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Article

Differences in Relevant Physicochemical Properties Correlate with Synergistic Activity of Antimicrobial Peptides

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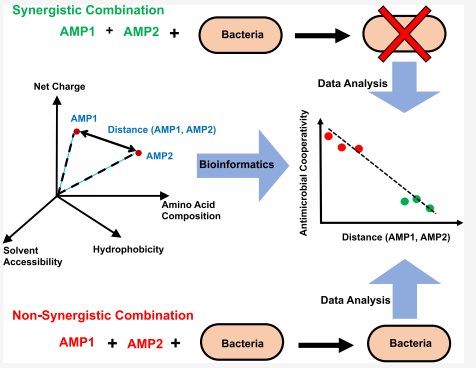
ABSTRACT: With the urgent need for new medical approaches due to increased bacterial resistance to antibiotics, antimicrobial peptides (AMPs) have been considered as potential treatments for infections. Experiments indicate that combinations of several types of AMPs might be even more effective at inhibiting bacterial growth with reduced toxicity and a lower likelihood of inducing bacterial resistance. The molecular mechanisms of AMP−AMP synergistic antimicrobial activity, however, remain not well understood. Here, we present a theoretical approach that allows us to relate the physicochemical properties of AMPs and their antimicrobial cooperativity. It utilizes correlation and bioinformatics analysis. A concept of physicochemical similarity is introduced, and it is found that less similar AMPs with respect to certain physicochemical properties lead to greater synergy because of their complementary antibacterial actions. The analysis of correlations between the similarity and the antimicrobial properties allows us to effectively separate synergistic from nonsynergistic AMP pairs. Our theoretical approach can be used for the rational design of more effective AMP combinations for specific bacterial targets, for clarifying the mechanisms of bacterial elimination, and for a better understanding of cooperativity phenomena in biological systems.



INTRODUCTION

One of the main achievements of modern medicine is the ability to efficiently eliminate various infections. This is currently accomplished by using several classes of specific small organic molecules that are generally called antibiotics.[1] However, in the last 30 years, we have witnessed an increasing resistance to antibiotics in bacteria, which threatens to severely decrease our ability to protect human health.[2−4] These alarming trends stimulated a broad search for novel antibacterial agents and techniques.[5,6] Antimicrobial peptides (AMPs), which are produced by multicellular organisms as part of their immune responses to external infections, came out as promising alternatives to antibiotics.[7−12] Some organisms, e.g., frogs, produce a wide variety of AMPs in their skin secretions.[13,14] AMPs are relatively short peptide-chain molecules with typically large fractions of separated positively charged and hydrophobic residues that exhibit activities against multiple classes of bacteria, fungi, viruses, and even cancer.[15−19] It was also observed that combinations of some specific types of AMPs frequently work much more efficiently than single-type peptides.[20−24] Although it is known that, in contrast to single AMPs, AMP combinations are less toxic, can better hinder the ability of bacteria to develop resistance, and can associate to bacterial cells faster, the microscopic mechanisms of AMP−AMP cooperativity remain not well understood.[23−26]

AMPs exhibit a wide spectrum of structures and mechanisms of bacterial removal.[8,9] Nonetheless, it is generally believed that the dominating antimicrobial pathway is the association of AMPs with bacterial membranes and the following pore formation that leads to the death of the bacterial cell.[17,27,28] The efficacy of antimicrobial peptides in eliminating infections is measured by the minimal inhibitory concentration (MIC), which is defined as the concentration required to inhibit the growth of the bacterial population.[26,29] It is interesting that some AMPs might also sensitize antibiotics in their action against the previously resistant bacterium.[9] In addition, like antibiotics, some AMPs have a broad spectrum of antimicrobial activity, targeting multiple different species of bacteria. Unlike antibiotics, however, AMP-based drugs are powerful against antibiotic-resistant strains. The combinations of different types of AMPs might be even more effective in their antibacterial activities.[21,22,24,30] They show higher efficacy, reduced toxicity, and a lower likelihood of inducing bacterial resistance compared to available antibiotics and even individual AMPs. The efficacy of AMP−AMP combinations is measured by fractional inhibitory concentrations (FIC), which reflect the extent to which MIC for a given AMP in

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combination with another AMP is reduced compared to the MIC of the same AMP when applied individually.[26]

The microscopic origin of antibacterial cooperativity between different types of AMPs is unclear. Bacterial resistance to individual AMPs is rare yet possible,[31−34] while bacterial resistance is less probable to AMP−AMP combinations.[23,35] This is one of the reasons why AMP−AMP combinations are more likely to not only be more effective than individual AMPs[36−38] but also serve as long-term antimicrobials in contrast to traditional antibiotics.[39−41] Several possible sources of cooperativity have been proposed. It was suggested, based on experimental findings, that two AMPs might be synergistic because they individually target different types of bacteria [e.g., Gram-positive and Gram-negative[42]. Thus, their combinations are more effective against both types of bacteria.[43] Furthermore, two AMPs might have different mechanisms of action to disrupt the bacterial function,[44−47,68] or they might perform different antimicrobial functions (e.g., one AMP sensitizes the bacterium cell to the other AMP). Then, the existence of multiple antimicrobial mechanisms can prevent the development of resistance to the combination since bacteria are not able to simultaneously respond to both of them.[49] Also, two AMPs with different secondary structures (for example, one has mostly α-helices and the other one has mostly β-sheets) might also better inhibit bacterial growth,[50−53] and the difference in their structures can prevent the peptide aggregation that is associated with a decrease in antibacterial properties.

It is clear that possible synergy between two different types of AMPs is a result of direct and/or indirect molecular interactions. It was already suggested that this interaction might appear due to similarity in the structures of the peptides.[54] However, recent experimental studies of individual AMPs determined that other physicochemical properties, and not structures, correlate better with higher antimicrobial activity and a lower likelihood of bacterial resistance.[55,56] In addition, for two AMPs to be a synergistic pair, it was shown that they must have very different degrees of hydrophobicity.[45,51,57] Furthermore, it was proposed that the larger differences in physicochemical properties for AMP combinations might be needed to prevent bacterial resistance. This is because bacteria are less likely to transfer resistance from an AMP in one class characterized by certain physicochemical features to an AMP in a different class (characterized by different physicochemical features than the first class), a phenomenon known as cross-resistance.[58−60] Nonetheless, experimental tests of this idea exhibit mixed results. Synergy and lack of cross-resistance are evident in the example of food-preservative AMPs curvaticin-13 and nisin,[61] while AMPs microcins showed cross-resistance and lack of synergistic antimicrobial activity.[62]

In this paper, we postulate that two classes of AMPs will cooperate if they have very different physicochemical properties relevant to their antimicrobial activity because they will complement each other in removing bacterial infections. To test this hypothesis, we introduce the concept of physicochemical similarity, defined as Euclidean distance in the space of more than 1500 physicochemical descriptors. This quantitative approach explicitly evaluates the correlations between physicochemical similarities and FICs of different AMP−AMP combinations. This allows us to select the most important features that contribute to synergistic antimicrobial activity. Applying principal component analysis (PCA), it is found that it is possible to distinguish between effective and ineffective AMP−AMP combinations only when similarity is calculated in terms of the selected features. We argue that greater physicochemical dissimilarity between AMPs in certain features is associated with stronger cooperative antimicrobial activity. Possible mesoscopic arguments to support these observations are presented. Our computational approach can be used to rationalize the design of effective and bacteria-specific AMP−AMP combinations as potential drugs, and it can also assist in clarifying a more microscopic picture of bacterial removal by AMPs.

MATERIAL AND METHODS

Physicochemical Similarity of AMP Pairs. There are 1547 descriptors in total that are broadly divided into the following groups:[63] autocorrelations (Moreau-Broto, Moran, and Geary coefficients for hydrophobicity, polarizability, free energy, and other features); amino acid compositions (single, for example, the percentage of valine in the peptide or dipeptide, for example, the percentage of valine adjacent to lysine); physicochemical compositions (composition and transition values for polarizability, charge, van der Waals forces, and other properties), pseudoamino acid compositions, and quasi-sequence order. Importantly, we view each peptide as a point in d-dimensional space of all physicochemical properties where each dimension represents a specific descriptor.

Data Normalization. Since the scales of physicochemical properties for each peptide are different, it is important as a first step to normalize them to have their values to be between 0 and 1. To normalize this quantity in the range 0 and 1, we use

$$\hat{z} = \frac{(z - z_{\min})}{(z_{\max} - z_{\min})} \tag{1}$$

Euclidean Distance as the Measure of Similarity between Two Peptides. There are different methods for defining the similarity between any two molecules.[64,65] We chose the Euclidean distance in the space of all physicochemical descriptors so that the properties with greater distance would have greater weight in the calculation of overall distance. For each AMP−AMP combination, the Euclidean distance is calculated in terms of N different sets of descriptors

$$d^{(\text{all } N \text{ features})}(A, B) = \sqrt{\sum_{i=1}^{N} (A_i - B_i)^2} \tag{2}$$

and for M selected features

$$d^{(\text{M selected features})}(A, B) = \sqrt{\sum_{i=1}^{M} (A_i - B_i)^2} \tag{3}$$

while for individual descriptors, we have

$$d^{(\text{individual feature})}(A, B) = \sqrt{(A_1 - B_1)^2} = |A_1 - B_1| \tag{4}$$

The Euclidean distance has already been successfully utilized in several bioinformatics investigations, including quantifying the relations between genes and proteins.[66] We postulate that the physicochemical similarity of two AMPs is inversely proportional to the Euclidean distance between them in the space of physicochemical properties

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$$\text{Similarity}(A, B) \equiv \frac{1}{d(A, B)} \tag{5}$$

Fractional Inhibitory Concentration. The quantitative measure of the efficiency of single-type AMPs against a bacterial species is MIC, which is the minimum concentration of an antimicrobial agent required to completely inhibit the growth of a bacterium population. The corresponding quantitative measure for AMP−AMP combinations is fractional inhibitory concentration (FIC), which is the sum of the ratios of each peptide's MIC in the combination to individual MIC

$$\text{FIC}_1 = \frac{\text{MIC}_{(1 \text{ in presence of } 2)}}{\text{MIC}_{(1 \text{ alone})}} = \frac{C_1}{C_{1,\text{MIC}}} \tag{6}$$

$$\text{FIC}_2 = \frac{\text{MIC}_{(2 \text{ in presence of } 1)}}{\text{MIC}_{(2 \text{ alone})}} = \frac{C_2}{C_{2,\text{MIC}}} \tag{7}$$

$$\text{FIC} = \text{FIC}_1 + \text{FIC}_2 \tag{8}$$

To better understand the cooperativity of two types of AMPs, it is convenient to look into a schematic view of a checkboard assay, shown in Figure 1, which is typically utilized

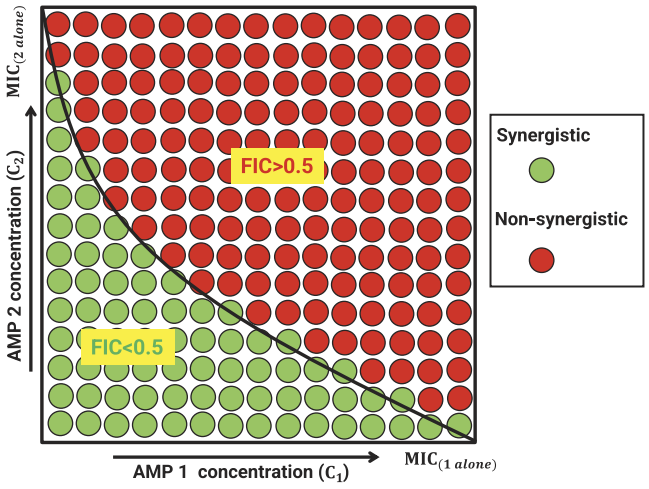

Figure 1. Schematic view of the checkerboard assay in which AMP−AMP combinations are tested. Each circle represents different sets of concentrations of AMPs. The solid black curve describes the conditions at which bacterial growth stops.

in experiments on measuring synergy of AMPs.[67] In this graph, the concentrations of peptides are presented as fractions of their corresponding MICs for single-type AMP measurements, and each circle corresponds to a specific combination of AMPs. Different curves describe the conditions at which bacterial growth is stopped.

For all curves below the black curve (e.g., green circles), we have FIC < 0.5, and this describes the synergistic action of AMP pairs in the elimination of bacterial infection. The presence of the second type of AMP enhances the antibacterial efficiency of the first type of AMP. For all curves above the black curve (red circles), we have FIC > 0.5, and these conditions are viewed in our method as nonsynergistic for AMP combinations. In other words, the presence of the second type of AMP lowers or does not affect the antimicrobial efficiency of the first type of AMP.

Spearman's Rank Correlation Coefficient for Calculating Correlations between FIC Values and Euclidean Distances. Each AMP−AMP combination is characterized by a Euclidean distance d (eq 4) and total FIC (eq 8 values). To elucidate the relationship between the Euclidean distance and FIC values of AMP−AMP pairs, we utilize the Spearman's rank correlation coefficient, which is defined as the Pearson's correlation coefficient between the ranks of distance, $R(d)$, and ranks of FIC values, $R(\text{FIC})$

$$r_s = \frac{\langle R(d)R(\text{FIC})\rangle - \langle R(d)\rangle\langle R(\text{FIC})\rangle}{\sqrt{\langle R^2(d)\rangle - \langle R(d)\rangle^2}\sqrt{\langle R^2(\text{FIC})\rangle - \langle R(\text{FIC})\rangle^2}} \tag{9}$$

The Spearman's rank correlation coefficient estimates how well the relationship between two variables can be described by a monotonic function, and, thus, it is a convenient measure to evaluate the correlations between two quantities. For computing Spearman's correlation, one has to sort the values from least to greatest, and rank is the position of each sorted value in the list.

RESULTS AND DISCUSSION

Feature Selection Procedure Based on Correlations between FIC Values and Physicochemical Similarity of AMP Pairs. For practical purposes, one is always searching for strong synergistic pairs of AMPs, and then some arbitrary thresholds are utilized to define this region of antimicrobial activities. Moreover, to simplify the statistical analysis, it is convenient to have similar numbers of synergistic and nonsynergistic pairs. In our work, FIC = 0.47 is chosen as the threshold for synergistic pairs, and AMP−AMP pairs with FIC > 0.47 are considered as effectively nonsynergistic, and pairs with FIC < 0.47 are viewed as synergistic. We collapsed data across weakly synergistic, additive, and antagonistic categories together because there was not enough data to examine each case separately, and our focus is on strongly synergistic pairs.[48,69] It is also important to note that the exact choice of the threshold value does not affect our main conclusions, as we explicitly checked in our calculations.

To obtain a quantitative description of the antibacterial efficiency of AMPs pairs, the data were extracted from the DBAASP database,[70] and we ensured that the data included in the analysis were collected under the same experimental conditions. These data contain FIC values for AMP−AMP combinations for three different bacterial species (*Escherichia coli*, *Micrococcus luteus*, and *Pseudomonas aeruginosa*) and in which both peptides in the combination consisted of only natural amino acids with an overall sequence length of at least 11 (see Table 1). The latter requirement is needed for the subsequent proper extraction of physicochemical descriptors of peptides.

We extracted a comprehensive set of 1547 physicochemical properties for each peptide sequence using a bioinformatic package *propy*.[63] These physicochemical features include amino acid compositions (percentage of each amino acid in the peptide), net charge, hydrophobicity, polarizability, polarity, van der Waals forces, solvent accessibility, and many others.

The first calculated quantity was the Euclidean distance between the AMPs in each pair in terms of all features to arrive at a single value.[64,65] In our analysis, it is viewed as the inverse of the similarity between two peptides. Then, we evaluated the

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Table 1. Overview of the Datasets for AMP−AMP Combinations Extracted from DBAASP Database[a]

Bacteria	Synergistic AMP-AMP combinations	Non-synergistic AMP-AMP combinations
E. coli	12	50
M. Luteus	6	12
P. Aeruginosa	4	11

[a]Specifically, the number of synergistic and non-synergistic AMP pairs for each bacterium is presented.

Table 2. (a) Various Tritrpticin and Temporin Peptides and Their Corresponding Sequences; (b) Various Magainin-2 Peptides and Their Corresponding Sequences

a)

Peptide	Sequence
Tritrpticin	VRRFPWWWPFLRR
TWF	VRRFPFFFPFLRR
TPA	VRRFAWWWAFLRR
Temporin A	FLPLIGRVLSGIL
Temporin B	LLPIVGNLLKSLL
Temporin L	FVQWFSKFLGRIL

b)

Peptide	Substituted position	Sequence
MAG2-WT	——	GIGKFLHSAKKFGKAFVGEIMNS
MAG2-F5A	Phe5	GIGK-Ala-LHSAKKFGKAFVGEIMNS
MAG2-L6A	Leu6	GIGKF-Ala-HSAKKFGKAFVGEIMNS
MAG2-F12A	Phe12	GIGKFLHSAKK-Ala-GKAFVGEIMNS
MAG2-G13	Gly13	GIGKFLHSAKKF-Ala-KAFVGEIMNS
MAG2-F16	Phe16	GIGKFLHSAKKFGKA-Ala-VGEIMNS
MAG2-V17	Val17	GIGKFLHSAKKFGKAF-Ala-GEIMNS
MAG2-G18	Gly18	GIGKFLHSAKKFGKAFV-Ala-EIMNS
MAG2-I20A	Ile20	GIGKFLHSAKKFGKAFVGE-Ala-MNS

Spearman's rank correlation coefficients[71,72] between the Euclidean distances and FIC values for each AMP pair (see Materials and Methods for details of the calculations).

Since there were no significant correlations between FIC and Euclidean distance for any pairs of peptides in terms of *all* descriptors, $p > 0.05$, we analyzed this relationship separately in terms of each of the 1547 physicochemical features. The corresponding histograms of correlation coefficients for three species of bacteria are shown in Figure 2. We decided to choose only those features for which the Spearman correlation coefficient was statistically significant, i.e., $|r_s|$ is large. Furthermore, a statistical analysis with a p-value of less than 0.005 was applied to filter out irrelevant features. This corresponds to features with r_s smaller than the red dashed lines for $r_s < 0$ and features with r_s larger than the red dashed lines for $r_s > 0$; see Figure 2. Based on these criteria, we obtained the features shown in Figure 3a for *E. coli*, in Figure 3b for *M. luteus*, and in Figure 3c for *P. aeruginosa*. Then, the physicochemical similarity for AMP pairs has been computed utilizing only the *selected* features to result in a single Euclidean distance value for each AMP pair, and the relationship between the FIC values and the distances has been analyzed (see Figure S1 in the Supporting Information). One can see that the distance in the space of selected physicochemical features between two peptides can be utilized to separate synergistic from nonsynergistic AMP pairs.

Our analysis suggests that most physicochemical features do not affect the correlations between the FIC and the similarity. The features that we selected are shown in Figure 3a for *E. coli* bacterium. More specifically, the distance between AMPs in the space of only selected features against the FIC values is presented in Figure S1. One can see that, in contrast to the case when all features are included (not shown), there is a visible separation between synergistic and nonsynergistic AMP pairs. Similar results are obtained for other analyzed bacterial species (see Figure S2 in the Supporting Information). It is interesting to note that most selected physicochemical properties are distinct for each bacterium (Figure 3), but there was also overlap in certain properties, including hydrophobicity and polarizability, suggesting that there might be some universal physicochemical features of AMPs that are important for eliminating any bacteria.

Analysis of Specific Selected Features: Autocorrelation Functions. Our theoretical method indicates that among the important selected physicochemical features for all types of investigated bacteria, there are several autocorrelation functions, which measure the variation of different physicochemical properties for any pair of amino acid residues along the peptide sequence. Three versions of autocorrelation functions, known as Geary, Moran, and Moreau-Broto, are considered here.[73,74] Although there is no fundamental difference between these functions, unlike Moreau-Broto, Geary and Moran autocorrelation parameters utilize averages and variances for each property. For example, the Moreau-Broto autocorrelation coefficient, which measures the correlation between physicochemical properties of residue i and residue $i + d$ (along the peptide contour), is given by[74]

$$\mathrm{MB}(d) = \frac{1}{N-d}\sum_{i=1}^{N-d} P_i P_{i+d} \tag{10}$$

where P_i and P_{i+d} are physicochemical properties of residue i and residue $i + d$, respectively. Alternatively, we can define Geary autocorrelation function[74] as

$$G(d) = \frac{\frac{1}{2(N-d)}\sum_{i=1}^{N-d}(P_i - P_{i+d})^2}{\frac{1}{N-1}\sum_{i=1}^{N}(P_i - \bar{P})^2} \tag{11}$$

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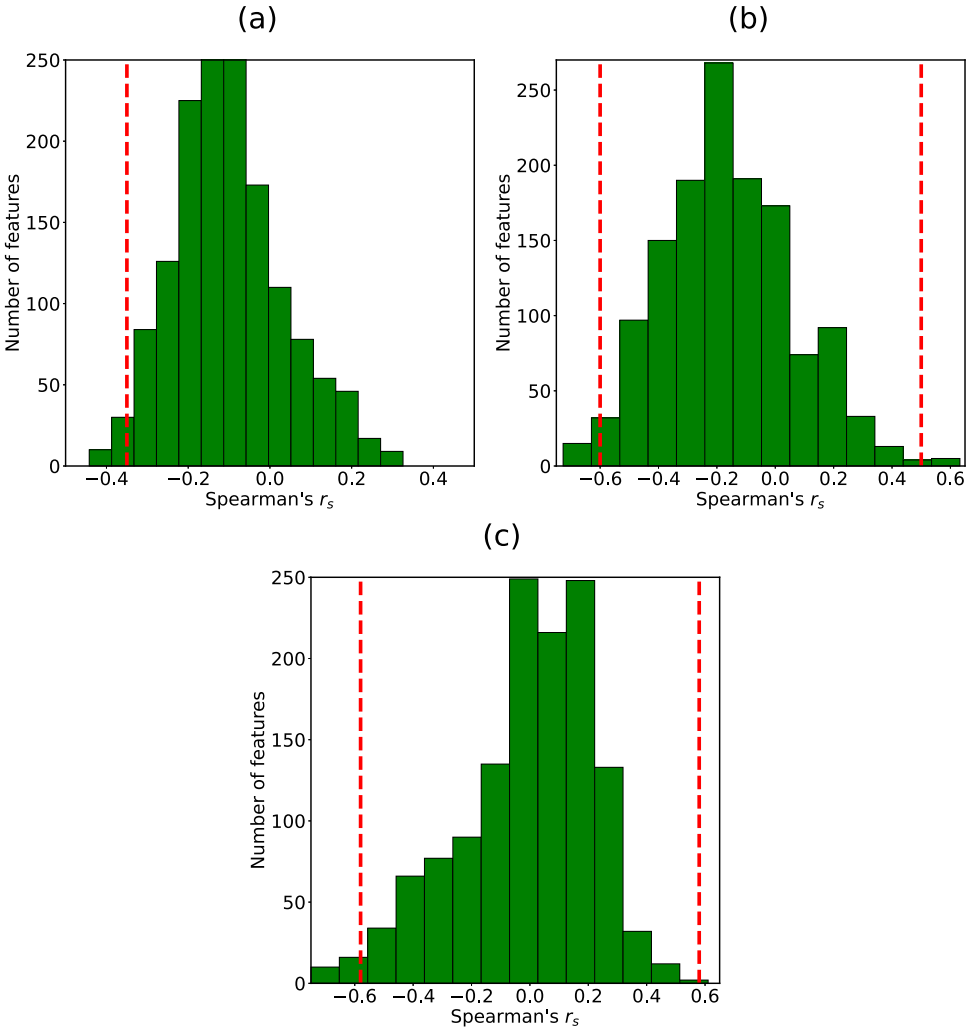

Figure 2. Distribution of Spearman correlation coefficients between fractional inhibitory concentration (FIC) values and the corresponding distances between paired AMPs in terms of individual physicochemical features $[d^{(\text{individual feature})}(A, B)]$ for (a) *E. coli*, (b) *M. luteus*, (c) *P. aeruginosa*. Vertical red dashed lines show threshold values of Spearman's correlations above which the corresponding features are selected.

where $\overline{P} = \frac{1}{N}\sum_{i=1}^{N} P_i$ is the average physicochemical property of the sequence. Finally, one can define the Moran autocorrelation function, which is similar to Pearson's correlation between the physicochemical property of residue i and residue $i + d$[74]

$$M(d) = \frac{\frac{1}{N-d}\sum_{i=1}^{N-d}(P_i - \overline{P})(P_{i+d} - \overline{P})}{\frac{1}{N}\sum_{i=1}^{N}(P_i - \overline{P})^2} \quad (12)$$

Considering physicochemical features important for *E. coli*, one can notice that the dissimilarity of two AMPs in terms of autocorrelation in hydrophobicity contributes to the synergistic activity of their combination. In this case, a positive autocorrelation corresponds to a peptide, which is either totally hydrophobic or totally hydrophilic, i.e., it has the same sign of hydrophobicity for different amino acids along the peptide chain. A negative autocorrelation describes amphiphaticity when one side of the peptide is hydrophobic and the other side is hydrophilic, i.e., a change of sign in hydrophobicity along the peptide chain. Accordingly, a large difference in hydrophobicity autocorrelation coefficients between two peptides in a combination suggests that in the synergistic combination one peptide is most probably amphipathic, while the other is predominantly hydrophobic or hydrophilic.

To illustrate the idea that for two AMPs to cooperate in removing the bacteria they must be very different in terms of autocorrelations in hydrophobicity, let us analyze a specific example of AMP−AMP combinations that target *E. coli* bacteria. Three different types of peptides, tritrpticin and its two derivatives labeled as TPA and TWF, are considered first.[75] Bacterial killing assays showed that the combination of tritrpticin and TPA was not synergistic, while tritrpticin−TWF and TPA−TWF pairs successfully cooperated against *E. coli*. To elucidate the relationship between the physicochemical similarity of these AMP pairs and their antibacterial activity, we computed the Moran autocorrelation parameter for hydrophobicity (which characterizes the degree of amphiphaticity of the peptide) at different distances along the peptide chain using the information from the *propy* package. The parameter d specifies the distance between amino acids along the peptide chain. As one can see in Figure 4a, tritrpticin is similar to TPA in terms of the lack of amphiphaticity, but it is different from TWF. Thus, the synergistic activity of TPA−TWF and tritrpticin−TWF combinations might be attributed to the fact that TWF is highly amphiphatic in contrast to both

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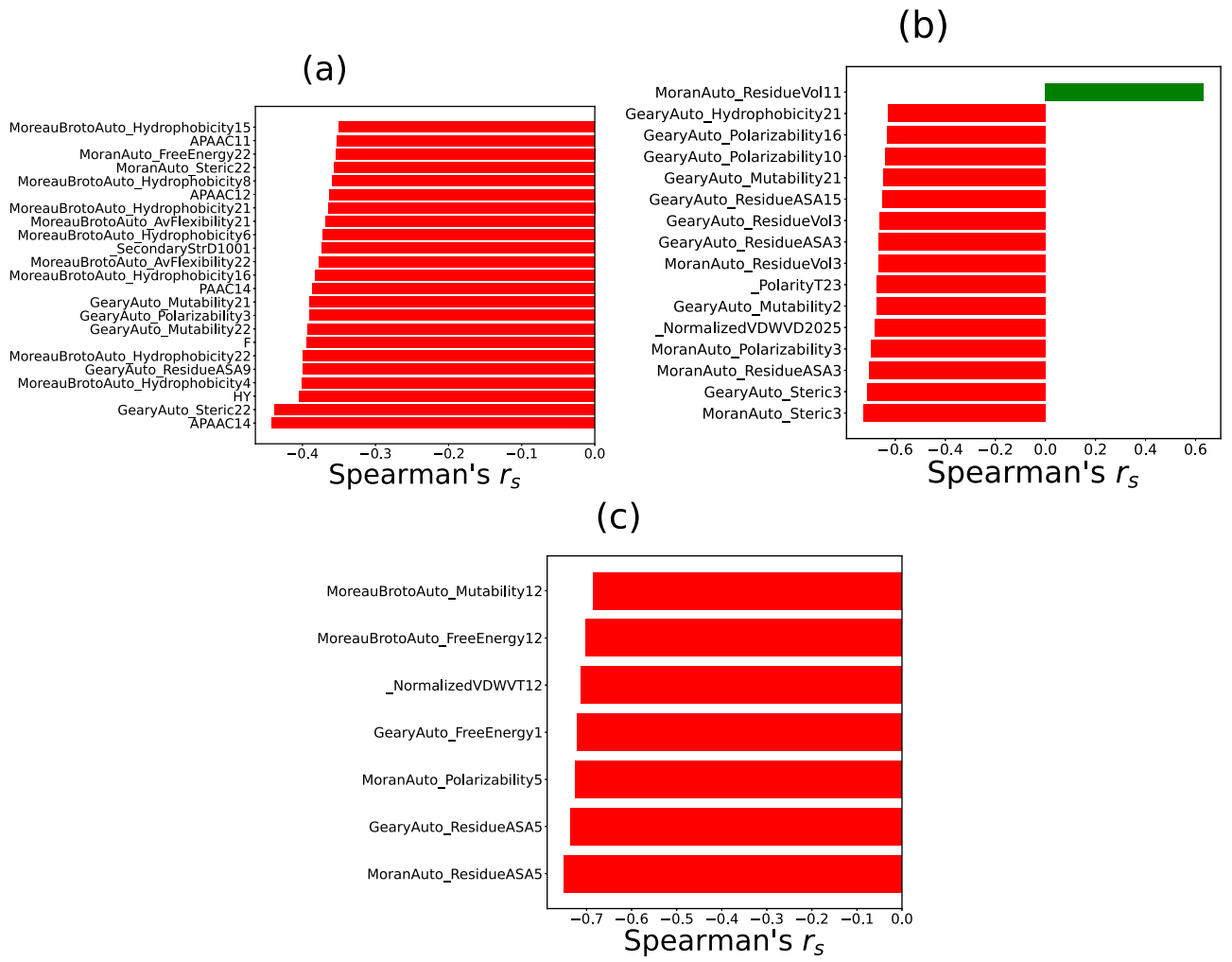

Figure 3. Relative importance of physicochemical features in terms of which AMP−AMP similarity is highly correlated with the corresponding FIC values (a) for *E. coli*, (b) for *M. luteus*, and (c) for *P. aeruginosa*.

tritrpticin and TPA. At the same time, the amphiphaticity is absent in both tritrpticin and TPA so that they are physicochemically more similar, making their combination ineffective against *E. coli*.

In another example, we consider AMPs in the temporin family, namely temporins A, B, and L.[76] Microbroth dilution assays to measure the inhibition of bacterial growth showed that against *E. coli*, temporin L was synergistic with temporins A and B separately, while temporins A and B together were not synergistic. As shown in Figure 4b, the Moran hydrophobicity autocorrelations are closer to each other for temporins A and B, and both of them exhibit relatively weak amphipathicity, as indicated by the slightly negative correlations at $d = 9$. However, there is a larger difference between the hydrophobicity autocorrelation parameters for temporin L and the other temporins A and B. The larger negative correlation reflects the greater amphipathicity for temporin L compared to the other temporins. These results suggest that the synergy between temporin L and the other temporins could be related to the differences in amphipathicity, while the similar levels of amphipathicity between temporins A and B lead to the lack of synergy.

In the final example, we consider the peptide magainin-2, originating from the *Xenopus laevis* frog, and several synthetic single-amino-acid-substituted analogues of magainin-2 that are all synergistic with the peptide PGLA in killing *E. coli* bacteria.[57] Figure 4c presents the Moran autocorrelation parameters for two distances d for all magainin-2 species and for the PGLA peptide. One can see that autocorrelation parameters for PGLA are very different from all other magainin-2 species. PGLA is nonamphipathic, while the negative correlations of different magainin-2 peptides demonstrate strong amphipathicity. Conversely, it can be predicted that when the magainin-2 peptides are combined in pairs, none of the pairs will be synergistic against *E. coli*. This is because the magainin-2 peptides are all strongly amphipathic and thus too similar in this important physicochemical characteristic. Therefore, PGLA and all considered magainin-2 peptides are very different physicochemically, and this explains the observed antibacterial cooperativity in this system.

SUMMARY AND CONCLUSIONS

Recent experimental studies revealed that bacteria are more susceptible to combinations of some specific types of antimicrobial peptides. In this work, we present a theoretical investigation that allows us to identify synergistic pairs of AMPs based on their physicochemical properties. It is proposed that cooperating AMPs are those peptides that are the most different in their physical−chemical properties relevant to bacterial elimination. In other words, the more

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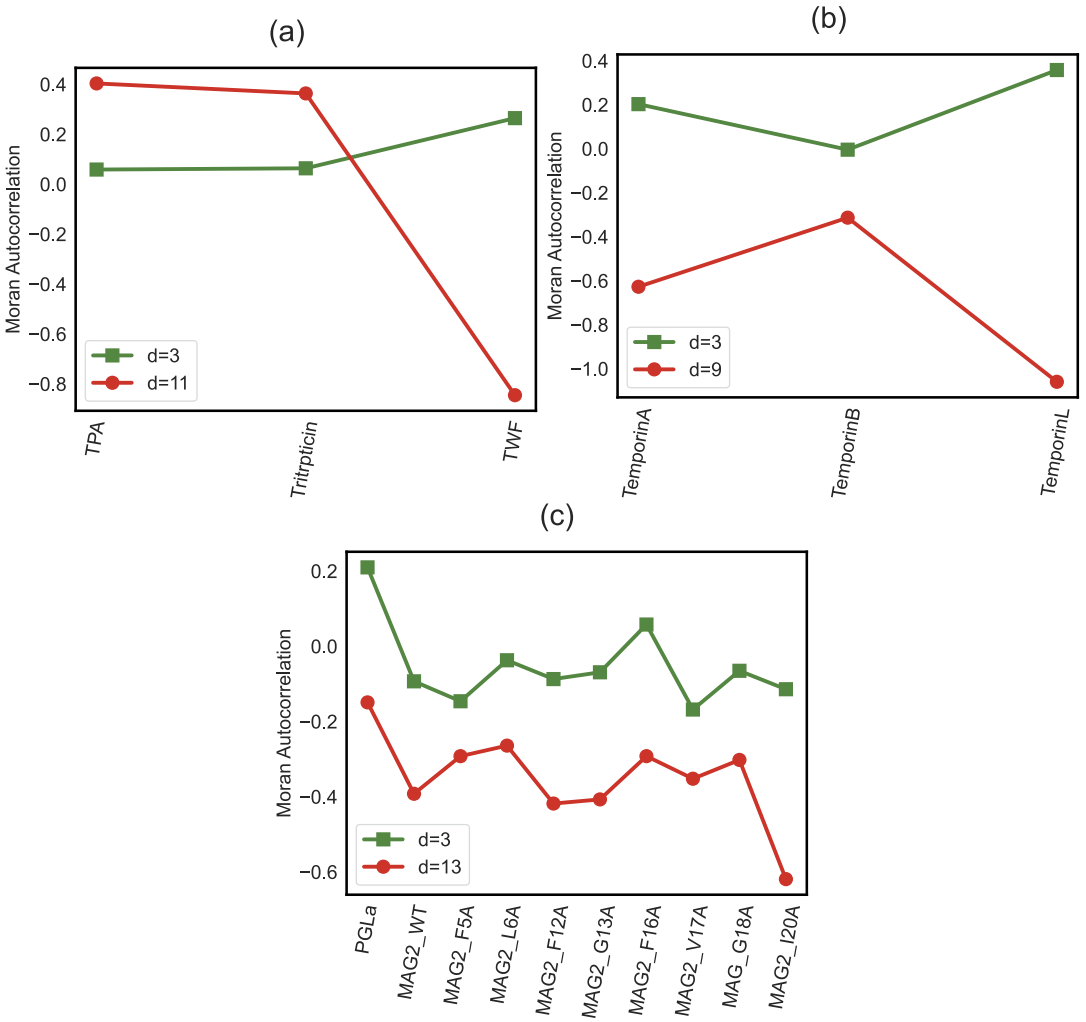

Figure 4. Calculation of Moran autocorrelation coefficient in hydrophobicity for (a) tritrpticin and its derivatives, TWF and TPA. TPA was synergistic with tritrpticin and TWF separately, but tritrpticin and TWF were not synergistic.[75] (b) Temporins A, B, and L. Temporins A and B are nonsynergistic, while temporin L is synergistic with temporins A and B separately. (c) PGLa and different mutants of magainin-2 (MAG2). PGLA was synergistic with all variants of MAG2, but the strongest synergy was between PGLA and MAG2-G13A and MAG2-G18A separately.[57] See Table 2 for more details.

dissimilar the AMPs are, the more cooperative they are in their antibacterial action. To test our hypothesis, we developed a computational framework that allowed us to quantify the physicochemical similarity and analyze its correlations with cooperativity in antibacterial activities. To illustrate our theoretical method, the synergy of AMP−AMP combinations acting against three different types of bacteria, namely *E. coli*, *M. luteus*, and *P. aeruginosa*, has been specifically considered.

A concept of physicochemical similarity between two peptides as inverse Euclidean distance in the space of properly normalized physicochemical features has been introduced and discussed. It has been found that there is a relatively small number of properties that is most relevant for supporting the synergy of AMP−AMP combinations. Theoretical analysis shows that measuring similarity using only the selected features inversely correlates with the antibacterial efficiency of AMP pairs, allowing us to separate synergistic and nonsynergistic AMP combinations. These observations clearly support our hypothesis that the most physicochemically dissimilar (in terms of the most relevant features) AMP pairs lead to cooperativity in the removal of bacterial infections, while similar AMP combinations do not exhibit cooperativity at all.

Generally, the selected physicochemical features for different bacteria do not coincide, although there are some common properties. It was found that several autocorrelation functions, including but not limited to hydrophobicity, play an important role in supporting the synergistic action of AMP combinations. For example, the autocorrelation in hydrophobicity is related to the amphipathicity of AMPs, which enables the AMP molecule to faster enter the bacteria cell.[77,78] Calculating these properties, such as autocorrelation in hydrophobicity, allowed us to explicitly illustrate the correlations between the physicochemical similarity and the antibacterial efficiency. The association between the selected physicochemical features for each species of bacteria and the cooperative activity of AMP−AMP combinations against the bacteria was also supported by additional analyses, including PCA, as shown in the Supporting Information (see Figures S1 and S2). In the several examples considered of AMP combinations acting against *E. coli* bacteria, cooperativity was observed only for peptides with very different autocorrelation parameters, while peptides with similar autocorrelation parameters never produced synergistic combinations. Similar results were found for antimicrobial dimers, specific types of AMPs in

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which two AMPs are linked to act as a single AMP: autocorrelation in hydrophobicity was related to the efficacy of dimers against *Enterococcus faecalis* (see Figure S3). These results again are fully consistent with our hypothesis about the relation between physicochemical similarity and antibacterial efficiency. We illustrated that this hypothesis can be used to generate predictions for untested AMP−AMP combinations because AMP−AMP combinations with greater distance in specific properties, such as autocorrelation in hydrophobicity, are more likely to be synergistic (see Figure S4).

Although our theoretical approach connects the selected physicochemical properties of AMPs with their ability to cooperate, it does not provide a microscopic picture of the underlying processes that lead to antibacterial synergy. At the same time, some of the obtained results allow us to present some speculations about possible molecular mechanisms of AMPs cooperativity. Our main idea is that this is a result of *complementarity* in the antimicrobial properties of peptides. The possible microscopic picture is that one type of AMPs is affecting the bacterial membrane in such a way that it makes it easier for the second type of AMPs to disrupt the membrane, though some AMPs might also act on intracellular targets such as ribosomes to disrupt bacteria cell functioning.[79] Since both activities are, in some sense, "orthogonal" to each other, this leads to stronger effective cooperativity. Those AMPs that are similar in their physicochemical properties do not exhibit complementarity because in this case peptides compete with each other for the same regions of bacterial cellular membranes in order to disrupt them.

Cooperativity is one of the main organizational principles in chemistry and biology required to support the functioning of living systems.[80,81] Examples include multiple phenomena ranging from ligand binding to cellular receptors and enzyme activities to molecular machines made of complex protein complexes. In many cases, the molecular mechanisms of the processes that lead to cooperativity are still not fully understood. Although our theoretical approach provides a possible mechanistic explanation for the synergistic action of AMPs, it seems reasonable to suggest that similar ideas can also be extended to other biological processes. We propose that in some systems, cooperating biological molecules might be complementary in their activities to perform their biological functions.

While our theoretical approach is successful in predicting the cooperativity of AMPs acting against bacteria, it is important to discuss its limitations. First, the method will work if there is enough data on synergistic and nonsynergistic combinations for the given bacteria to identify the most relevant features and evaluate the physicochemical similarity. Second, it does not clarify the microscopic origin of cooperativity since it only detects the correlations but not their sources. Despite these limitations, however, this theoretical approach provides a powerful method for designing more efficient AMP drugs, and it also gives the starting point for uncovering what molecular forces are responsible for the synergetic effects of peptides. In addition, this method can be easy to extend to other biological systems, e.g., to investigate cooperativity in protein−protein systems.

■ ASSOCIATED CONTENT

Data Availability Statement

The data obtained in this work and the in-house scripts are available on figshare at the following URL: https://figshare.com/s/fa1408174d816c222b01.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpcb.3c07663.

> Physicochemical features distinguish between synergistic and nonsynergistic pairs; principal component analysis; feature selection based on correlations between FIC values and physicochemical similarity of dimers; and predictions for untested AMP−AMP combinations against *E. coli* (PDF)

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Author Contributions

A.M. and H.T. designed the research. A.M. and H.T. performed the research. A.M., H.T., and A.B.K. wrote the article.

Notes

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