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Structural congruence among membrane-active host defense polypeptides of diverse phylogeny

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Abstract

A requisite for efficacious host defense against pathogens and predators has prioritized evolution of effector molecules thereof. A recent multidimensional analysis of physicochemical properties revealed a novel, unifying structural signature among virtually all classes of cysteine-containing antimicrobial peptides. This motif, termed the γ-core, is seen in host defense peptides from organisms spanning more than 2.6 billion years of evolution. Interestingly, many toxins possess the γ-core signature, consistent with discoveries of their direct antimicrobial activity. Many microbicidal chemokines (kinocidins) likewise contain iterations of the γ-core motif, reconciling their antimicrobial efficacy. Importantly, these polypeptide classes have evolved to target and modulate biomembranes in protecting respective hosts against unfavorable interactions with potential pathogens or predators. Extending on this concept, the current report addresses the hypothesis that antimicrobial peptides, kinocidins, and polypeptide toxins are structurally congruent and share a remarkably close phylogenetic relationship, paralleling their roles in host–pathogen relationships. Analyses of their mature amino acid sequences demonstrated that cysteine-stabilized antimicrobial peptides, kinocidins, and toxins share ancient evolutionary relatedness stemming from early precursors of the γ-core signature. Moreover, comparative 3-D structure analysis revealed recurring iterations of antimicrobial peptide γ-core motifs within kinocidins and toxins. However, despite such congruence in γ-core motifs, the kinocidins diverged in overall homology from microbicidal peptides or toxins. These findings are consistent with observations that chemokines are not toxic to mammalian cells, in contrast to many antimicrobial peptides and toxins. Thus, specific functions of these molecular effectors may be governed by specific configurations of structural modules associated with a common γ-core motif. These concepts are consistent with the hypothesis that the γ-core is an archetype determinant in polypeptides that target or regulate with biological membranes, with specific iterations optimized to unique or cognate host defense contexts. Quantitative and qualitative data suggest these protein families emerged through both parallel and divergent processes of modular evolution. Taken together, the current and prior findings imply that the γ-core motif contributes to conserved structures and functions of host defense polypeptides. The presence of this unifying molecular signature in otherwise diverse categories of membrane-active host defense peptides implies an ancient and essential role for such a motif in effector molecules governing host–pathogen relationships.

Keywords: Antimicrobial peptide; Toxin; Chemokine; Host defense; Structure; Evolution

1. Introduction

Host–pathogen relationships have undergone co-evolution for as long as organisms have interacted with one another. Yet, the basic definitions of host and pathogen are relative. To humans, pathogens are most often considered microbial. To lower organisms, pathogens include microbes, but also include predators. For example, scorpions contain antimicrobial effector molecules, but have also evolved host defense toxins designed to protect against challenges by higher organisms. If the molecules that confer such parallel functions derived from a shared or common ancestry, it would be reasonable to predict they would also exhibit significant structural conservation.

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Recently, we discovered a structural signature common to all of the major classes of disulfide-stabilized antimicrobial peptides. This motif, termed the γ-core, has several physicochemical features that are associated with membrane-active antimicrobial efficacy [1]. Remarkably, the sequence and 3-dimensional formulae defining this signature also led us to identify the γ-core in other host defense polypeptides, such as toxins and microbicidal chemokines (kinocidins). Importantly, while the γ-core motif is highly conserved, structural modules associated with this motif vary considerably among these classes of host defense peptides. It should also be noted that while the γ-core motif is common to the vast majority of cysteine-containing antimicrobial peptides, exceptions exist, including the predominantly α-helical "rana-box" peptides in frogs [2,3], certain antimicrobial peptide fragments derived from larger proteins, such as the lactoferricins [4,5], or those with non-cysteine bridging [6].

Extending upon discovery of the γ-core, we proposed an immunorelativity model that unifies the structure–activity relationships shared among such host defense effector molecules [7]. Several concepts comprise the cornerstones of this model: (1) *functional multiplicity*: the γ-core motif may confer direct membrane-activity and serve as a greater structural scaffold; (2) *modular configuration*: distinct structural modules with specific functions appear to be configured to the γ-core in various types of host defense peptides; (3) *context specificity*: host defense peptides have evolved to function optimally in specific contexts, where they may have one or more functions in their native form or via deployment of structural modules to act against cognate pathogens; and (4) *immunopotentiation*: integration of the above extends the repertoire of host defense capabilities. For example, kinocidins have direct microbicidal efficacy, but also potentiate the antimicrobial mechanisms of leukocytes. Thus, host defenses are optimized by virtue of synergistic interactions among the multiple functions of host defense polypeptides.

The current report focuses on comparative structural and phylogenetic relationships among host defense peptides containing γ-core motifs. Multiple analyses were used to assess the structural similarities among three major groups of host defense molecules containing the γ-core: antimicrobial peptides, kinocidins, and polypeptide toxins. Statistical methods were also employed to measure the phylogenetic relatedness among these molecules, and predict whether this molecular signature has been retained through convergent, parallel, or divergent evolutionary mechanisms. As will be considered in the ensuing discussion, results suggest cysteine-stabilized host defense polypeptides including antimicrobial peptides, kinocidins, and certain toxins have remarkable structural congruence revolving around their common γ-core signature. Moreover, these classes of molecules appear to have derived from a process of modular evolution, where complementary structural modules such as α-helical and/or β-sheet domains are interchangeably configured to the γ-core. Thus, while the γ-core motif appears to be a shared determinant among polypeptides that target or modify biological membranes, structural modules associated with this scaffold likely influence the potential for toxicity and target cell specificity. Collectively, the striking degree of structural congruence among host defense polypeptides suggests that the γ-core motif is an archetypal determinant in effector molecules that govern immune syntax and mediate host–pathogen relationships.

2. Methods

2.1. Investigational polypeptides

Phylogenetic relatedness among amino acid primary sequences was examined in cysteine-containing antimicrobial peptide, kinocidin, and toxin prototypes representing taxa spanning a cumulative evolutionary distance of 2.6 billion years (BY; estimated divergence of fungi and plants from higher organisms [8]). Representative prototypes from each class were included in these analyses [1], with specific inclusion criteria being: (1) eukaryotic origin; (2) published antimicrobial activity; (3) non-enzymatic mechanism of action; (4) mature protein sequence; and (5) less than 75 amino acids. Peptides for which structures have been determined were used in the structural analyses [1]. The resulting study set included host defense polypeptides encompassing a broad distribution in source (i.e., biological kingdoms ranging from microbes to man), amino acid sequence, and conformation class. As in our prior studies, amino acid sequence data were prioritized for these analyses, since not all nucleotide sequences have been fully characterized, and saturation of nucleotide sequence data occurs among non-mitochondrial sequences over evolutionary timescales.

2.2. Phylogenetic comparisons

Potential evolutionary relationships among the study peptides were assessed by multiple methods. The relatedness among primary structures was analyzed using multiple sequence alignment (MSA) and Clustal W tool [9]. In this method, amino acid sequence alignment similarity was prioritized based on overall and motif-specific congruence. This approach yielded average distance dendograms quantifying evolutionary distances between individual molecular species (nodes) and molecular groups (clades). The phylogenetic relationships among 3-D structures of study molecules were then assessed using the neighbor joining method [10]. The neighbor joining method employs cluster analysis, but does not require ultrametric data; thus, it allows comparisons among molecules with different rates of mutation and is especially well suited for analyses comparing sequences separated by vast evolutionary distances.

2.3. Structural analyses

A panel of prototypic antimicrobial peptides, kinocidins, and toxins representing each of the respective groups were structurally analyzed as previously described [1]. Polypeptide structure data were derived from the Protein Data Bank (PDB), and visualized using Protein Explorer [11]. Three-dimensional structural alignments were carried out using combinatorial extension as previously detailed [12].

2.4. Computational modeling

To complement the above structure analyses, 3-D models of some polypeptides were generated using complementary methods as previously described [1,13–17]. For selected comparisons, net surface charge was assessed using a combination of molecular modeling techniques. In brief, 3-D models were constructed using their published molecular coordinates, along with COMPOSER and AMBER force field applications [13,14,16]. The net charge was then estimated and projected onto molecular 3-D solvent accessible surface areas using algorithms detailed previously [13,14,17,18].

3. Results

3.1. Primary structure congruence in polypeptides containing a γ-core signature

Our prior multidimensional analyses identified a unifying sequence and structural signature present in all classes of

cysteine-stabilized antimicrobial peptides [1]. Multiple sequence alignment of diverse families of cysteine-stabilized antimicrobial peptides revealed alternate consensus formulae:

$NH_2...[X_{1-3}]-[GXC]-[X_{3-9}]-[C]...COOH$
(dextromeric)

$NH_2...[C]-[X_{3-9}]-[CXG]-[X_{1-3}]...COOH$
(levomeric isoform 1)

$NH_2...[C]-[X_{3-9}]-[GXC]-[X_{1-3}]...COOH$
(levomeric isoform 2)

These conserved sequence patterns were found in antimicrobial peptides from highly divergent phylogenetic sources. Several themes emerged upon examination of this sequence motif. First, the consensus formulae are present in otherwise highly diverse structure classes of antimicrobial peptides. In some cases, these patterns were only evident when the peptide primary sequence was inverted with respect to conventional sequence orientation; such sequences are referred to as the levomeric isoforms 1 and 2 [1]. Additionally, the consensus characteristics of charged termini and a central hydrophobic domain in γ-core motifs among antimicrobial peptides were discovered in these prior analyses.

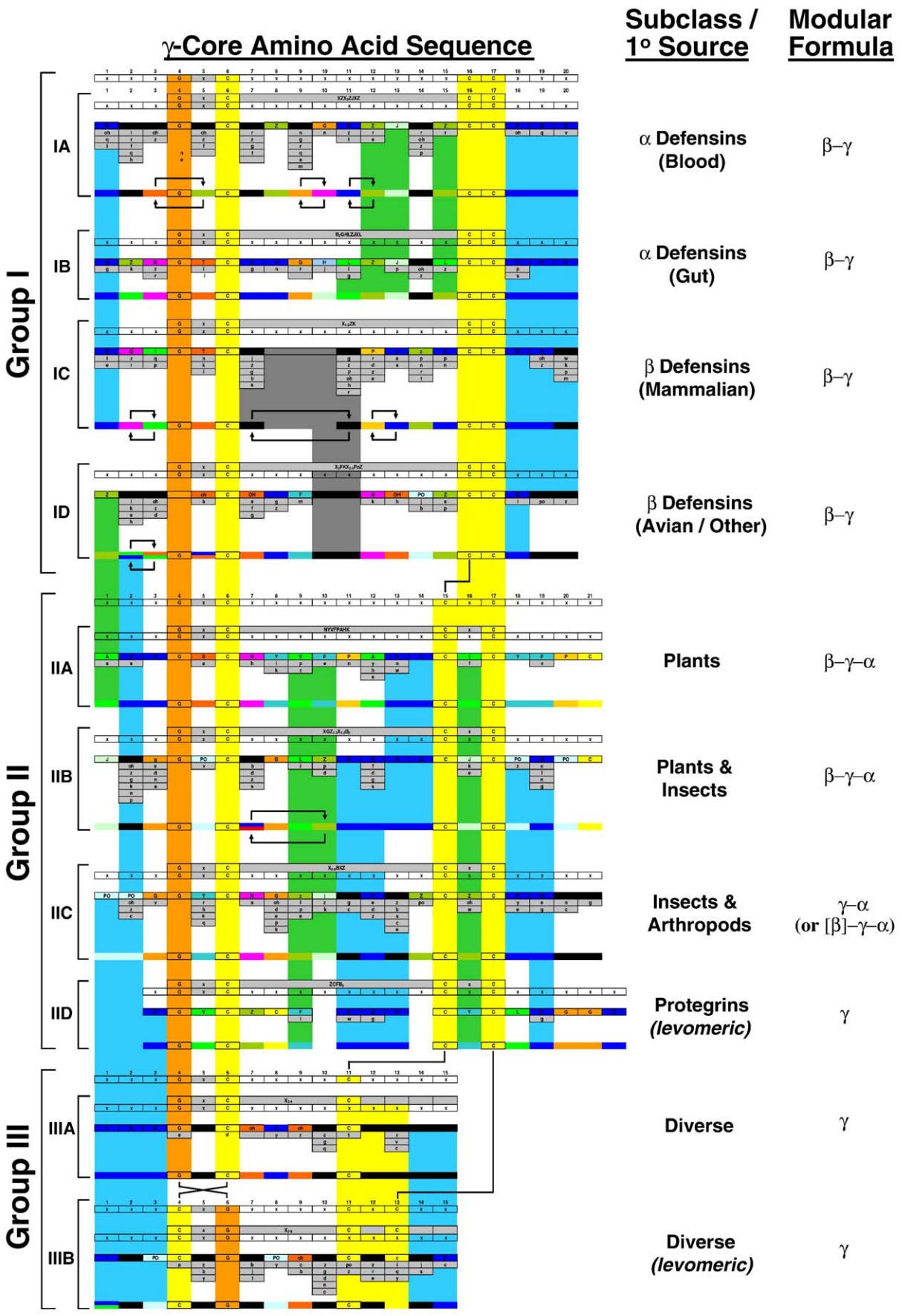
The next step in discovery of the multidimensional signature in antimicrobial peptides was to search for unifying 3-D motifs within antimicrobial peptides containing the primary sequence formulae. This analysis identified a hallmark structural element common to broad classes of cysteine-containing antimicrobial peptides. This structural motif consisted of an anti-parallel β-hairpin typically 12–18 residues in length, a 3-D conformation reminiscent of the Greek letter gamma (γ), and a central location in many antimicrobial peptides. Therefore, this multidimensional signature has been termed the γ-core motif [1]. In many antimicrobial peptides, α-helical or β-sheet domains flank the γ-core, yielding molecular structures with an apparently modular variety of α–γ, β–γ, γ–α, γ–β, α–γ–β, and other compound configurations.

As a part of our previous studies, we carried out sequence and structural alignments to probe in silico for novel unidentified antimicrobial peptides. This approach revealed that several other protein families for which antimicrobial activity had not been described also exhibited a γ-core signature as in antimicrobial peptides. These findings also revealed that many mammalian kinocidins possessed iterations of the γ-core, corresponding to their direct antimicrobial efficacy as we and others had established earlier [1,19–22]. Importantly, all of the polypeptides predicted by the γ-core signature to have antimicrobial activity did so upon testing against human bacterial and fungal pathogens in vitro [1].

The specific primary structures comprising the γ-core sequence motif of antimicrobial peptides are compared in Fig. 1. A comprehensive inspection of the amino acid patterns in antimicrobial peptides that contain the γ-core signature reveals potential relationships that suggest broader themes in phylogenesis. For example, distinct antimicrobial peptide families sort based upon structural and host source relatedness. Illustrating this theme is a remarkable similarity among γ-core primary structures within defensin and related molecules from higher eukaryotes (Fig. 1, Group I). However, further specification appears to differentiate such molecules associated with host contexts. For instance, defensins principally corresponding to hematogenous function (Group IA), and those most closely associated with gastrointestinal source (Group IB), differ in the variable amino acid positions within their respective γ-core sequence patterns.

Detailed analysis of γ-core sequences in diverse antimicrobial peptides also suggests potentially broader co-evolutionary relationships. The fact that γ-core motifs in antimicrobial peptides from plants and insects share a particularly close structural congruence exemplifies this concept (Fig. 1, Group II). Ostensibly, these hosts necessarily developed effector molecules optimized to defend against common pathogens, such as fungi. Reinforcing this point, recent studies have demonstrated that such antimicrobial peptides are elaborated by way of toll-like receptors in response to common signals of microbial infection, including fungal cell wall constituents as well as bacterial lipopolysaccharide (LPS) [23,24]. This continuum of amino acid patterns relates plants, insects, and arthropods (Fig. 1, Groups IIA, B, and C). Yet, context specificity also distinguishes subgroups of peptides from these distinct host sources, equivalent to the theme observed in mammals (see above). Finally, it is worth noting that the inverted dextromeric or levomeric orientations of primary sequences in antimicrobial peptides comprised solely of a γ-core motif trace phylogenetically to the most diverse host sources — from mammals (e.g., protegrins) to microbes (Fig. 1, Groups IID and IIIA and B). Thus, Nature appears to have allowed plasticity within otherwise highly conserved 3-D structures (see below) of the γ-core within molecules optimized to function in specific host defense contexts.

Our prior studies uncovered that a number of additional protein families possessed the multidimensional γ-core signature, including certain subclasses of protease inhibitors, sweet proteins (e.g., brazzein), and polypeptide toxins [1]. The molecules comprising this latter group were especially predominant, and will be considered here for purposes of illustration. The γ-core domain of toxins (γ_T-core) is essentially invariant from that found in antimicrobial peptides (γ_{AP}-core). As in the γ_{AP}-core, the γ_T-core is approximately 12–18 residues in length, and contains a central hydrophobic domain flanked by charged termini. Interestingly, as the γ_T-core and γ_{AP}-core motifs themselves are highly congruent, differences in overall structure between these molecule classes likely relate to amino acid domains or structural modules beyond their γ-core regions. Similarly, the kinocidin γ-core domain (γ_κ-core) is an iteration of the γ-core motif observed in antimicrobial peptides and toxins. As observed in its γ_{AP}-core and γ_T-core relatives, the γ_K-core is an anti-parallel β-hairpin approximately 15 residues in length, and has a central hydrophobic region that is typically flanked by charged residues. However, in kinocidins, the GXC consensus pattern of the γ_{AP}-core in antimicrobial peptides is varied such that a GX_3C pattern is often observed. Importantly, analyses of more than 100 kinocidins from species as phylogenetically diverse as teleost fish and man identify the most


γ-Core Amino Acid Sequence
Subclass / 1° Source
Modular Formula
Group I
IA
α Defensins (Blood)
β–γ
IB
α Defensins (Gut)
β–γ
IC
β Defensins (Mammalian)
β–γ
ID
β Defensins (Avian / Other)
β–γ
Group II
IIA
Plants
β–γ–α
IIB
Plants & Insects
β–γ–α
IIC
Insects & Arthropods
γ–α (or [β]–γ–α)
IID
Protegrins (levomeric)
γ
Group III
IIIA
Diverse
γ
IIIB
Diverse (levomeric)
γ

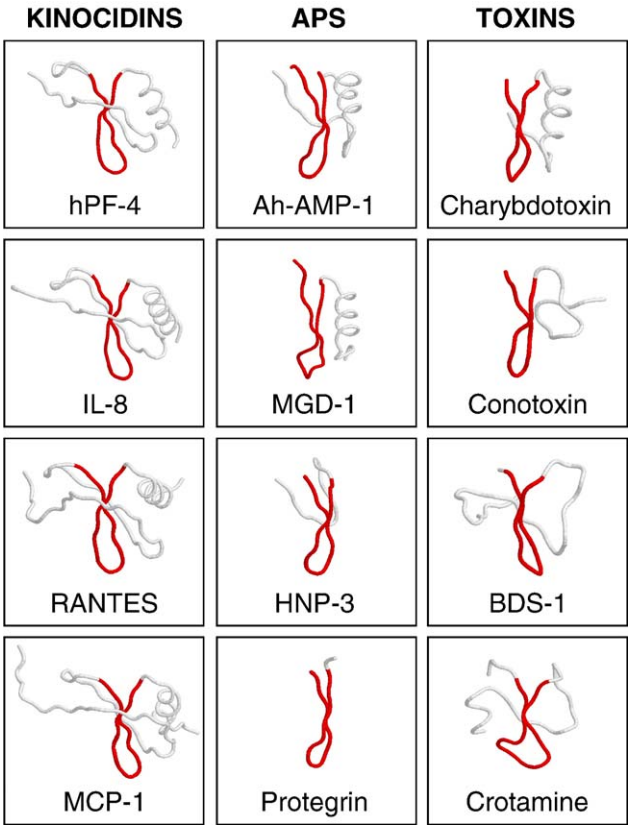


Fig. 2. Conserved γ-core motif among diverse disulfide-containing host defense peptides. The 3-dimensional conformations of peptides with structures previously defined experimentally were visualized and compared using Protein Explorer. The γ-core motifs are indicated in red for kinocidins (microbicidal chemokines), antimicrobial peptides, and toxins spanning roughly 2.6 BY of evolutionary distance. A non-exhaustive panel of prototypic peptides depicted for illustration includes: kinocidins—human platelet factor-4 (hPF4; 1RHP); interleukin-8 (IL-8; 1IL8); releasable upon activation normal T-cell expressed and secreted (RANTES; 1RTO); and monocyte chemotactic protein-1 (MCP-1; 1DOM); antimicrobial peptides—chestnut antifungal peptide (Ah-AMP-1; 1BK8); mussel antimicrobial peptide (MGD-1; 1FJN); human neutrophil defensin (HNP-3; 1DFN); porcine protegrin-1 (1PG1); toxins—scorpion charybdotoxin (2CRD); sea snail conotoxin (1AG7); sea anemone toxin BDS (1BDS); and the rattlesnake toxin crotamine (1H5O). Note how structural modules such as β-sheet or α-helical domains are configured to the common γ-core in different molecules.

highly conserved domains in the mature portion of these proteins localize to the γ_{κ}-core.

3.2. 3-dimensional congruence among polypeptides containing a γ-core signature

The structural and biophysical identities among antimicrobial peptides, kinocidins and toxins are significant. At the 3-D level, congruence in γ-core domains of such diverse protein classes is striking (Fig. 2). Within these polypeptides, the γ-core is typically centrally localized. In addition, this motif often interposes adjacent α-helical or β-sheet domains, yielding modular architectures revolving around congruent permutations of the γ-core.

The degree to which these peptide classes share structural identity can be quantified using combinatorial extension to measure root mean squared deviation (RMSD) values between carbon-backbone atoms [Table 1; [12]]. By convention, RMSD values of less than 3.0 Å are indicative of a highly significant degree of structural identity between protein comparators. As might be expected, comparing peptides with similar modular architectures, such as representative α–γ peptides (e.g., 1E4S; HBD-1), yields the greatest identity (lowest RMSD; Table 1). Peptide toxins generally share a high degree of identity with prototypic β–γ–α antimicrobial peptides of plants (e.g., 1BK8; Ah-AMP-1), although even lower RMSD values may be obtained if comparisons are made within structural subclasses. Kinocidins share the greatest identity with structurally austere γ-core peptides (e.g., 1PG1; protegrin-1), likely due to variances in flanking domains in other γ-core polypeptides having compound modular configurations.

In certain cases, the degree of 3-D identity in peptides that contain a multidimensional γ-core signature is remarkable. For example, some toxin and antimicrobial peptide pairs are essentially superimposable 3-dimensionally, and share a striking degree of identity at their primary sequence level (Fig. 3). Notably, specific residues contributing to secondary structure (e.g., Cys, Pro) and amphipathicity (hydrophobic and charged residues) are most highly conserved within the γ-core. The fact that such peptides are separated by as much as 2.6 billion years of evolutionary distance (e.g., chestnut tree antimicrobial

Fig. 1. Amino acid sequence pattern map illustrating structural themes in iterations of the γ-core motif conserved across diverse classes of disulfide-containing antimicrobial peptides. Three major structural groups are identified based on their GXC, CXG and $CX_{0-1}C$ sequence pattern motifs within the γ-core: Group I: $G_4X_5C_6\bullet\bullet\bullet C_{16}C_{17}$; Group II: $G_4X_5C_6\bullet\bullet\bullet C_{15}X_{16}C_{17}$; Group III: $G_4X_5C_6\bullet\bullet\bullet C_{11}$ (IIIA) or $C_4X_5G_6\bullet\bullet\bullet C_{11}X_{12}C_{13}$ (IIIB). Note that Groups IID and IIIB are aligned corresponding to levomeric orientations. From top to bottom for each subgroup, amino acid position, consensus formula, motif pattern, degrees of freedom at each position (with amino acids listed in descending order of frequency), and overall consensus map are indicated for comparison. The primary sources of each subgroup are also indicated, along with their modular formulae relative to the γ-core (also see Figs. 2 and 5). The sources of peptides within Group III are denoted as diverse, including primates, mollusca, arachnids, insects, plants, and microorganisms. In some cases, the levomeric pattern formulae reflect a reduced length as compared with their dextromeric counterparts. Coloration reflects the most conserved residue (threshold, > 50% frequency) at the position indicated, as adapted from the RASMOL color schema: cysteine (C), yellow; glycine (G), orange; variable residue within the GXC or CXG motif (X), gray; yellow; lysine or arginine, royal blue; serine or threonine, orange; leucine, isoleucine, alanine or valine, dark green; aromatic, mint green; polar, aqua; and highly variable positions (no more than 25% consensus in a subgroup), black. In some sequences, gaps have been inserted to maximize alignments. Residues denoted with two colors (e.g., Group ID, residues 2 and 5) depict positions in which there is approximately equal frequency of the two amino acid categories indicated. Other specific amino acid residues or residue classes are also denoted in standard single letter code, with the following additions: OH, hydroxylated (S, T, Y); B, cationic (K, R, H); Z, hydrophobic/non-aromatic (L, I, A, V); J, aromatic (W, F); Po, polar. Note that some amino acids may apply to more than one class (e.g., Y is aromatic and hydroxylated). Upper and lower case amino acid designations indicate positions of conserved (frequency > 50%) or common (frequency 20–50%) residues, respectively. Intergroup shading indicates common themes preserved across all iterations: yellow, cysteine array; orange, glycine; blue, cationic charge; green, hydrophobicity; gray, high variability. For example, note that with the exception of subgroups ID and IIA, cationic amino acid residues are polarized to the termini of the γ-core motifs. Arrows indicate positions in which amino acid motifs are commonly interchanged. Note the striking overall conservation among amino acid patterns related to γ-core signature across subgroups encompassing vast phylogenetic diversity.

Table 1
Structural congruence among antimicrobial peptides, toxins, and kinocidins

Modular form	Known antimicrobial peptides	AA	γ			γα			αγ			βγ			βγα		
			vs. Protegrin			vs. MGD-1			vs. hBD-2			vs. HNP-3			vs. Ah-AMP-1		
			%	RMSD	A/G	%	RMSD	A/G	%	RMSD	A/G	%	RMSD	A/G	%[a]	RMSD	A/G[b]
γ	**Protegrin** (*Sus*; Pig; 1PG1)	19	–	–	–										18.8	1.2	16/0
	Gomesin (*Acanthoscurria*; Spider; 1KFP)	19	25.0	1.6	16/0										6.2	1.2	16/0
	Thanatin (*Podisus*; Soldier Bug; 8TFV)	21	12.5	2.7	16/0										12.5	2.2	16/0
	RTD-1 (*Macaca*; Rhesus Macaque; 1HVZ)	18	37.5	1.7	16/0										25.0	2.3	16/0
	Tachyplesin (*Tachypleus*; Horseshoe Crab; 1MA2)	17	18.8	1.9	16/0										6.2	2.6	16/0
	Polyphemusin I (*Tachypleus*; Horseshoe Crab; 1RKK)	19	31.2	1.7	16/0										12.5	2.3	16/4
γα	**MGD-1** (*Mytilus*; Mussel; 1FJN)	39				–	–	–							26.5	2.0	34/1
	Sapecin (*Sarcophaga*; Flesh Fly; 1L4 V)	40				9.4	4.1	32/11							18.8	3.5	32/4
	Plectasin (Pseudoplectania; Mycoplasma; 1ZFU)	40				54.2	1.4	24/1							21.9	3.1	32/2
	Heliomycin (*Heliothis*; Moth; 1I2U)	44				12.5	3.9	32/4							6.2	3.0	32/8
	Drosomycin (*Drosophila*; Fruit Fly; 1MYN)	44				34.4	2.4	32/3							29.3	1.2	6 5/6
	Insect Defensin A (*Protophormia*; Flesh Fly; 1ICA)	40				45.8	1.8	24/3							18.8	5.3	32/4
αγ	**hBD-2** (*Homo*; Man; 1FD3)	37							–	–	–				3.1	5.3	4
	hBD-1 (*Homo*; Man; 1IJV)	36							41.7	1.3	36/0				9.4	5.7	32/6
	hBD-3 (*Homo*; Man; 1KJ6)	45							35.7	1.8	34/0				12.5	4.4	32/17
	mBD-8 (*Mus*; Mouse; 1E4R)	35							25	2.6	32/5				0.0	3.4	24/13
	mBD-7 (*Mus*; Mouse; 1E4 T)	37							35.5	1.9	31/2				12.5	4.4	32/17
βγ	**HNP-3** (*Homo*; Man; 1DFN)	30										–	–	–	8.3	3.2	24/17
	RK-1 (*Oryctolagus*; Rabbit; 1EWS)	32										29.2	2.3	24/2	4.2	3.1	24/19
	BNBD-12 (*Bos*; Cow; 1BNB)	38										33.3	2.1	24/2	12.5	6.0	32/9
	Spheniscin-2 (*Aptenodytes*; Penguin; 1UT3)	38										16.7	2.5	24/2	6.2	4.4	1 8/9
	Cryptdin-4 (*Mus*; Mouse; 1TV0)	32										25	2.6	24/2	0.0	3.6	24/19
	Tachystatin (*Tachypleus*; Horseshoe Crab; 1CIX)	44										16.7	4.4	24/2	0.0	4.3	32/20
βγα	**Ah-AMP-1** (*Aesculus*; Chestnut Tree; 1BK8)	50													–	–	–
	Rs-AFP-1 (*Raphanus*; Radish; 1AYJ)	51													51	1.7	49/0
	Psd-1 (*Pisum*; Pea; 1JKZ)	46													39.5	1.7	43/4
	γ -1-P-thionin (*Triticum*; Wheat; 1GPT)	47													23.9	1.7	46/3
	γ-1-H-thionin (*Hordeum*; Barley; 1GPS)	47													26.1	1.8	46/3
	Brazzein (*Pentadiplandra*; J'oublie berry; 1BRZ)	54													17.4	2	46/2
	Mean	36.5		1.9			2.7			1.9			2.8			3.1	
	± S.D.	10.8		0.4			1.2			0.5			0.9			1.4	
Variable	**Toxins**																
	Huwentoxin-IV (*Selenocosmia*; Spider; 1MB6)	35	12.5	2.9	16/0										6.2	4.2	32/8
	Erabutoxin B (*Laticauda*; Sea Snake; 3EBX)	62	0.0	1.0	16/4										9.4	4.2	32/9

Table 1 (*continued*)

Modular form	Known antimicrobial peptides	AA	γ			γα			αγ			βγ			βγα		
			vs. Protegrin			vs. MGD-1			vs. hBD-2			vs. HNP-3			vs. Ah-AMP-1		
			%	RMSD	A/G	%	RMSD	A/G	%	RMSD	A/G	%	RMSD	A/G	%[a]	RMSD	A/G[b]
Variable	Anthopleurin-B (*Anthopleura*; Sea Anenome; 1APF)	49	18.8	2.9	16/7										9.4	4.1	32/16
	Hanatoxin 1 (*Grammostola*; Spider; 1NIX)	34				12.5	2.8	24/10							18.8	3.9	32/9
	Hainantoxin-1 (*Selenocosmia*; Spider; 1D1H)	35				20.8	2.8	24/10							21.9	3.7	32/11
	Omega Grammotoxin (*Grammostola*; Spider; 1KOZ)	36				25	2.3	24/10							25	3.5	32/9
	DLP-1 (*Ornithorhynchus*; Platypus; 1B8W)	42							21.9	2.6	32/3				9.4	4.7	32/16
	Crotamine (*Crotalus*; Snake; 1H5O)	42							28.1	2.3	32/3				9.4	3.6	32/14
	BDS-1 (*Anemonia*; Anemone; 1BDS)	43										4.2	3.2	24/3	8.3	2.9	24/5
	Charybdotoxin (*Leiurus*; Scorpion; 2CRD)	37													34.4	1.9	32/4
	BeKm-1 (*Buthus*; Scorpion; 1J5J)	36													15.6	2.1	32/3
	Tstx-K (*Tityus*; Scorpion; 1HP2)	37													28.1	2.1	32/4
	Mean	39.3		2.3			2.6			2.5			–			3.4	
	± S.D.	3.3		1.1			0.3			0.2						0.9	
ββββγα	**Kinocidins**																
	IL-8 (*Homo*; Human; 1IL8)	72	0	1.4	16/3										9.4	4.3	32/29
	PF-4 (*Homo; Man*; 1RHP)	70	0	1.4	16/3										12.5	4.2	32/26
	RANTES (*Homo*; Human; 1RTO)	68	6.2	1.8	16/1										6.2	4.1	32/26
	MCP-1 (*Homo*; Human; 1DOM)	76	0	1.7	16/2										9.4	3.8	32/29
	GRO-α (*Homo*; Human; 1MSG)	72	0	1.4	16/3										12.5	4.6	32/29
	Lymphotactin (*Homo*; Human; 1J9O)	93	18.8	1.4	16/1										12.5	4.6	32/27
	MIP-1α (*Homo*; Human; 1B53)	69	18.8	1.4	16/1										12.5	4.2	32/27
	CTAP-III (*Homo; Human*; (1F9P)	85	6.2	1.3	16/1										6.2	4.5	32/25
	Mean	75.6		1.5												4.3	
	± S.D.	8.9		0.2												0.3	

The comparative 3-D structure identities were calculated between peptides using combinatorial extension [12], and are expressed as RMSD values. Each peptide is compared to a prototypic βγα peptide (Ah-AMP-1; 1BK8), and to specific structure classes: γ, (protegrin; 1PG1); γα, (MGD-1; 1FJN); αγ, (HBD-2; 1FD3); and βγ, (HNP-3; 1DFN). Peptides are listed in the following nomenclature: peptide name (*Genus*; common name; PDB code).

peptide, Ah-AMP-1, and scorpion charybdotoxin; Fig. 3, panels A and B) highlights the significance of these observations. The potential evolutionary mechanisms driving this extent of structural and compositional identity, with concomitant functional diversity, are under investigation.

At a biophysical level, antimicrobial peptides, kinocidins and toxins have significant degrees of homology, in theory reflecting the fact that members of each of these polypeptide classes target and interact with biomembranes. All three classes are typically small (2–10 kDa), cationic and amphipathic. In addition, most antimicrobial peptide, kinocidin and toxin isoelectric points are 8.0 or greater, indicative of a net positive charge at neutral pH. However, the distribution of charge within these molecules is not uniform, with the most frequently cationic domains including the C-terminal α-helix (if present) and termini of the β-hairpin conformation of the γ-core. Molecular modeling of prototypic antimicrobial peptides, kinocidins and toxins indicates they typically have one or more electropositive domains and surface facets (Fig. 4; panels A, C, E, G, I, K). Importantly, the segregation of positive and negative charge appears to be a recurring theme in these classes of molecules. Furthermore, polypeptides representing these classes have overall as well as domain-specific regions of amphipathicity (Fig. 4; panels B, D, F, H, J, L). In general, they typically have one surface facet that is significantly more hydrophobic than the other. Not surprisingly, it is this hydrophobic face that is often oriented toward solvent-inaccessible interiors of those peptide multimers that have been characterized structurally [25,26]. Taken together, such attributes are likely integral to the membrane-targeting properties that confer antimicrobial, cytotoxic, or receptor-activating functions of these molecules.

3.3. Phylogenetic relationships among polypeptides containing a γ-core signature

Given the structural and biophysical congruence between antimicrobial, kinocidin and toxin families of peptides, it was of

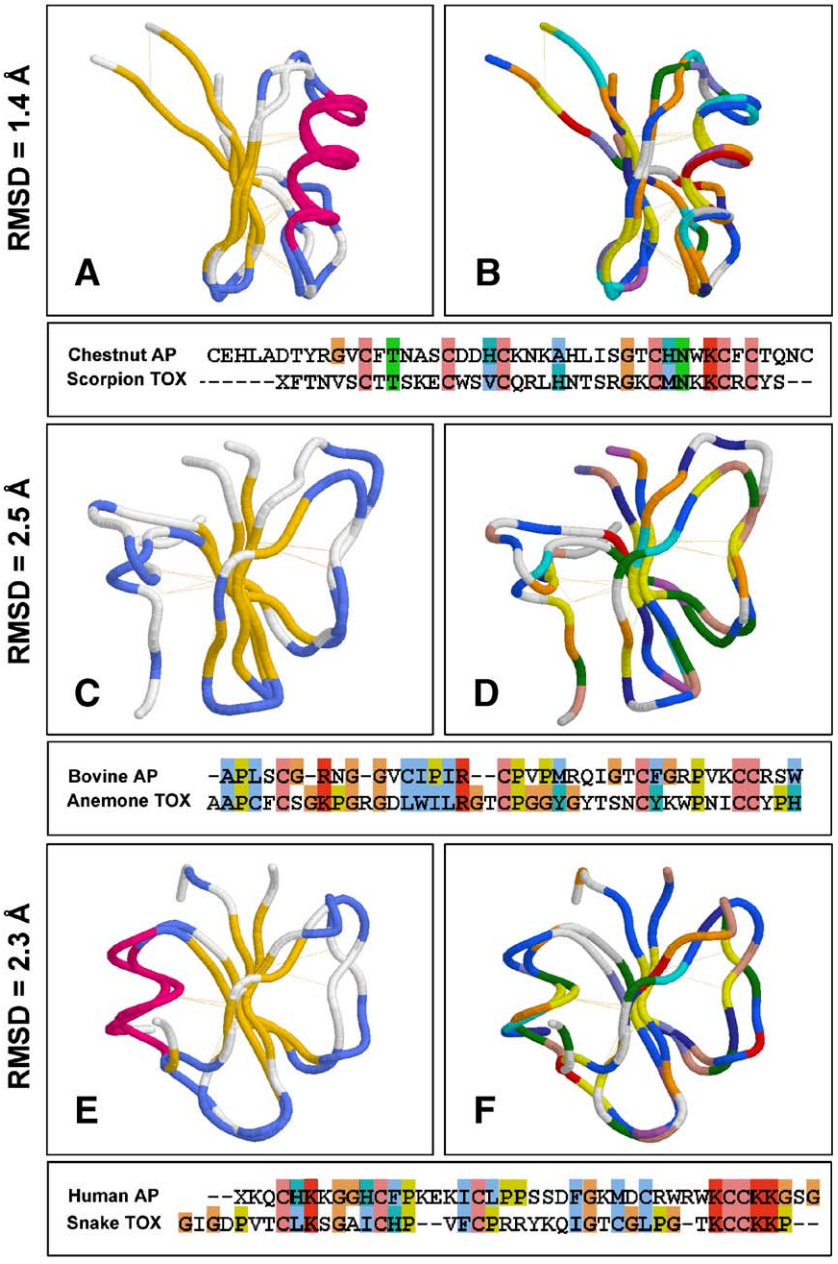


Fig. 3. Conservation of 3-D structure among antimicrobial peptides and toxins encompassing vast evolutionary distances. Illustrated are 3-D and primary sequence alignments of structurally conserved antimicrobial peptide/toxin comparators. Alignments are: chestnut antifungal peptide (Ah-AMP-1; 1BK8) with scorpion charybdotoxin (2CRD) (panels A and B); antimicrobial peptide bovine beta-defensin-12 (BNBD-12; 1BNB) with sea anemone toxin BDS (1BDS) (panels C and D); and antimicrobial peptide human beta-defensin 2 (1FD3) with rattlesnake toxin crotamine (1H5O) (panels E and F). Coloration in panels A, C, and E is secondary structure-red (helix), gold (sheet), blue (turn); coloration in panels B, D, and F is that of amino acid (Rasmol) schema based on Protein Explorer [11]. Sequence alignments were via CLUSTAL W [9], with coloration as per the amino acid conservation scheme of Jalview (www.ebi.ac.uk/jalview). Alignment identity scores are given as RMSD values [12] and expressed in angstroms.

interest to examine their potential evolutionary relatedness. Phylogenetic analysis of representative peptides from these three superfamilies revealed a number of interesting findings (Fig. 5). As might be anticipated among antimicrobial peptides, plant defensins sorted as being the most distant. Consistent with a shared ancestral lineage, insect defensins, mammalian α- and β-defensins, and arachnid toxins co-localized to a single group. However, each defensin group formed its own subclade, with toxins forming a node interposing mammalian α- and β-defensin groups, and distinctly separated from the insect defensin group. Of special interest was a localization of the kinocidin group to a node between the plant and animal defensins. This pattern suggests that arachnid toxins may be more closely related to mammalian β-defensins than to insect defensins. Additionally, these data imply that kinocidins may have evolved from a common ancestor, but appear to have diverged from either insect or

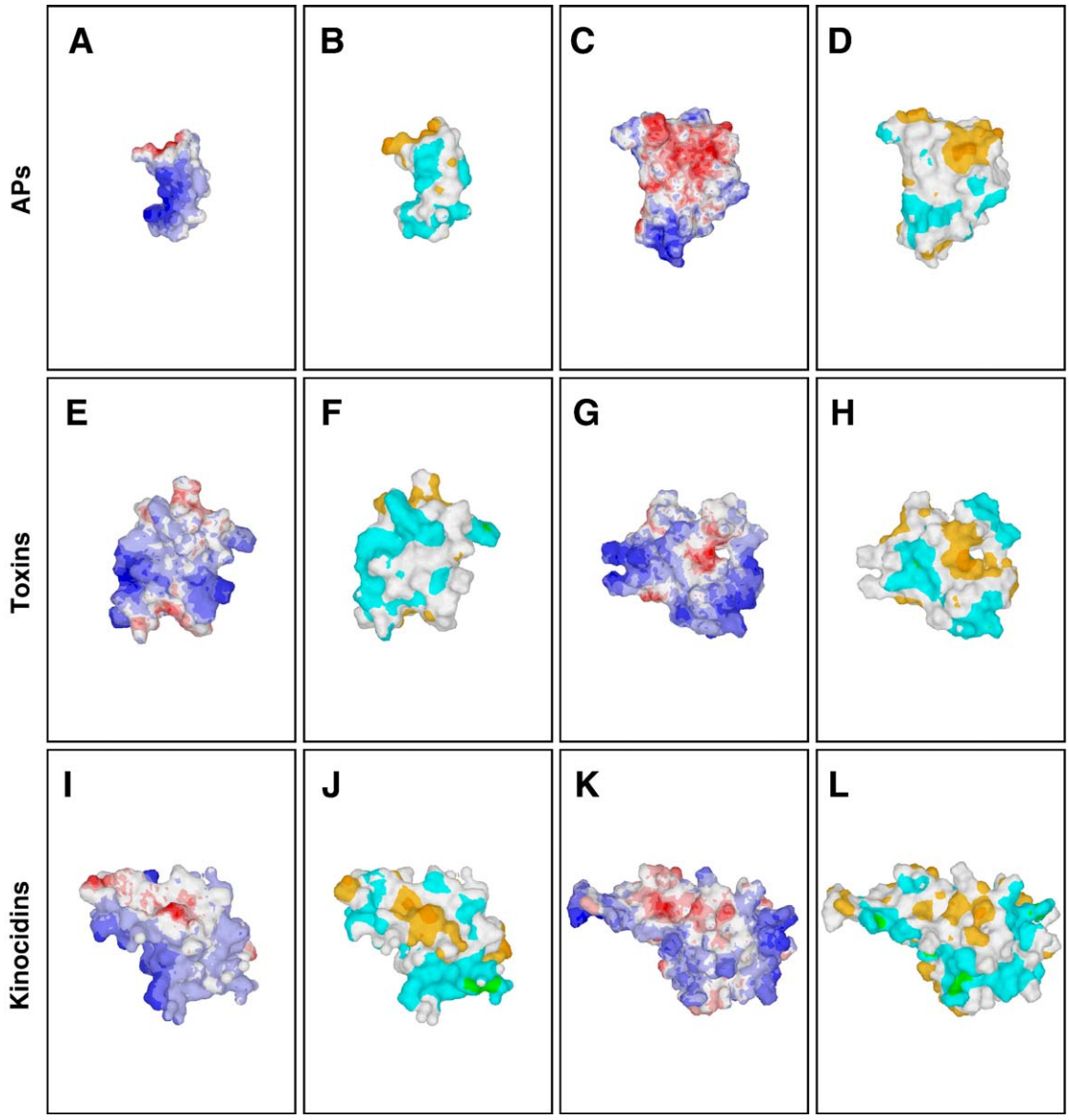


Fig. 4. Molecular surface projections illustrating the relative electrostatic and lipophilic potential of comparative polypeptide families all containing the γ-core motif. Molecular surfaces were generated as detailed in Methods for the following peptide prototypes: antimicrobial peptides (APs) — (A, B) human neutrophil defensin-3 (HNP-3; 1DFN); and (C, D) chestnut antimicrobial peptide (Ah-AMP-1; 1BK8); toxins — (E, F) scorpion charybdotoxin (2CRD); and (G, H) rattlesnake toxin crotamine (1H5O); kinocidins — (I, J) human platelet factor-4 (hPF4; 1RHP); and (K, L) interleukin-8 (IL-8; 1IL8). Coloration is per Rasmol [11]: relative electrostatic potential, blue — positive, red — negative; absolute lipophilic potential, yellow, orange, red — polar; blue, green — non-polar; white — intermediate.

mammalian defensins, or arachnid toxins. Further refinement of potential evolutionary relationships among antimicrobial peptides, toxins and kinocidins will benefit from identification of kinocidins in host sources more ancient than vertebrates if they exist.

Beyond phylogenetic relatedness, the current observations emphasize the importance of considering evolutionary procession in host defense polypeptide structural complexity relative to the γ-core signature. As previously described [1], the γ-core appears to play a multifunctional role. For example, in antimicrobial peptides consisting only of the γ-core (e.g., protegrins or tachyplesins), this motif is the sole determinant of membrane interaction. However, as illustrated in Fig. 6, the γ-core also provides a consistent scaffold to which increasingly complex modular forms may be configured in diverse host defense polypeptides. Such a lineage of complexity in structural configuration suggests key biological milestones favoring modular evolution. It is intriguing to note that compound configurations associated with the γ-core can be traced back to earliest eukaryotes, including plants or fungi (e.g., AFP; *Aspergillus*). Moreover, a distinction between γ-core configurations that do or do not contain α-helical and β-sheet structural modules becomes apparent from such phylogenetic analyses (Fig. 6). Such diverse configurations are found in host organisms of profoundly different phylogenetic ancestry, classification, and niche. These observations are consistent with the hypothesis that the γ-core motif is an archetypal signature that developed early in the course of polypeptide

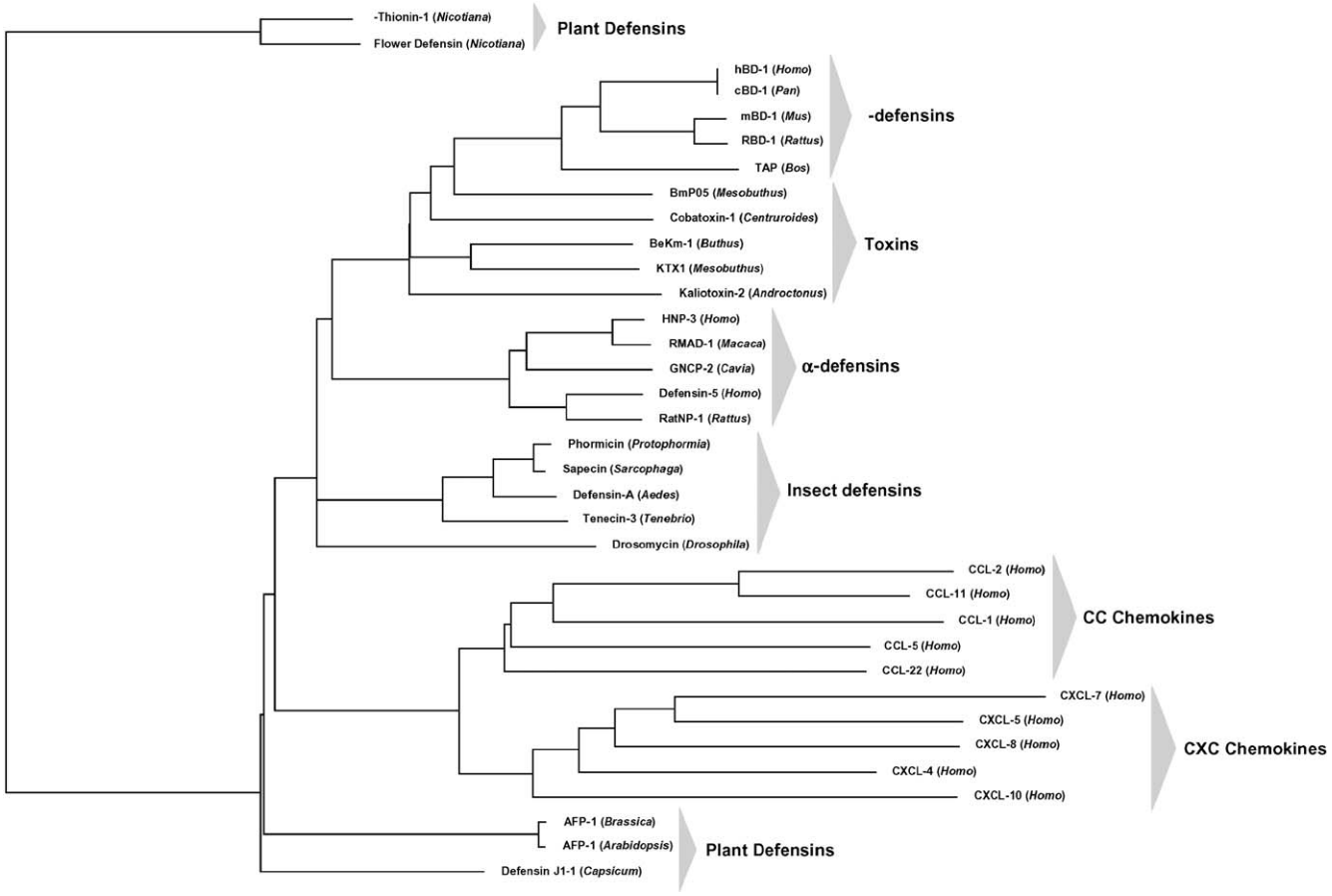


Fig. 5. Phylogenetic relatedness among prototypic antimicrobial peptides, toxins and kinocidins. Sequences were aligned via CLUSTAL W [9] with generation of a neighbor joining phylogenetic tree [10] using Jalview (www.ebi.ac.uk/jalview). Nomenclature of peptides is common name and (*Genus*).

interactions with biological membranes in governing potential host–pathogen relationships.

4. Discussion

Mechanisms for defending against detrimental interactions with other organisms have been subject to natural selection since primordial stages in co-evolution. Be they endogenous microbes or exogenous predators, each host has by necessity evolved molecular effectors that are designed to protect against the threat of unfavorable interaction. From these perspectives, host defense molecules include direct and indirect effector molecules such as antimicrobial peptides, kinocidins, and toxins.

4.1. Structural congruence of antimicrobial peptides, kinocidins, and toxins

The fact that these types of molecules are relatively similar in size, composition, net charge, and biologic function initially spurred our hypothesis that they share broader structure–activity congruence (Fig. 7). Our recent discovery that disulfide-stabilized antimicrobial peptides, kinocidins, toxins, and related host defense molecules contain recurring iterations of the multidimensional γ-core signature substantiated this hypothesis [1]. Moreover, while γ-core motifs in these polypeptides are highly conserved, other structural modules associated with this motif may be specialized or unique to a given molecule and its cognate host defense roles. Based on these considerations, we recently proposed an immunorelativity model that reconciles the structure–activity congruence observed among host defense effector molecules spanning vast phylogenetic diversity and evolutionary time [1,27]. The cardinal features of molecules encompassed in this model include a capacity for multiple functions through modular structural forms, context specificity, and potentiation of complementary protective mechanisms amplifying the net host defense repertoire. Thus, host defenses may be optimized by way of synergistic interactions among the multiple functions of such host defense effector molecules and the cells they influence.

The present report was designed to shed additional light on the comparative structural and phylogenetic relationships among host defense peptides that contain the γ-core motif. In addition, the fact that these peptides all target, permeabilize, depolarize, actuate receptors, or otherwise interact with biomembranes reinforces their common structural and phylogenetic themes. Several important insights emanated from these studies.

The current findings extend our prior reports that antimicrobial peptides, kinocidins, and toxins contain versions of the multidimensional γ-core signature motif [1]. Importantly, the 3-D attributes integral to this signature exist despite their diverse primary sequences and host sources. For example, as in

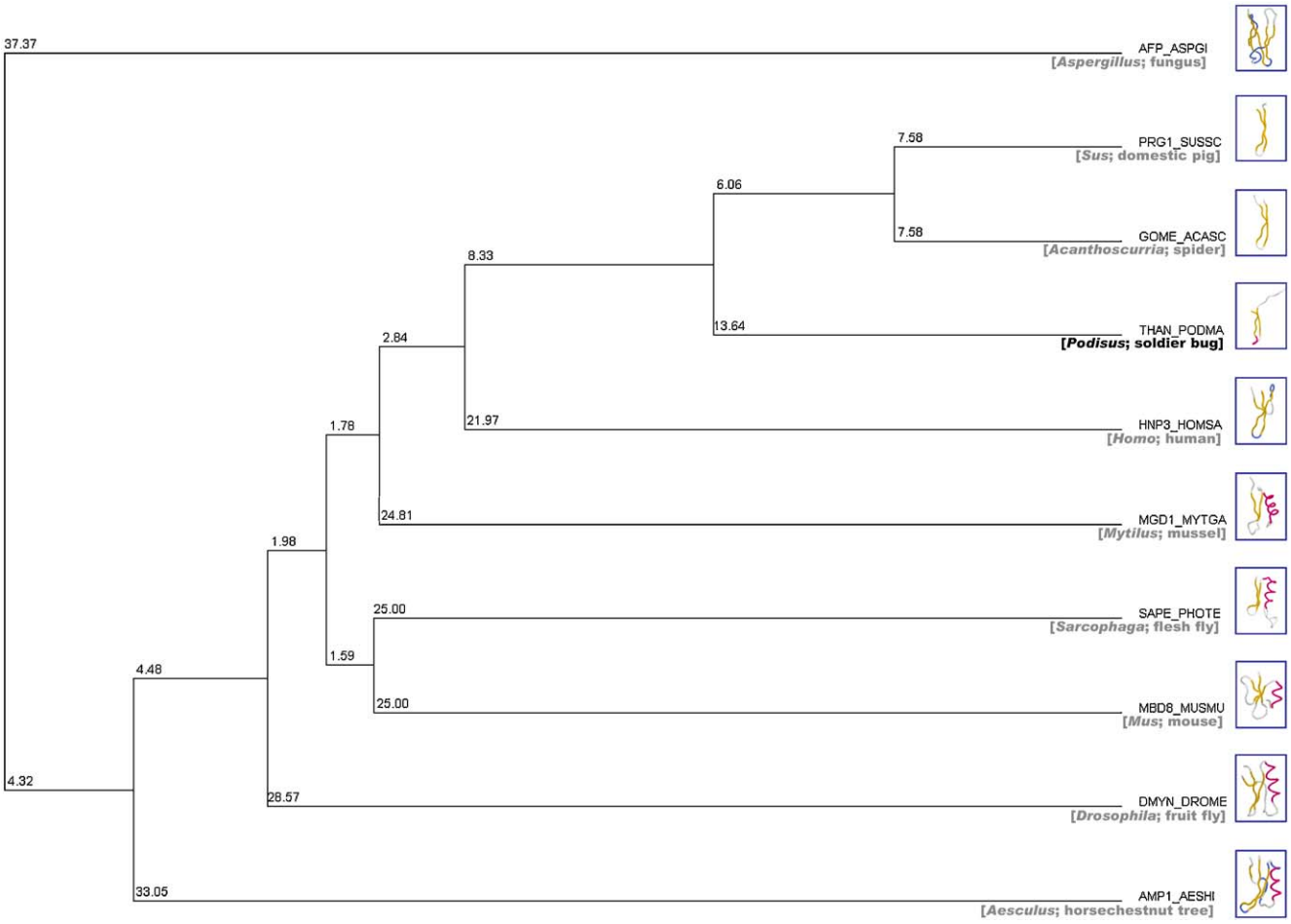


Fig. 6. Structural relationships among prototypical antimicrobial peptides containing a γ-core motif. The relative differences in amino acid sequence are indicated at branch nodes in this average distance dendogram. Representative study peptides for which structures have been determined are (descending order): AFP (AFP-1; *Aspergillus*, fungal); PRG1 (protegrin-1; *Sus*, pig); GOME (gomesin; *Acanthoscurria*, spider); THAN (Thanatin; *Podisus*, soldier bug); HNP3 (human neutrophil peptide-3; *Homo*, human); MGD1 (MGD-1; *Mytilus*, mussel); SAPE (sapecin; *Sarcophaga*, flesh fly); MBD8 (murine β-defensin-8; *Mus*, mouse); DMYN (drosomycin; *Drosophila*, fruit fly); AMP1 (AMP-1; *Aesculus*, horsechestnut tree). Note the appearance of a structural procession in peptides of increasing complexity through addition of α-helical and/or β-sheet modules configured to the γ-core motif. These data corroborate our hypothesis of modular evolution in host defense polypeptides.

antimicrobial peptides, sequence formulae of the signature in toxins and kinocidins may be derived from dextromeric or levomeric amino acid sequence orientations. These findings reinforce structural congruence in γ-core motifs of diverse host defense polypeptides, and transcend any peptide class-specific motifs identified previously.

Disulfide-stabilized host defense polypeptides exhibit structural features that suggest several configurations relative to the γ-core. Examples of this modular theme are plentiful across biological kingdoms. For example, the crustacean antimicrobial peptide MGD-1 and the arachnid charybdotoxin share a highly conserved γ-core motif, each being associated with an adjacent α-helical module (Fig. 2). More diverse configurations relative to the γ-core motif are illustrated by the antimicrobial peptide protegrin-1 (comprised solely of a γ-core) and the kinocidin RANTES (a compound configuration of α-helical and β-sheet modules linked to the γ core). Numerous permutations upon such modular themes are present among naturally-occurring host defense polypeptides. This fact supports our hypothesis that distinct structural modules configured to the γ-core optimize the function(s) of host defense effector molecules in cognate physiologic or environmental niches and host defense scenarios.

As previously reported [1], a critical effect of the conformation of the γ-core appears to be distribution of charge, steric bulk, and hydrophobicity in 3-D space. Optimal spatial relationships may be governed by the γ-core, relative to α-helix or β-sheet domains that may confer distinct yet complementary functions. Such properties are likely crucial to membrane targeting or perturbation by antimicrobial peptides, kinocidins, or toxins. Thus, the highly conserved γ-core signature extant in these otherwise diverse host defense molecules reflects a consilience in structure and function that may have been critical for survival in environments rich in potential microbial pathogens or macrobial predators.

The current results also provide important new insights into structural relationships among membrane-active polypeptides that contain a γ-core signature motif. For example, on a macromolecular scale, the location of the γ-core motif in compound antimicrobial peptides, toxins, and kinocidins is relatively invariant, immediately prior to their C-terminal α-helical domains. Such proximity of the γ-core to the α-helical domain

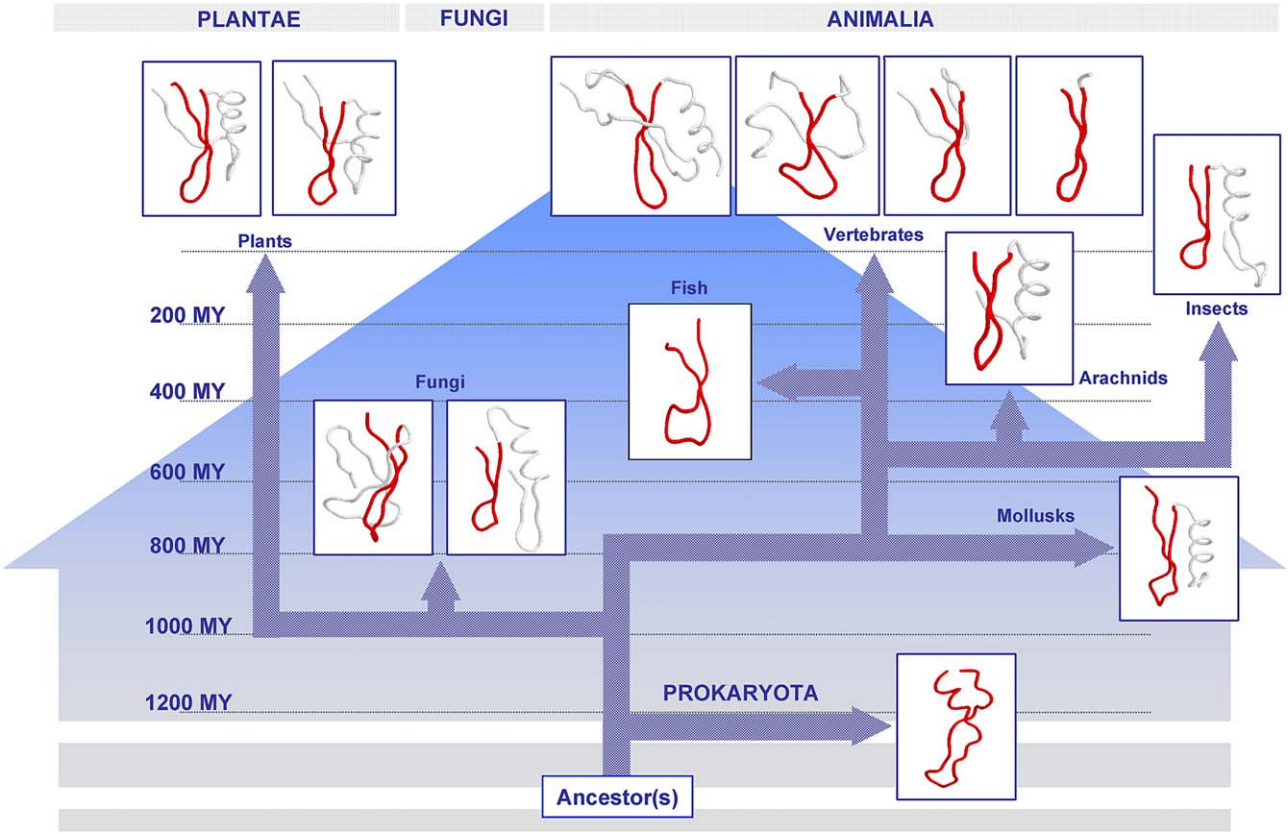


Fig. 7. Illustrative evolutionary lineage in cysteine-stabilized antimicrobial peptides, kinocidins, and toxins. Example peptides were visualized with Protein Explorer [11] with γ-core motifs highlighted in red. Representative polypeptides shown: plants-chestnut Ah-AMP-1 (1BK8; antifungal peptide), and γ-1-P-thionin (1GPS; antimicrobial peptide); mammals (left to right)—human PF-4 (1RHP; kinocidin), hBD-2 (1FD3; antimicrobial peptide); HNP-3 (1DFN; antimicrobial peptide), and protegrin (1PG1; antimicrobial peptide); reptiles—rattlesnake crotamine (1H5O; toxin); fishes—bass hepcidin (1S6W; antimicrobial peptide); insects—sapecin (1L4V; antimicrobial peptide); arachnids—charybdotoxin (2CRD; toxin); mollusks—MGD-1 (1FJN; antimicrobial peptide); fungi—AFP-1 (1AFP; antifungal peptide); plectasin (1ZFU; antimicrobial peptide); and bacteria (prokaryota)—subtilosin A (1PXQ; antimicrobial peptide). Estimates of evolutionary divergence dates are approximated for relative comparison.

resembles the configuration seen within the cysteine-stabilized-αβ (CS-αβ) family of antimicrobial peptides commonly found in plants and insects [28–30]. Modular architecture of the CS-αβ family of peptides consists of a central β-hairpin motif tethered to a C-terminal α-helical domain. Of note, many of the CS-αβ peptides exert potent antifungal activity, though the specific structural or compositional features conferring this activity are not yet known. Recent reports likewise demonstrate that certain kinocidins [19–21] or domains thereof [15,22] have remarkably potent antifungal activity. Similar themes can readily be identified in comparisons among other classes of host defense molecules, such as toxins and antimicrobial peptides.

It is noteworthy that the γ_{κ}-core appears to be divergent from its γ_{AP}-core or γ_{T}-core relatives. Kinocidins are organized to contain an N-terminal chemotactic domain, separated from their γ_{κ}-core and C-terminal α-helical domains by an interposing β-sheet-rich region. Hence, kinocidins are typically larger and structurally more complex than most toxins and antimicrobial peptides. However, the fact that kinocidins have direct antimicrobial activity [19–22] is consistent with their possession of a γ-core as is congruent with antimicrobial peptides and toxins. The localization of the antimicrobial aspect of kinocidin antimicrobial activity to the C-terminal hemimer [22], which contains the γ_{κ}-core and ultimate α-helical domain, further suggests structural and functional identity with classical antimicrobial peptides.

4.2. Phylogenetic congruence of antimicrobial peptides, kinocidins, and toxins

The present data support our hypothesis that the γ-core emerged early in the process of host pathogen co-evolution as a result of increasing requirements for effective protein–membrane interactions. For example, antimicrobial peptides isolated from hosts ranging from microbes to man clearly interact with and modify functions of biomembranes. Likewise, polypeptide toxins of prokaryotic and eukaryotic origin often target and perturb functions of membranes. Recognition of their common phylogeny and structure as summarized herein reconciles the duality that many antimicrobial peptides exert toxicity toward eukaryotic cells, and many eukaryotic toxins exert antimicrobial efficacy [1]. Similarly, kinocidins bind to and modify membrane-receptors on host defense cells of higher organisms. As detailed above, the γ-core motifs of antimicrobial peptides, toxins, and

kinocidins from evolutionarily distant organisms such as plants and crustacea recur in higher organisms including humans (Fig. 7). In these respects, the current findings support our hypothesis that the γ-core motif emerged as a result of its critical functions in mediating protein–membrane interactions.

A particularly striking result of the current studies was the phylogenetic interposition of kinocidins between invertebrate (plant, insect) and mammalian (e.g., α and β) defensins (Fig. 5). That kinocidins map to such an evolutionary intermediary is consistent with the fact that these molecules retain chemoattractant as well as antimicrobial host defense functions [31]. Evolutionarily, such molecules may represent a bridge in the phylogenetic transition between unicellular and multicellular immune mechanisms.

Extending the above concepts is the intriguing fact that most eukaryotic toxins have direct antimicrobial activity, and that most antimicrobial peptides and toxins are injurious to mammalian cell membranes. This striking interrelationship reinforces the hypothesis that the γ-core motif common among these molecules has been retained from early evolutionary time. The concept of modular evolution is consistent with the finding that virtually every structural mosaic predicted in such host defense polypeptides (e.g., [γ], [γα], [γβ], [γαβ], [βγα], etc.) is represented along the phylogenetic spectrum. Over time, natural selection has likely favored specific modular configurations that are optimally effective in certain host defense contexts. Thus, Nature appears to have allowed plasticity within and beyond the γ-core motif to afford optimal host defense capability. Current data suggest that vertical and horizontal acquisition of genes, along with their recombination to yield mosaic structural configurations, may account for the diverse repertoire of cysteine-stabilized host defense now recognized.

4.3. Summary and prospectus

The current findings demonstrate remarkable structural and phylogenetic relationships related to γ-core multidimensional signatures in host defense polypeptides originating in organisms separated by vast evolutionary distances. As detailed through evidence presented above, it is clear there are numerous structural and functional parallels linking antimicrobial peptide, kinocidin, and toxin families of host defense effector molecules, including: (1) γ-core signature; (2) relatively small size; (3) net cationic charge, with regions of charge intensification; (4) global and local amphipathicity; (5) ability or propensity to interact with membrane targets in monomeric or multimeric forms; and (6) elaboration in contexts of host defense. Considering these similarities, it is perhaps not surprising that this and other recent reports [19–22] emphasize that many kinocidins and toxins [1] exert direct antimicrobial activity, and the majority of antimicrobial peptides examined to date are toxic to mammalian or other eukaryotic cells.

The current findings also suggest important themes relating to broader issues in the evolution of host defense peptides and strategies. For example, antimicrobial peptides have evolved to defend highly diverse tissue and niche repertoires in vastly different types of hosts (e.g., skin of mammals, hemolymph of insects, cuticle of plants) against an equally diverse repertoire of pathogens. Thus, it is possible that host defense polypeptides have undergone convergent evolution within specific subclasses [32], but divergent evolution between such classes. The evolution of a molecular effector and scaffold system such as the γ-core motif may have facilitated host defense against this breadth of circumstances. Potential biological pressures favoring this remarkable degree of structural and functional conservation likely include a genetic selection against structural variants, and the prioritization of specific molecular determinants resulting from recurring themes in host–pathogen interactions.

Structure–activity data also support our hypothesis that modular conformations in host defense polypeptides orchestrate multiple and complementary homeostatic functions, without autotoxicity [7]. Therefore, certain structural domains may be optimized to exert specific, autonomous functions in specific contexts of host defense. The following example illustrates how modular structures of polytopic kinocidins hypothetically convey multiple host defense functions [7]: (1) cationic C-terminal facets avidly target kinocidins to anionic microbial membranes – but not charge-neutral mammalian host cells – intensifying these peptides to microbicidal levels in close proximity to infection (i.e., antimicrobial peptide roles); (2) in the context of microbial phospholipids and/or acidic microenvironments (e.g., phagolysosomes), specific kinocidin domains undergo conformation phase transitions that amplify microbicidal effects; (3) in contrast, diffusion to lower concentrations, neutral pH, or in the absence of microbial lipids favor kinocidin properties that modulate the antimicrobial mechanisms of neutrophils (i.e., chemokine roles), with minimal host cell toxicity; and (4) proteolysis in the context of infection may deploy specific kinocidin functional modules such as their α-helical domains to act independently in coordinating complementary microbicidal and neutrophil-enhancing activities. Such concepts suggest the need for a reinterpretation of the traditional roles ascribed to these peptide superfamilies, to address their role(s) as multifunctional host-defense effector molecules that contribute directly and indirectly to preservation of the host in the face of potentially harmful interactions with other organisms.

Finally, structural and functional congruence among host defense effector molecules may also provide insights for developing new generation membrane-active therapeutics, such as anti-infective agents, beneficial cytotoxins, or leukocyte modulators. Thus, discovery of multidimensional signatures in these peptides may advance the discovery and development of improved agents and strategies to augment endogenous or exogenous host defense.

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