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Functional Synergy between Antimicrobial Peptoids and Peptides against Gram-Negative Bacteria▽†

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Antimicrobial peptides (AMPs) are integral components of innate immunity and are typically found in combinations in which they can synergize for broader-spectrum or more potent activity. Previously, we reported peptoid mimics of AMPs with potent and selective antimicrobial activity. Using checkerboard assays, we demonstrate that peptoids and AMPs can interact synergistically, with fractional inhibitory concentration indices as low as 0.16. These results strongly suggest that antimicrobial peptoids and peptides are functionally and mechanistically analogous.

Cationic antimicrobial peptides (AMPs) comprise a diverse class of natural antibiotics produced by a vast array of organisms, including prokaryotes, insects, plants, amphibians, and mammals, forming an integral component of their innate immunity (33). This universal presence across the kingdoms of life and broad-spectrum activity against multiple pathogens, including drug-resistant strains, has created substantial interest in developing them for clinical applications (7, 9, 10, 21). Due to rising rates of drug resistance, the need for novel antibiotics is acute (1), but many AMPs suffer from high dose-limiting toxicity. One potential solution to the problems of both resistance and toxicity is to use a synergistic combination of antimicrobial compounds, an approach that is ubiquitous in anticancer therapy and is receiving increasing attention in the treatment of infectious diseases (2). Many species produce AMPs with known synergistic interactions, including bacteria (14), insects (20), amphibians (16, 31), and humans (3, 27, 28), with synergy arising through a variety of mechanisms (2, 14, 29).

Although AMPs have the potential to be developed into a new class of clinically useful antibiotics, peptides are susceptible to proteolytic degradation and are thus poorly bioavailable. Therefore, we have developed mimics of AMPs using peptoids (poly-*N*-substituted glycines), which are protease-resistant (15) isomers of peptides (Fig. 1) with broad-spectrum antibacterial activity comparable to, and in some cases better than, that of antimicrobial peptides (4, 5, 11, 12, 19).

We hypothesized that as true mechanistic analogs of AMPs, antimicrobial peptoids should also be able to interact synergistically with peptides and with each other. Using checkerboard antibacterial assays, we determined fractional inhibitory concentrations for a panel of nine cationic, helical antimicrobial peptoids and peptides against both Gram-negative and Gram-positive bacteria, which revealed highly synergistic interactions.

***In vitro* antibacterial and hemolytic activities of individual oligomers.** For these studies, we selected a panel of two AMPs and seven antimicrobial peptoids with a range of hydrophobicities and selectivities for bacterial versus mammalian cells. Peptoids were synthesized as previously reported (34), and peptides were synthesized using conventional 9-fluorenylmethoxy carbonyl (Fmoc) chemistry. The names, sequences, hydrophobicities, antibacterial activities against Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria, and hemolytic activities of these nine compounds are shown in Table 1 (values in μg/ml are provided in Table S1 in the supplemental material). The antibacterial activities are reported as MICs and were determined according to standard CLSI M7-A6 protocols (6); hemolytic activities, determined as previously reported (5), serve as a measure of antimicrobial peptide/peptoid toxicity (9), which is commonly used to optimize antimicrobial peptide/peptoid therapeutic performance (4, 5, 17, 19, 22–24).

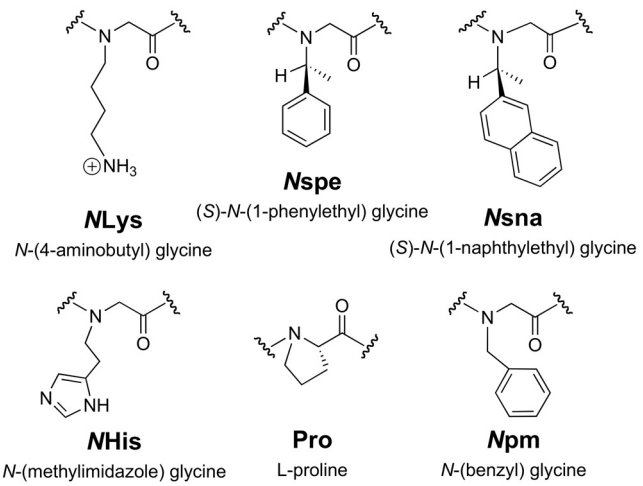

FIG. 1. Guide to peptoid monomers.

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TABLE 1. *In vitro* activities of individual peptoids and peptides for synergy studies

Compound[a]	Sequence[b]	% ACN at RP-HPLC elution[c]	MIC (μM) for		HD_{10}/HD_{50} (μM)	SR[d]
			E. coli ATCC 35218	*B. subtilis* ATCC 6633		
Pexiganan	GIGKFLKKAKKFGKAFVKILKK-NH_2	50.2	3.1–6.3	1.6	73/>200	12
1-*N*Lys$_{5,11}$	H-(*N*Lys-*N*spe-*N*spe-*N*Lys-***N*Lys**-*N*spe)$_2$-NH_2	51.2	50	0.78	>200/>200	>4.0
1-*N*His$_{6,12}$	H-(*N*Lys-*N*spe-*N*spe-*N*Lys-*N*spe-***N*His**)$_2$-NH_2	51.4	50	0.78–1.6	>200/>200	>4.0
$1_{achiral}$	H-(*N*Lys-***N*pm**-***N*pm**)$_4$-NH_2	60.4	12.5	1.6	180/>200	14
1-Pro$_6$	H-*N*Lys-*N*spe-*N*spe-*N*Lys-*N*spe-**Pro**-(*N*Lys-*N*spe-*N*spe)$_2$-NH_2	62.2	12.5	1.6	83/>200	6.6
1	H-(*N*Lys-*N*spe-*N*spe)$_4$-NH_2	65.1	6.3	1.6	14/62	2.2
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	65.2	12.5	1.6	1/6	0.16
1-*N*sna$_{6,12}$	H-(*N*Lys-*N*spe-*N*spe-*N*Lys-*N*spe-***N*sna**)$_2$-NH_2	68.1	25–50	0.78–1.6	7/27	0.28
1_{17mer}	H-*N*spe-*N*spe-(*N*Lys-*N*spe-*N*spe)$_5$-NH_2	70.1	25–50	0.78–1.6	3/15	0.06

[a] Compounds are listed in order of increasing molecular hydrophobicity, as measured by reverse-phase high-performance liquid chromatography (RP-HPLC) retention time.
[b] Boldface indicates residues that differ from the sequence of peptoid 1.
[c] Determined using a gradient of 5 to 95% acetonitrile (ACN) over 45 min on a C_{18} column at 0.2 ml/min. The average of three replicates is reported.
[d] Calculated as HD_{10}/*E. coli* MIC.

We calculated selectivity ratios (SRs) for each compound, which we defined as the quotient of the 10% hemolytic dose (HD_{10}) and the *E. coli* MIC. All seven peptoids were based on the previously reported dodecamer 1 (19), which contains one-third lysine-like *N*Lys monomers and two-thirds phenylalanine-like *N*spe residues (Fig. 1).

Checkerboard antibacterial assays. We used checkerboard antibacterial assays to determine fractional inhibitory concentrations (FICs) and FIC indices ($\sum$FICs) for interactions between peptoids and peptides (as described in the supplemental material) (8). A $\sum$FIC of 1 is defined as additive with no synergy, and values of $\leq$0.5 indicate increasing degrees of synergy. Lowest $\sum$FICs for combinations of the compounds in Table 1 are shown in Tables 2 and 3 (for *E. coli* and *B. subtilis*, respectively).

Against *E. coli* (Table 2), 21 of 36 combinations tested (excluding controls) demonstrated $\sum$FICs of $\leq$0.50, indicating synergy; 7 combinations yielded $\sum$FICs of $\leq$0.25, indicating highly synergistic interactions with at least an 8-fold decrease in the MIC of each compound in the presence of the other. These highly synergistic interactions comprised all three possible classes of combinations: peptide-peptide, peptide-peptoid, and peptoid-peptoid. In contrast, no synergy was observed against *B. subtilis* (Table 3), as further discussed in the supplemental material.

Mechanistic implications. Very broadly, two possible mechanisms may account for synergy between two compounds: the compounds associate to form a third entity with more potent antimicrobial activity, or the two compounds operate through complementary mechanisms. We propose that the latter situation is true for the compounds tested for several reasons. First, although the seven peptoids were all derived from the sequence of peptoid 1, many of the most synergistic combinations involved both peptoids and peptides that differ considerably in sequence. Were these compounds forming synergistic dimers, they might be expected to share common structural (i.e., dimerization) motifs. Second, intermolecular associations should give rise to both antagonistic and synergistic interactions—it is likely that dimerization would, in some cases, inhibit the normal action of each molecule, with antibacterial activity of the dimer being worse than that of its constituents. The conspicuous absence of antagonism in both Tables 2 and 3 (i.e., $\sum$FICs $\geq$ 4.00) implies that intermolecular associations are not responsible for synergy in these cases. Notably, PGLa, which is well known for its synergistic interaction with magainin-2 (31), has been shown to interact antagonistically with AMPs other than magainin-2 (30). Third, if heterodimerization were responsible for synergy, synergistic combinations would be expected to exhibit 1:1 stoichiometry. In-

TABLE 2. Lowest FIC indices for binary combinations of peptoids and peptides against *E. coli* ATCC 35218[a]

Compound	$\sum$FIC ([A]/[B]) of compound (% ACN) for *E. coli* ATCC 35218[b]								
	PEX (50.2)	1-*N*Lys$_{5,11}$ (51.2)	1-*N*His$_{6,12}$ (51.4)	$1_{achiral}$ (60.4)	1-Pro$_6$ (62.2)	1 (65.1)	MEL (65.2)	1-*N*sna$_{6,12}$ (68.1)	1_{17mer} (70.1)
1_{17mer}	**0.16 (0.20/3.1)**	**0.16 (1.6/6.3)**	**0.25 (6.3/6.3)**	**0.31 (3.1/3.1)**	**0.50 (3.1/6.3)**	0.63 (6.3/3.1)	0.75 (3.1/12.5)	1.00 (12.5/25)	
1-*N*sna$_{6,12}$	**0.19 (0.20/3.1)**	**0.25 (6.3/3.1)**	**0.31 (12.5/1.6)**	**0.31 (3.1/3.1)**	**0.50 (3.1/6.3)**	0.51 (3.1/0.20)	0.75 (6.3/6.3)		
MEL	**0.16 (0.20/1.6)**	**0.16 (1.6/1.6)**	**0.31 (3.1/3.1)**	**0.31 (3.1/0.78)**	**0.50 (3.1/3.1)**	0.52 (3.1/0.20)	0.75 (3.1/6.3)		
1	**0.38 (0.78/1.6)**	**0.50 (0.20/3.1)**	**0.50 (12.5/1.6)**	**0.50 (3.1/1.6)**	**0.50 (3.1/1.6)**				
1-Pro$_6$	**0.50 (1.6/3.1)**	1.00 (25/6.3)	0.63 (6.3/6.3)	0.75 (3.1/6.3)	1.00 (6.3/6.3)				
$1_{achiral}$	0.52 (3.1/0.20)	0.63 (25/25)	0.56 (25/0.78)						
1-*N*His$_{6,12}$	0.63 (0.39/25)	1.00 (25/25)							
1-*N*Lys$_{5,11}$	1.00 (1.6/25)								
PEX	0.53 (3.1/0.20)								

[a] $\sum$FICs of $\leq$0.50 are shown in bold type. Additionally, $\sum$FICs of $\leq$0.25 are underlined. The molar compositions of the lowest-FIC wells are shown in parentheses beside each $\sum$FIC. Compounds are organized in order of increasing molecular hydrophobicity horizontally and decreasing molecular hydrophobicity vertically.
[b] "A" denotes the compound listed across the top, and "B" denotes the compound listed on the left side.

TABLE 3. Lowest FIC indices for binary combinations of peptoids and peptides against *B. subtilis* ATCC 6633[a]

Compound	ΣFIC ([A]/[B]) of compound (% ACN) for *B. subtilis* ATCC 6633[b]								
	PEX (50.2)	1-*N*Lys$_{5,11}$ (51.2)	1-*N*His$_{6,12}$ (51.4)	1$_{achiral}$ (60.4)	1-Pro$_6$ (62.2)	1 (65.1)	MEL (65.2)	1-*N*sna$_{6,12}$ (68.1)	1$_{17mer}$ (70.1)
1$_{17mer}$	1.13 (0.20/1.6)	0.63 (0.20/0.78)	1.00 (0.78/0.39)	1.00 (0.78/0.78)	1.00 (0.78/0.78)	1.00 (0.78/0.39)	0.63 (0.78/0.20)	1.25 (0.20/0.78)	
1-*N*sna$_{6,12}$	1.00 (0.78/0.78)	0.75 (0.20/0.39)	0.75 (0.39/0.78)	0.75 (0.78/0.39)	0.75 (0.39/0.39)	0.63 (0.39/0.78)	0.75 (0.78/0.20)		
MEL	1.00 (0.78/0.78)	0.75 (0.39/0.39)	0.75 (0.78/0.39)	0.63 (0.20/0.78)	1.00 (0.78/0.78)	1.00 (0.78/0.78)	0.75 (0.39/0.78)		
1	1.00 (0.78/0.78)	1.00 (0.39/0.78)	0.75 (0.39/0.78)	1.00 (0.78/0.78)	1.00 (0.78/0.78)				
1-Pro$_6$	1.13 (0.20/1.6)	1.00 (0.39/0.78)	1.00 (0.78/0.78)	0.63 (0.20/0.78)	1.06 (0.10/1.6)				
1$_{achiral}$	0.75 (0.78/0.39)	1.00 (0.39/0.78)	1.13 (0.20/1.6)						
1-*N*His$_{6,12}$	0.75 (0.39/0.78)	0.63 (0.78/0.20)							
1-*N*Lys$_{5,11}$	0.75 (0.78/0.20)								
PEX	1.00 (0.78/0.78)								

[a] Compounds are organized in order of increasing molecular hydrophobicity horizontally and decreasing molecular hydrophobicity vertically.
[b] "A" denotes the compound listed across the top, whereas "B" denotes the compound listed on the left side.

stead, the majority of the highly synergistic pairs worked most efficiently in molar ratios other than 1:1 (Tables 2 and 3). Notably, Yan and Hancock found that antimicrobial peptides from distinct species and structural classes effect synergistic antibacterial activity (32), suggesting that intermolecular associations may not be required, since unrelated peptides have not coevolved and are thus less likely to form synergistic dimers. Thus, while this has not been proven experimentally, it is unlikely that a dimerization is occurring.

If complementary mechanisms are indeed responsible for the observed synergy, then several important mechanistic hypotheses may be deduced. In previous work, we showed that low molecular hydrophobicity corresponds to selective antibacterial activity, whereas high hydrophobicity correlates with nonselective activity for both peptides and peptoids (5, 19). As seen in Table 2, highly synergistic interactions ($\sum$FIC $\leq$ 0.25, corresponding to at least an 8-fold decrease in the MIC for each compound in the presence of the other) between these nine oligomers against *E. coli* occurred exclusively in combinations containing one selective (less hydrophobic) compound and one nonselective (more hydrophobic) compound. It is therefore likely that the members of synergistic pairs in Table 2 employ distinct but complementary mechanisms.

Notably, these synergy data are highly consistent with mechanistic analogy between antimicrobial peptoids and peptides. One of the most synergistic combinations consists of the peptides pexiganan and melittin ($\sum$FIC = 0.16). A high degree of synergy is maintained either if the nonselective melittin is replaced by a nonselective peptoid (e.g., 1$_{17mer}$) or if pexiganan is replaced by a highly selective peptoid (e.g., 1-*N*Lys$_{5,11}$), or both. The robustness of synergy to these substitutions implies that the mechanisms used by peptoids are fully analogous to those used by AMPs of similar hydrophobicity and selectivity. Although we did not explicitly investigate mechanism in this work, the aforementioned trends bear a notable resemblance to the spectrum of mechanisms defined at either extreme by the barrel stave and carpet mechanisms, as described in several reviews by Shai and Oren (18, 25, 26).

Hemolysis and therapeutic potential. We determined the hemolytic activities of the nine most synergistic pairs in Table 2 by combining them in the molar ratios present in lowest-$\sum$FIC wells and serially diluting them. The resulting HD$_{10}$ and HD$_{50}$ for each combination, as well as the molar ratio used, are shown in Table 4. In addition, we calculated the theoretical HD$_{10}$ and HD$_{50}$ for each combination from the individual hemolysis data (Table 1) by assuming an additive hemolytic interaction; i.e., we averaged the individual percent hemolysis curves, weighted according to the molar composition of the combinations, and determined the hemolytic doses from the averaged curves (Table 4). We found a close correspondence between experimentally determined hemolytic doses and those theoretically calculated assuming that hemolysis was nonsynergistic, demonstrating that hemolytic activities are the result of additive, rather than synergistic, interactions (Table 4). This is not particularly deleterious, however, since much current development of antimicrobial peptides is for topical applications (13), and synergy can be maximized while hemolytic activity is minimized by using two moderately selective peptoids, as in the combination 1–1-Pro$_6$.

In summary, we have demonstrated highly synergistic interactions between antimicrobial peptoids and peptides. The observed synergy strongly suggests mechanistic analogy between these two classes of compounds. Furthermore, the tendency of hydrophobic oligomers to synergize with relatively hydrophilic oligomers suggests that selective and nonselective antimicrobial peptides and peptoids kill bacteria via distinct but complementary mechanisms, offering a pathway to further optimize both for therapeutic applications.

TABLE 4. Theoretical and experimentally determined hemolytic activities of synergistic combinations

Combination (compound A–compound B)	Molar ratio (mol A/mol B)	HD$_{10}$/HD$_{50}$ (μM)	
		Theoretical[a]	Experimental
Pexiganan–melittin	1:8	1/7	2/8
Pexiganan–1-*N*sna$_{6,12}$	1:16	7/29	7/28
Pexiganan–1$_{17mer}$	1:16	3/17	4/18
1-*N*Lys$_{5,11}$–melittin	1:1	2/24	2/10
1-*N*Lys$_{5,11}$–1-*N*sna$_{6,12}$	2:1	17/>100	19/76
1-*N*Lys$_{5,11}$–1$_{17mer}$	1:4	4/21	5/21
1-*N*His$_{6,12}$–melittin	1:1	2/24	3/12
1-*N*His$_{6,12}$–1-*N*sna$_{6,12}$	8:1	59/>100	65/>200
1-*N*His$_{6,12}$–1$_{17mer}$	1:1	6/79	9/38
Peptoid 1–1-Pro$_6$	1:2	31/180	31/140
Peptoid 1–1$_{achiral}$	1:2	34/>200	46/190

[a] Assumes additive interaction—calculated as the weighted average of percent hemolysis curves.

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