positive bacteria than Gram-negative bacteria, while synthetic temporin B molecules displayed similar relatively weak antimicrobial activities against both Gram-positive and Gram-negative bacteria. Remarkably, the combination of these two types of AMPs resulted in a very strong synergy against both Gram-positive and Gram-negative bacteria. This means that a lower concentration of AMPs was needed in combination to kill the same number of bacteria in comparison with the concentration of individual pure AMPs. Similar synergistic results have been also observed for the combinations of AMPs fighting biofilm formation of bacteria *Pseudomonas* aeruginosa,[26] and for the mixtures of prokaryotic and eukaryotic AMPs that eliminate *Escherichia coli* bacteria.[27]

Although the use of combinations of AMPs has been shown experimentally as a promising route in the development of new medical treatments,[16,25,26] there is a limited understanding regarding the molecular mechanisms of such synergistic activities for the mixtures of different AMPs.[21,28−30] In addition, currently there is a short range of theoretical methods that can be used to analyze the cooperativity phenomena for AMPs.[18,31−33] In this paper, we present a possible mechanism that can quantitatively explain the cooperativity phenomena in AMPs. Our idea is that the effective interactions between peptides of different types accelerate the association AMPs to bacterial membranes, leading to a faster elimination of pathogens. Using the corresponding chemical-kinetic model, we are able to quantify the synergistic activities of AMPs, and our method is successfully applied for several experimental systems of AMP combinations, clarifying some fundamental aspects of AMP functions.

THEORETICAL MODEL

Because membrane-active AMPs can only kill bacteria after binding to their cellular membranes, we postulate that their synergistic activities are related to the ability to bind faster to the membrane for a mixture of AMPs in comparison with pure single peptides. Our hypothesis is that the AMPs of one type stimulate the association of another type of AMPs to the membrane due to beneficial inter-molecular interactions when they are on the membrane. The schematic view of these processes is presented in Figure 1. We note that the process of the removal of bacterial pathogens by AMPs is dynamic. Without AMPs, the amount of bacteria is growing exponentially, and the sooner AMPs can stop the bacterial growth the more efficient they are. Our idea is that the combination of AMPs associates quicker to the membranes because of inter-molecular interactions between different peptide species, allowing them also to kill the bacteria faster. In our theoretical approach, this is the main reason for the appearance of synergy for AMPs. Below, we present a chemical-kinetic model of the process that provides a quantitative realization of this idea.

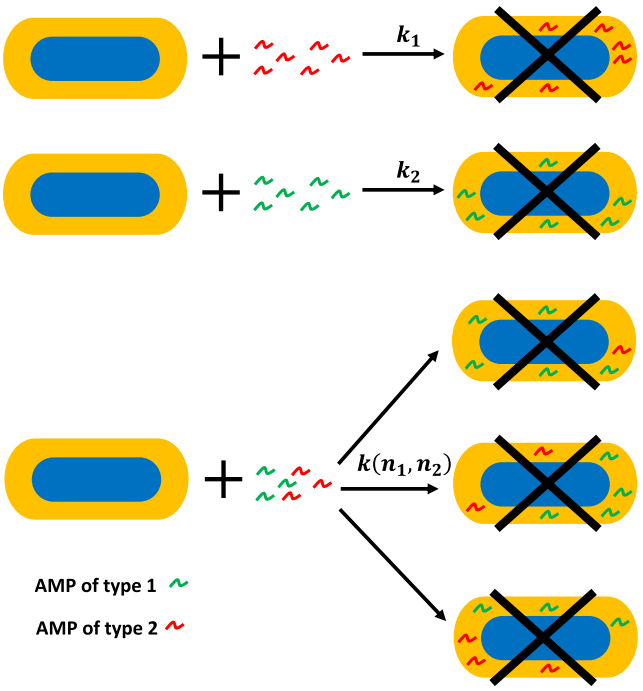

Figure 1. Schematic of the association of AMPs to the bacterial cell membranes that kills the bacteria. AMPs of type 1 bind to the membrane with an associate rate constant k_1. AMPs of type 2 bind to the membrane with an associate rate constant k_2. Mixture of AMPs of type 1 and type 2 associate with a rate constant $k(n_1,n_2)$ that contains all the information about the possible cooperativity. See the text for more explanations.

Let us start the analysis by comparing two different scenarios for the clearance dynamics of bacterial population by AMPs. First, we consider a situation when the bacteria are exposed to only one type of AMP. We define $B(t)$ and $C(t)$ as concentrations of bacteria and AMPs, respectively, at time t. There are two main processes that work in opposite directions and determine the overall outcome in the system: bacterial exponential growth or elimination of bacteria by AMPs. Figure 2 schematically shows these processes. The number of bacterial cells increases via cell division with a rate constant λ. At the same time, the amount of bacterial cells decreases with a rate

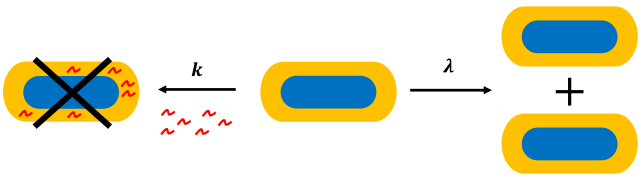

Figure 2. Chemical-kinetic view of the processes related to the functioning of AMPs. The number of bacteria can increase with a rate constant λ due to cell divisions, or it can decrease with a rate constant k due to AMPs killing them.

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constant k due to the killing action of AMPs. The temporal evolution of concentrations of bacteria and AMPs can be described by the following chemical-kinetic equations

$$\frac{dB(t)}{dt} = \lambda B(t) - k[C(t)]^N B(t) \quad (1)$$

and

$$\frac{dC(t)}{dt} = -Nk[C(t)]^N B(t) \quad (2)$$

where N is the average number of AMPs that is needed to neutralize a single bacterial cell. Note, that this is not necessary for this number to be always the same. It has been shown experimentally that this parameter varies widely, $N \sim 10^4$ to 10^8, corresponding to AMP concentrations that range from nanomolar to millimolar ranges.[1,34]

A critically important property of the system that quantifies the efficiency of AMPs is a minimal inhibitory concentration (MIC). It is defined as the concentration of AMPs at which the bacterial growth stops, that is, when $dB/dt = 0$. From eq 1, it can be seen that in our chemical-kinetic description for the single-AMP system the MIC is given by

$$C_{MIC} = \left(\frac{\lambda}{k}\right)^{1/N} \quad (3)$$

which clearly reflects the balance between the bacterial growth and killing.

Now, let us consider a situation when the bacteria are exposed to a combination of two different types of AMPs. We define $C_1(t)$ and $C_2(t)$ as the corresponding concentrations of AMPs of different types at time t. Assuming that the total number of AMPs needed to kill the single bacterium is still equal to N, the chemical-kinetic equations for the system with the combination of AMPs can be written as

$$\frac{dB(t)}{dt} = \lambda B(t) - \sum_{n_1=0}^{N} \binom{N}{n_1} k(n_1, n_2)[C_1(t)]^{n_1}[C_2(t)]^{n_2} B(t) \quad (4)$$

$$\frac{dC_1(t)}{dt} = -\sum_{n_1=0}^{N} n_1 \binom{N}{n_1} k(n_1, n_2)[C_1(t)]^{n_1}[C_2(t)]^{n_2} B(t) \quad (5)$$

$$\frac{dC_2(t)}{dt} = -\sum_{n_2=0}^{N} n_2 \binom{N}{n_1} k(n_1, n_2)[C_1(t)]^{n_1}[C_2(t)]^{n_2} B(t) \quad (6)$$

where n_1 and n_2 are the numbers of AMPs of types 1 and 2, respectively, that are simultaneously bound to the bacterial membrane (with the condition that $n_1 + n_2 = N$). In addition, $k(n_1, n_2)$ are the rate constants for the association of n_1 AMP molecules of type 1 and n_2 AMP molecules of type 2 to the membrane.

The physical meaning of eqs 4−6 can be explained using the following arguments. We assumed that to kill the bacteria, one needs to have the total N AMP molecules of both types in the membrane. Then, for a given composition of membrane-associated AMPs (n_1, n_2), there are $\binom{N}{n_1} = \frac{N!}{n_1! n_2!}$ possible spatial arrangements, leading to $\binom{N}{n_1}$ different association reactions that lead to the specific composition (n_1, n_2). The summation over all possible values of n_1 and n_2 finally accounts for all different compositions of AMPs that can kill the bacteria. As one can see, there are multiple association processes because there are different compositions of the AMPs can be bound to the membrane.

In our theoretical approach, the effective association rate constant $k(n_1, n_2)$ contains all the information about the possible synergy between different types of AMPs. It can be presented in the following form

$$k(n_1, n_2) = k_1^{n_1/N} k_2^{n_2/N} \varepsilon(n_1, n_2) \quad (7)$$

where k_1 and k_2 are rate constants for the association of only AMPs of types 1 and 2, respectively, and a phenomenological parameter ε reflects the cooperativity in the system. When $\varepsilon = 1$, the effect of using the combination of AMPs is simply additive. For $\varepsilon > 1$, there is a positive cooperativity and the mixture of AMPs binds faster to the membrane than the single-component AMPs. The case of $\varepsilon < 1$ corresponds to a negative cooperativity when the mixture of AMPs binds slower to the membrane than the single-component AMPs.

While the details of the molecular mechanisms that might lead to cooperativity between different types of AMPs remain not well understood, we can propose the following theoretical picture of the synergistic activities. It is assumed that the binding of the AMP molecule of type 2 (1) to the membrane together with the AMP molecule of type 1 (2) lowers the free-energy of the system, on average, by amount of ΔE per each pair of bound AMPs. Now, the cooperativity parameter ε can be approximated as

$$\varepsilon \simeq \exp\left[\left(\frac{n_1}{N}\right)\left(\frac{n_2}{N}\right)\left(\frac{\Delta E}{k_B T}\right)\right] \quad (8)$$

This is because the quantities $\left(\frac{n_1}{N}\right)$ and $\left(\frac{n_2}{N}\right)$ are the fractions of the AMPs of type 1 or 2, respectively, associated to the membrane. Then, the product $\left(\frac{n_1}{N}\right)\left(\frac{n_2}{N}\right)$ can be viewed as the probability that any two bound neighboring AMPs are of the different types, contributing to the interactions. The physical meaning of eq 7 can be now understood better. It suggests that the effective association rates to the configurations that lower the overall free-energy of the system are higher, and this is the main source of synergy between different membrane-associated AMPs since the bacterial killing can happen sooner. If the molecular configurations of associated AMPs increase the free-energy of the system, then the association rates to such configurations are slower, producing the antagonistic effect. In the case of no change in the free-energy of the system, the effect of AMPs is simply additive. Note that the evaluation of the free-energy changes associated with AMP association to the bacterial membranes remains a difficult task.[33]

For the combination of AMPs, the conditions that lead to stopping the bacterial growth, $dB/dt = 0$, are now specified not by the single specific concentrations (MIC) but multiple pairs of concentrations of AMPs of types 1 and 2. From eq 4, one might conclude that for any concentrations C_1 and C_2 that satisfy the relation

$$\lambda = \sum_{n_1=0}^{N} \binom{N}{n_1} k(n_1, n_2)[C_1]^{n_1}[C_2]^{n_2} \quad (9)$$

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the bacterial growth rate will be zero. All these combinations (C_1, C_2) can be viewed as corresponding MICs for the mixture of AMPs.

Another convenient way to quantify the synergy between AMPs is by using so-called fractional inhibitory concentration (FIC) indexes.[35] For two antimicrobial agents, labeled as 1 and 2, acting individually or in combination, the FIC indexes are defined as

$$\mathrm{FIC}_1 = \frac{\mathrm{MIC}_{(1\text{ in presence of }2)}}{\mathrm{MIC}_{(1\text{ alone})}} = \frac{C_1}{C_{1,\mathrm{MIC}}}$$
$$\mathrm{FIC}_2 = \frac{\mathrm{MIC}_{(2\text{ in presence of }1)}}{\mathrm{MIC}_{(2\text{ alone})}} = \frac{C_2}{C_{2,\mathrm{MIC}}} \quad (10)$$

The combined FIC index is then given by $\mathrm{FIC} = \mathrm{FIC}_1 + \mathrm{FIC}_2$. The value of the combined FIC index conveniently quantifies the functioning of the AMP combination as follows: FIC < 1 indicates the synergism, FIC = 1 corresponds to additivity, and FIC > 1 indicates the antagonism.

We also propose a different way of measuring the effect of cooperativity for AMPs. It is based on evaluating how faster is the association rate of the mixture of AMPs in comparison with the binding rates of single-components AMPs. For this purpose, we introduce a dimensionless parameter R_i $(i = 1, 2)$

$$R_i = \frac{\sum_{n_1=0}^{N}\binom{N}{n_1}k_1^{n_1/N}k_2^{n_2/N}\exp\left[\left(\frac{n_1}{N}\right)\left(\frac{n_2}{N}\right)\left(\frac{\Delta E}{k_BT}\right)\right]\left(\frac{C_1}{2}\right)^{n_1}\left(\frac{C_2}{2}\right)^{n_2}}{k_iC_i^N} \quad (11)$$

In this expression, the numerator is proportional to the overall association rate for the system that is obtained by mixing equal volumes of the single-component AMPs with the concentrations C_1 and C_2, as typically done in experimental studies.[25] This lowers the concentrations of AMPs in the mixture to $C_1/2$ and $C_2/2$, respectively. The denominator in eq 11 is proportional to the overall association rate for the single-component AMPs of type $i = 1$ or 2. Because labeling of different types of AMPs is arbitrary, for convenience, we consider only $i = 1$ and assume that $R_1 = R$. The positive cooperativity (synergism) is observed for $R > 1$, while the negative cooperativity (antagonism) is realized for $R < 1$. The case of $R = 1$ corresponds to neutral cooperativity or additivity. Note also that a similar expression can be written for mixing different (not equal) volumes of the single-component AMPs.

RESULTS AND DISCUSSION

To test our theoretical approach, let us start with the simplest situation of no cooperative interactions between AMPs of different types, that is, $\Delta E = 0$ and $\varepsilon = 1$. Then, using the binomial formula, eq 9 simplifies into

$$\lambda = (k_1^{1/N}C_1 + k_2^{1/N}C_2)^N \quad (12)$$

which can be rewritten as

$$C_2 = \left(\frac{\lambda}{k_2}\right)^{1/N} - \left(\frac{\lambda}{k_1}\right)^{1/N} C_1 \quad (13)$$

Thus, our theoretical approach predicts that concentrations of AMPs that stop the bacterial growth in the mixture are linearly related if there are no cooperativity in the system. However, when interactions are present, $\varepsilon \neq 1$, the relations between the concentrations of C_1 and C_2 are more complex. In Figure 3, we

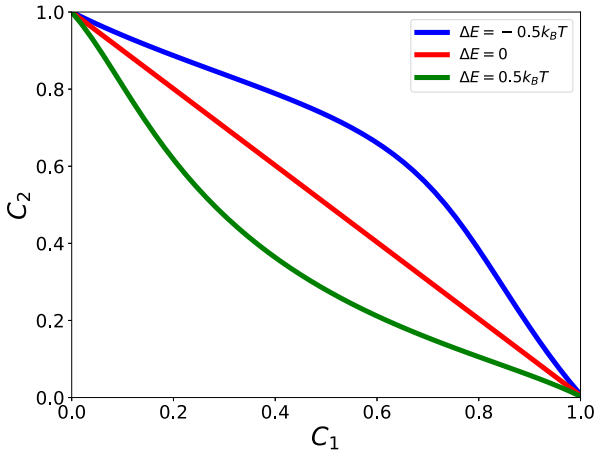

Figure 3. Concentrations of AMPs of types 1 and 2 at which the bacterial growth stops for different inter-molecular interactions. The concentrations C_1 and C_2 are normalized with respect to the corresponding MIC of the single-component AMP. In calculations, the following parameters have been used: $N = 10$, $\lambda = 3/60\ \mathrm{min}^{-1}$, $k_1 = 0.75\lambda$, and $k_2 = 0.5\lambda$.

presented the results of our explicit calculations using eq 9 for curves that specify MICs for the combination of AMPs. The concave curves that deviate from the linear dependence correspond to the positive cooperativity ($\Delta E > 0$), while the convex curves correspond to the negative cooperativity ($\Delta E < 0$).

Varying the degree of cooperativity by changing the inter-molecular interactions also modifies the parameter FIC. It can be written as

$$\mathrm{FIC} = \frac{C_1}{(\lambda/k_1)^{1/N}} + \frac{C_2}{(\lambda/k_2)^{1/N}} = \frac{k_1^{1/N}C_1 + k_2^{1/N}C_2}{\lambda^{1/N}} \quad (14)$$

One can see then from eq 12 that when there are no interactions ($\Delta E = 0$, $\varepsilon = 1$), we always have FIC = 1. However, as shown in Figure 4, our calculations suggest that FIC < 1 for the positive cooperativity ($\Delta E > 0$, $\varepsilon > 1$), while the negative cooperativity ($\Delta E < 0$, $\varepsilon < 1$) will always produce FIC > 1.

It is also important to consider the parameter R that specifies how fast is the combination of AMPs in associating to the bacterial membrane in comparison with the single-component AMPs. If we start with the solutions of single-component AMPs that separately can stop the bacterial growth, $C_1 = C_{1,\mathrm{MIC}} = (\lambda/k_1)^{1/N}$ and $C_2 = C_{2,\mathrm{MIC}} = (\lambda/k_2)^{1/N}$, then mixing the solutions in the ratio 1:1 will produce the AMP combination with the concentrations $C_1/2$ and $C_2/2$. For such a system, eq 11 modifies into

$$R_i = \frac{\sum_{n_1=0}^{N}\binom{N}{n_1}\exp\left[\left(\frac{n_1}{N}\right)\left(\frac{n_2}{N}\right)\left(\frac{\Delta E}{k_BT}\right)\right]}{2^N} \quad (15)$$

One can clearly see then that $R = 1$ for the case of neutral cooperativity ($\Delta E = 0$) because $\sum_{n_1=0}^{N}\binom{N}{n_1} = 2^N$; $R > 1$ will be the case of the positive cooperativity ($\Delta E > 0$); and the

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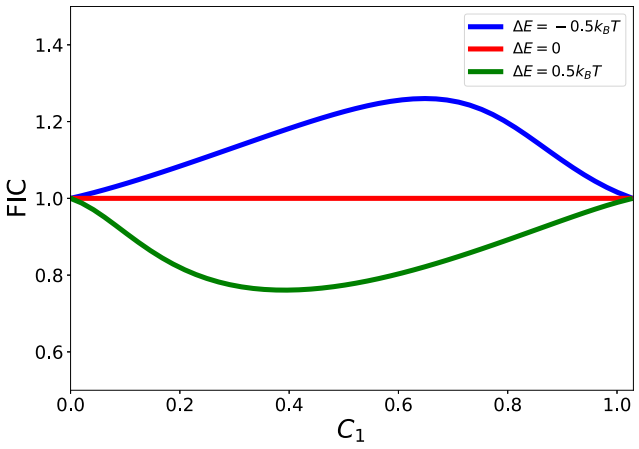

Figure 4. FIC indexes for the combination of AMPs for different interactions. The concentration C_1 is normalized with respect to the corresponding MIC of the single-component AMP. In calculations, the following parameters have been used: $N = 10$, $\lambda = 3/60\ \mathrm{min}^{-1}$, $k_1 = 0.75\lambda$, and $k_2 = 0.5\lambda$.

negative cooperativity ($\Delta E < 0$) leads to $R < 1$. The results of specific calculations for the acceleration parameter R for different interactions are presented in Figure 5.

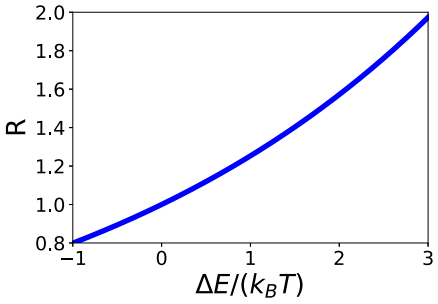

Figure 5. Association acceleration parameter R as a function of the inter-molecular interactions. In calculations, the following parameters have been used: $N = 10$, $\lambda = 3/60\ \mathrm{min}^{-1}$, $k_1 = 0.75\lambda$, and $k_2 = 0.5\lambda$.

To better understand our theoretical approach, let us consider in detail the limiting situations. We start with the simplest case of $N = 2$, when only two AMP molecules are needed to kill the bacterial cell. This is not a very realistic situation, but it allows us to understand better the mechanisms of cooperativity for AMPs. From eq 9, we obtain the curve of MICs for the combination of AMPs

$$\lambda_{N=2} = k_1 C_1^2 + 2\sqrt{k_1 k_2}\, C_1 C_2 x + k_2 C_2^2 \tag{16}$$

where $x = \exp\left[\frac{1}{4}\frac{\Delta E}{k_B T}\right]$. For the situation with zero cooperativity, $\Delta E = 0$ and $x = 1$, there is a linear relation between the concentrations of AMPs that lead to killing the bacteria, while the positive cooperativity, $\Delta E > 0$ and $x > 1$, produces the convex curves in coordinates (C_1, C_2); and the negative cooperativity, $\Delta E < 0$ and $x < 1$, leads to the concave curves for MIC of AMPs in coordinates (C_1, C_2), similarly to the behavior presented in Figure 3.

For the FIC parameter, from eq 14, it can be derived for the $N = 2$ model

$$\mathrm{FIC}_{N=2} = \frac{k_1^{1/2} C_1 + k_2^{1/2} C_2}{\sqrt{[(k_1^{1/2} C_1 + k_2^{1/2} C_2)^2 + 2\sqrt{k_1 k_2}\, C_1 C_2 (x-1)]}} \tag{17}$$

We can see that no cooperativity ($x = 1$) corresponds to FIC = 1, the synergism between AMPs ($x > 1$) leads to FIC < 1, and the antagonism ($x < 1$) would make FIC > 1. Similar analysis for the acceleration parameter R yields a very simple relation

$$R(N = 2) = \frac{1 + x}{2} \tag{18}$$

suggesting that increasing the interactions (larger ΔE) exponentially increases the overall association rate of the AMP combination in comparison with the single-component AMP association rates.

Another limiting case, which is, however, much more realistic, is the situation when $N \gg 1$. The experimentally observed range $N \sim 10^4$ to 10^8 corresponds to this limit. Then, we can argue that the dominating association process is the one that binds to the membrane exactly $n_1 = N/2$ AMPs of type 1 and $n_2 = N/2$ AMPs of type 2. This is because it is associated with the largest number of spatial arrangements, $N!/(N/2!)\,(N/2!) \simeq 2^N$, and the largest overall interaction energy (in units of $k_B T$), $\left[\frac{1}{4}\frac{\Delta E}{k_B T}\right] = \ln x$.

In this limit, the condition to determine that concentrations at which the bacterial growth stops [eq 9] can be written as

$$\lambda_{N \gg 1} \simeq 2^N x k_1^{1/2} k_2^{1/2} C_1^{N/2} C_2^{N/2} \tag{19}$$

Now, recalling that single-component AMPs MIC are given by $\lambda = k_1 [C_{1,\mathrm{MIC}}]^N = k_2 [C_{2,\mathrm{MIC}}]^N$, this expression can be simplified into

$$\left(\frac{C_{1,\mathrm{MIC}}}{2C_1}\right)\left(\frac{C_{2,\mathrm{MIC}}}{2C_2}\right) \simeq x \tag{20}$$

One can see that the larger the positive inter-molecular interactions ($\Delta E > 0$ and $x > 1$), the smaller the concentrations of AMPs that are needed to stop the bacterial growth. The opposite effect is observed for repulsive interactions between AMPs, ($\Delta E < 0$ and $x < 1$), when the larger quantities of AMPs are required to kill the bacteria. This expression is also important because it allows us to evaluate the effective interaction energy from experimental data that measure MIC for single component AMPs and for the combination of AMPs

$$\Delta E \simeq 4 k_B T \ln\left(\frac{C_{1,\mathrm{MIC}}}{2C_1}\right)\left(\frac{C_{2,\mathrm{MIC}}}{2C_2}\right) \tag{21}$$

For $N \gg 1$, the FIC coefficient can be also easily evaluated from eq 14, yielding

$$\mathrm{FIC}(N \gg 1) \simeq \frac{k_1^{1/N} C_1 + k_2^{1/N} C_2}{2x (k_1^{1/N} C_1)^{1/2} (k_2^{1/N} C_2)^{1/2}} \tag{22}$$

This suggests that the minimum for the parameter FIC can be achieved when $k_1^{1/N} C_1 \sim k_2^{1/N} C_2$, which gives the value $1/x$ for this minimum, providing another route to estimate inter-molecular interactions ΔE from experimental observations that report FIC. Similarly, we can evaluate the acceleration parameter R in the limit of large number of AMPs using eq 15

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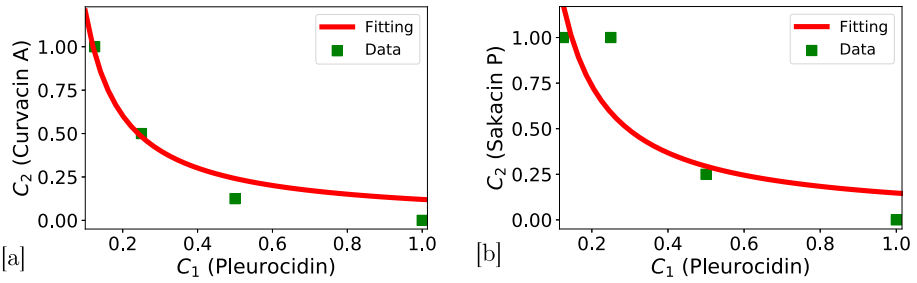

Figure 6. Fitting of experimental data[27] of synergistic activities of combinations of AMPs using eq 20. (a) MIC curves for the mixture of curvacin A and pleurocidin. The effective inter-molecular interaction for this combination is $\Delta E = 2.9\ k_BT$ (b) MIC curves for the mixture of sakacin P and pleurocidin. The effective inter-molecular interaction for this combination is $\Delta E = 2.1\ k_BT$.

$$R(N \gg 1) \simeq x \tag{23}$$

Because our theoretical approach is fully quantitative, it can be applied to estimate the degree of cooperativity in real systems. We choose the experiments that measured the antimicrobial effect of the combinations of prokaryotic AMPs (sakacin P and curvacin A) obtained from lactic acid bacteria with eukaryotic AMP pleurocidin obtained from fish.[27] In these experiments, it was found that such combinations acted highly synergistically against the Gram-negative *E. coli* bacterial strains. As shown in Figure 6, fitting these data for the combination of curvacin A and pleurocidin produces the effective energy of interactions per pair of bound AMPs $\Delta E \simeq 2.9\ k_BT$, while for the combination of sakacin P and pleurocidin, we obtain $\Delta E \simeq 2.1\ k_BT$. Our analysis suggests that the combination of curvacin A and pleurocidin is more synergistic than the combination of sakacin P and pleurocidin. This shows that this theoretical method can not only explain and describe the experimental data but also quantify the degree of synergistic activities, providing useful information for future more microscopic investigations of such effects.

Our theoretical approach indicates that relatively weak interactions, several k_BT per pair of bound AMP molecules, are able to generate significant synergy between different types of AMPs in killing pathogens. This suggests that the anti-bacterial properties of AMP combinations can be further improved by tuning the inter-molecular interactions. It can be done via mutational analysis of AMPs or via adding other chemicals that can attach to the bacterial membranes. This is because the inter-molecular interactions between AMPs might be not only the direct interactions but also due to indirect interactions with the membrane.

SUMMARY AND CONCLUSIONS

We presented a possible theoretical mechanism of synergistic action of AMP combinations in killing the bacterial cells. It is argued that the cooperativity in the association to bacterial membranes stimulates AMPs to bind quicker to the membrane and to remove the infection sooner. Based on this idea, we developed a chemical-kinetic model that assumes that AMPs of different types interact during the binding process, increasing the association rates, which eventually leads to faster killing of bacteria. Our theoretical approach allows us to quantify the degree of cooperativity between different types of AMPs. Specific calculations evaluate the concentrations that stop the bacterial growth and the acceleration in the association rates for the combinations of AMPs in comparison with single-component AMPs. The method is successfully applied then to analyze the degrees of synergy between AMPs in several real systems. It is found that relatively weak interactions (order of several k_BT per bacterial cell) make the association process faster by ∼3−4 times, which leads to significant synergy between AMPs. It is interesting to note that our estimates of the effective interactions are consistent with recent theoretical efforts to evaluate the binding energies of AMPs to bacterial membranes.[33] In light of the proposed theoretical mechanism, possible ways to improve the efficiency of AMP combinations are also discussed. It was suggested that due to a complex nature of interactions this might be done via changes in AMPs or via changes in the membranes.

Although the proposed theoretical method provides a clear physical-chemical picture of how the synergy in eliminating the bacterial pathogens by combinations of AMPs can appear, it is important to discuss its limitations. Our theoretical approach implicitly assumes that the rate-limiting step in the overall dynamics of bacterial killing is the association to the membranes. It is likely, however, that other biochemical and biophysical processes might be more important in regulating the efficiency of AMP combinations. We also assumed that the mechanisms of bacterial killing are the same for single-component AMPs and for the combinations of peptides. It is reasonable to expect that interactions between different types of AMPs might open new pathways in elimination the bacterial infections. More detailed investigations are needed to confirm these possibilities. In addition, our theoretical approach is effectively a mean-field approximation where homogeneous average inter-molecular interactions are assumed over the whole bacterial membrane. Clearly, the biological processes are typically much more heterogeneous.

However, despite these shortcomings, our theoretical method provides a simple quantitative approach to explain and to measure the cooperativity in AMP functioning. Importantly, it gives predictions that can be tested experimentally. Future studies could test AMP activity in model membrane systems rather than in live cells, for example, and the theoretical predictions could be probed by using dye leakage assays.[36] Our theoretical method suggests that the combinations of AMPs is powerful tool in fighting against infections that can be effectively tuned and optimized further by varying the inter-molecular interactions.

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Notes

The authors declare no competing financial interest.

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