Supplementary Information

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"High-throughput Discovery of Broad-spectrum Peptide Antibiotics"

Table S1: Comprehensive data table for all the peptides discussed in this work. Antimicrobial activity, membrane permeabilization, toxicity, hemolysis. Includes all control peptides and control samples.

Table S2: Data table for antibacterial activity at higher cell counts.

Table S1. Biological Activities of the peptides described in this work.

Assay[a]	Peptide[b]	Antimicrobial activity[c] MSC μM (SE ± 0.1 to 2.0)				Hemolytic activity (%)[c]		Cytotoxic activity (%)[c]
		E. coli	S. aureus	P. aeruginosa	C. neoformans	Human RBC	Sheep RBC	HEK293[d]
MOS	*GRVY*	1.2	2.2	5.9	4.9	45 ± 6	2 ± 2	3 ± 2
	NATT	1.6	3.3	4.0	4.9	13 ± 4	0 ± 1	12 ± 2
	TNTN	1.8	2.4	2.9	4.3	8 ± 2	2 ± 2	10 ± 2
SBS	*ARNY*	1.4	2.2	2.9	4.2	22 ± 5	0 ± 1	11 ± 2
	NRRV	1.5	1.8	1.7	2.2	11 ± 6	2 ± 2	35 ± 12
	RNNY	1.5	2.0	2.5	4.5	24 ± 7	0 ± 1	82 ± 2
Vesicle-based Screen	*VDVY*	1.9	2.9	2.4	9.0	25 ± 2	7 ± 2	15 ± 2
	VAVY	4.9	1.8	2.4	8.5	41 ± 4	4 ± 2	6 ± 2
	VAVR	1.9	1.4	2.1	3.6	28 ± 3	8 ± 3	8 ± 3
	VAYR	2.1	1.5	1.6	4.1	23 ± 5	6 ± 1	2 ± 5
	*VRAA	1.5	3.3	1.3	2.7	11 ± 2	6 ± 3	2 ± 6
	*ARVA	1.9	2.9	2.4	2.6	18 ± 2	6 ± 2	3 ± 1
	*RVAV	1.4	2.4	1.7	5.4	28 ± 6	5 ± 1	2 ± 3
	YTTG*	3.1	2.8	2.9	6.2	43 ± 5	6 ± 3	1 ± 3
	ARYV	3.8	2.8	1.9	6.1	12 ± 2	4 ± 2	3 ± 2
	VVRG	1.7	3.6	2.9	10.6	26 ± 3	8 ± 3	9 ± 4
Controls	*ARVA (dL_{10})[e]	9.7	8.8	>10	6.5	4 ± 3	10 ± 12	4 ± 1
	all D *ARVA[f]	1.3	2.8	2.0	6.5	12 ± 5	1 ± 1	ND
	Indolicidin[g]	1.2	2.9	5.3	4.7	14 ± 2	19 ± 5	2 ± 4
	Melittin[h]	1.5	1.3	0.9	1.1	100 ± 12	93 ± 7	93 ± 4
	PMSD[i]	>15	>15	>15	>15	2 ± 1	4 ± 1	4 ± 6
	Ampicilin	0.4	0.1	4.4	>15.0	ND	ND	ND
	Triton-X	ND	ND	ND	ND	100	100	100

Table S1 (continued) Biological Activities of the peptides described in this work.

Assay[a]	Peptide[b]	Liposome leakage P:L 1:50 (%)[c]	Sytox Green Permeation into cells (%)[c]			
			E.coli	S.aureus	C. neoformans	HEK293[d]
MOS	*GRVY*	24 ± 9	55 ± 2	71 ± 8	5 ± 2	18±1
	NATT	3 ± 3	88 ± 6	79 ± 14	7 ± 1	1±1
	TNTN	5 ± 5	83 ± 4	84 ± 3	11 ± 1	22 ± 1
SBS	*ARNY*	3 ± 2	74 ± 3	68 ± 4	20 ± 4	35 ± 1
	NRRV	2 ± 2	81 ± 2	38 ± 4	69 ± 11	10 ± 1
	RNNY	2 ± 1	104 ± 1	65 ± 3	43 ± 7	30 ± 1
Vesicle-based Screen	*VDVY*	72 ± 20	61 ± 9	22 ± 12	3 ± 5	1 ± 10
	VAVY	67 ± 20	6 ± 4	65 ± 25	1 ± 13	7 ± 4
	VAVR	65 ± 20	91 ± 14	52 ± 6	12 ± 2	6 ± 4
	VAYR	93 ± 15	94 ± 11	47 ± 11	16 ± 5	2 ± 13
	*VRAA	25 ± 17	89 ± 14	71 ± 7	33 ± 5	3 ± 6
	*ARVA	39 ± 25	80 ± 11	76 ± 2	32 ± 13	5 ± 3
	*RVAV	69 ± 2	100 ± 10	72 ± 11	1 ± 8	7 ± 13
	YTTG*	79 ± 6	17 ± 4	84 ± 5	10 ± 4	14 ± 24
	ARYV	50 ± 21	24 ± 3	97 ± 12	9 ± 1	4 ± 1
	VVRG	55 ± 22	16 ± 2	89 ± 12	4 ± 6	3 ± 2
Controls	*ARVA (dL_{10})[e]	1 ± 1	10 ± 8	ND	0 ± 1	1 ± 1
	all D *ARVA[f]	4 ± 4	93 ± 4	24 ± 10	2 ± 1	1 ± 1
	Indolicidin[g]	24 ± 10	14 ± 5	23 ± 18	7 ± 2	4 ± 2
	Melittin[h]	98 ± 7	100	100	100	100
	PMSD[i]	ND	0	3 ± 1	3 ± 1	ND
	Ampicilin	ND	0	0	0	ND
	Triton-X	100	ND	ND	ND	ND

[a]MOS: multiorganisms screen, see main text; SBS: stringent biological screen, see main text; Vesicle based screen: Tb/DPA leakage from Large Unilamellar vesicles composed of 90% palmitoyloleoylphosphatidylcholine (POPC) and 10% palmitoyloleoylphosphatidylglycerol (POPG).

[b]All peptides from the library are 9, 12 or 15 residues in length. The library has the form {RRG}WOLOLOLOY{RRG}-amide where the {RRG} terminal basic cassettes are randomly

present or absent and the O residues can be one of the following amino acids: NDTRGAVY. The W,L and C-terminal Y residues are fixed. The simplified peptide notation uses an asterisk for the terminal basic

cassette, when present, and identifies the only the four O-residues directly. For example the peptide RRGWRLVLALAY is referred to as **RVAA*.

[c] Experiments are described in detail in recent publications(*1-4*)

[d] Human embryonic kidney cell line 293.

[e] The peptide RRGWALRLVLAY in which the C-terminal-most L-leucine is replaced with a D-leucine.

[f] The peptide RRGWALRLVLAY in which the entire sequence is composed of D-amino acids

[g] The bovine neutrophin antimicrobial peptide indolicidin has the sequence ILPWKWPWWPWRR-amide.

[h] The Honey Bee venom peptide melittin is a 22-residue, alpha helical pore forming peptide which potently permeabilizes all lipid bilayer membranes, synthetic or biological. In all assays, 5 μM melittin had the same permeabilizing effect as membrane dissolution with detergents (e.g. Triton X-100).

[i] The perfringolysin membrane spanning domain (PMSD) is a sequence that contributes to a membrane permeabilizing beta barrel in the context of the whole multimeric perfringolysin O protein toxin. But the membrane-spanning sequence alone is inactive and we use it for a control peptide.

Table S2. Antimicrobial activities at higher cell density.

Peptide	E. coli	S. aureus	E. coli	S. aureus
	5 x 10^5 cells/ml		**1 x 10^3 cells/ml**	
GRVY	11.6	9.4	1.2	2.2
NATT	3.3	12.5	1.6	3.3
TNTN	3.6	8.1	1.8	2.4
ARNY	3.4	7.3	1.4	2.2
NRRV	2.0	9.7	1.5	1.8
RNNY	5.9	11.0	1.5	2.0
VDVY	6.5	9.7	1.9	2.9
VAVY	ND	9.7	4.9	1.8
VAVR	2.9	2.0	1.9	1.4
VAYR	ND	4.3	2.1	1.5
*VRAA	5.2	9.7	1.5	3.3
*ARVA	2.4	6.5	1.9	2.9
*RVAV	8.2	4.3	1.4	2.4
YTTG*	6.5	6.5	3.1	2.8
ARYV	9.7	9.7	3.8	2.8
VVRG	9.7	9.7	1.7	3.6
Indolicidin	5.1	9.7	1.2	2.9
Melittin	2.4	1.3	1.5	1.3
PMSD	>15	>15	>15	>15
Ampicilin	9.7	1.3	0.4	0.1

Hancock and colleagues have recently given guidelines for assessing antimicrobial activities of peptides(5). Among the guidelines was the use of $5x10^5$ cells per ml rather than the 10^3 or 10^4 commonly employed. Previously we had found little or no difference between 10^3 or 10^4 cells/ml. In Table S2 we compare the antimicrobial activities of the selected antimicrobial peptides at low and high cell density. All peptides tested retain potent activity (MSC < 15 μM) at higher cell density, although MSC values are an average of 3 fold larger at $5x10^5$ cells/ml compared to $1x10^3$ cells/ml. Sequences and descriptions of the peptides are given above for Table S1. ND = not done. All MSC values have standard errors of 0.5 to 2.5 μM.

Supplemental Reference List

1. Rathinakumar, R., Walkenhorst, W. F., & Wimley, W. C.(2009) Broad-spectrum Antimicrobial Peptides by Rational Combinatorial Design and High-throughput Screening: The Importance of Interfacial Activity *J. Am. Chem. Soc.* **131**, 7609-7617.

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3. Rausch, J. M., Marks, J. R., Rathinakumar, R., & Wimley, W. C.(2007) Beta-sheet pore-forming peptides selected from a rational combinatorial library: mechanism of pore formation in lipid vesicles and activity in biological membranes *Biochemistry* **46**, 12124-12139.

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5. Wiegand, I., Hilpert, K., & Hancock, R. E.(2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances *Nat. Protoc.* **3**, 163-175.