Design potent antimicrobial peptides against the ESKAPE pathogens based on human cathelicidin LL-37

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UNMC

Outline

I. Why bother with peptides?

II. How to identify peptide leads?

III. What's the state of the art of human LL-37 engineering?

IV. Summary

Part I: Why peptides?

Why bother peptides?

Small molecules: not specific enough;

Large biologics: limited oral bioavailability.

Consequently, there is a great interest in developing **peptide drugs**.

Peptide drug market

Lupron (Abbot lab) for prostate cancer sold over 2.3 billion in 2011.

Over 60 FDA-approved peptide drugs (e.g., daptomycin, colistin);

140 under clinical trials;

500 under preclinical development.

Drug Discovery Today 2015; 20:122-128.

Goals of peptide drug development

To identify proper leads and overcome the hurdles toward practical applications.

Drug development stages

Lead identification (Novel?)

↓

Optimization in vitro (SAR)

↓

In vivo efficacy test (PK and PD);

↓

Clinical trials (Safe, effective, afforadable?)

↓

Therapeutic use/withdrawal from the market

Methods for lead identification

(1)Library screening

in the lab;

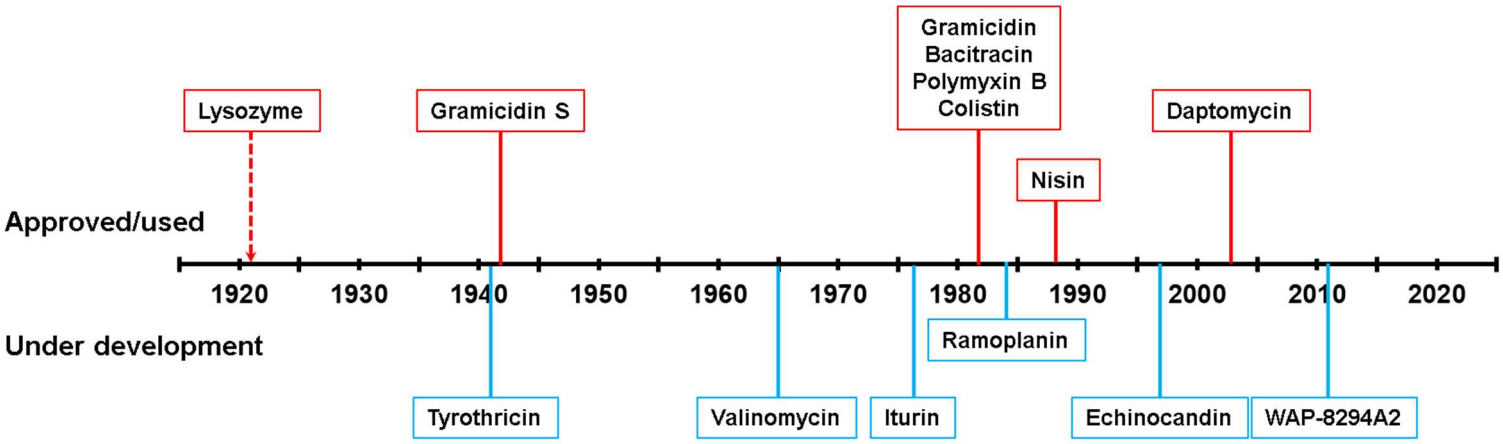
in the field;

and in silico;

(2) Structure-based design

(Rational design).

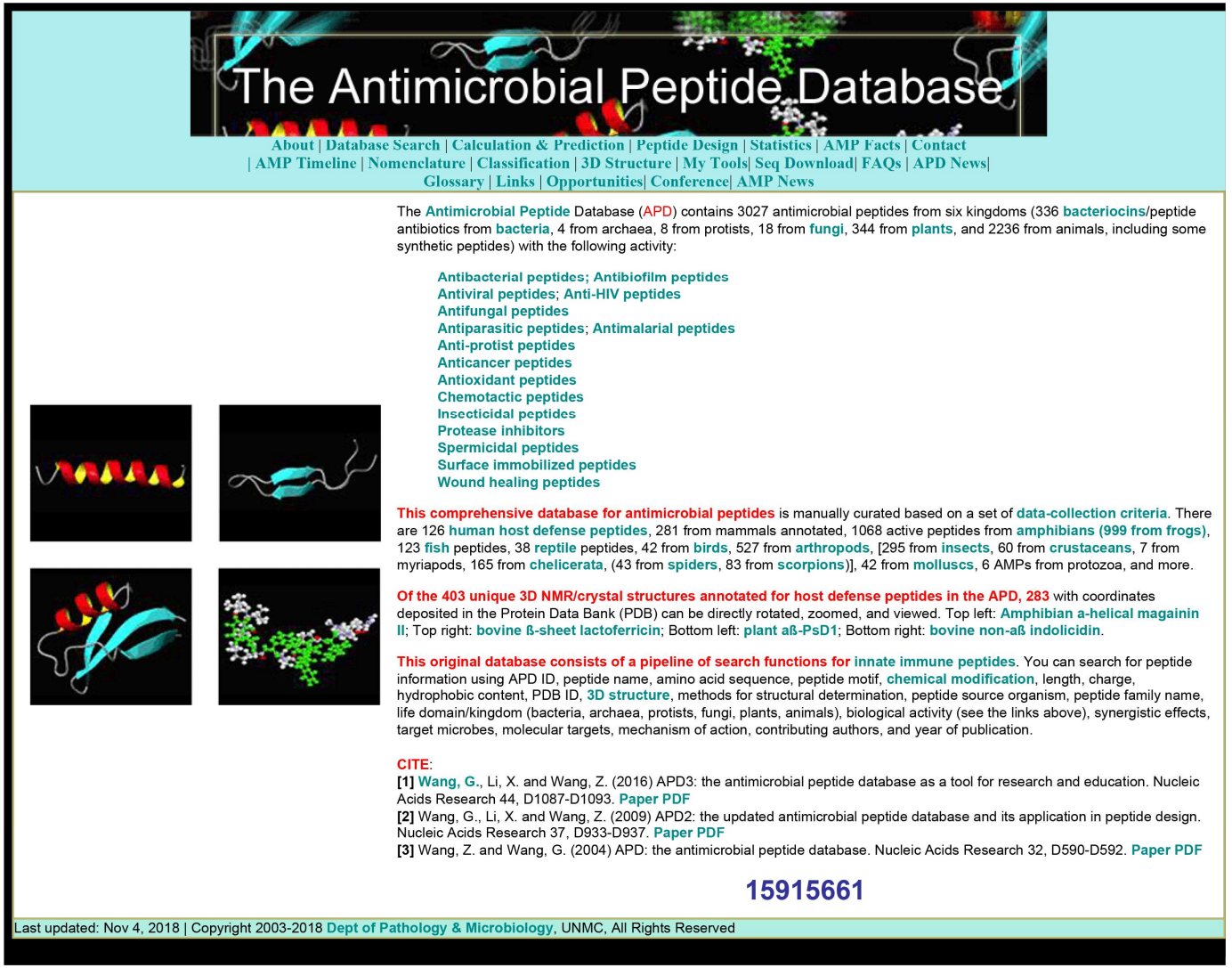
Select antimicrobial peptides (AMPs) *in practical use (red) and under development (blue)*



Note that lysozyme is regarded as the first AMP and the beginning of innate immunity.

Mishra, B., Reiling, S., Zarena, D., Wang, G. (2017). Host defense antimicrobial peptide as antibiotics: design and application strategies. *Curr. Opin. Chem. Biol. 38*, 87-96.

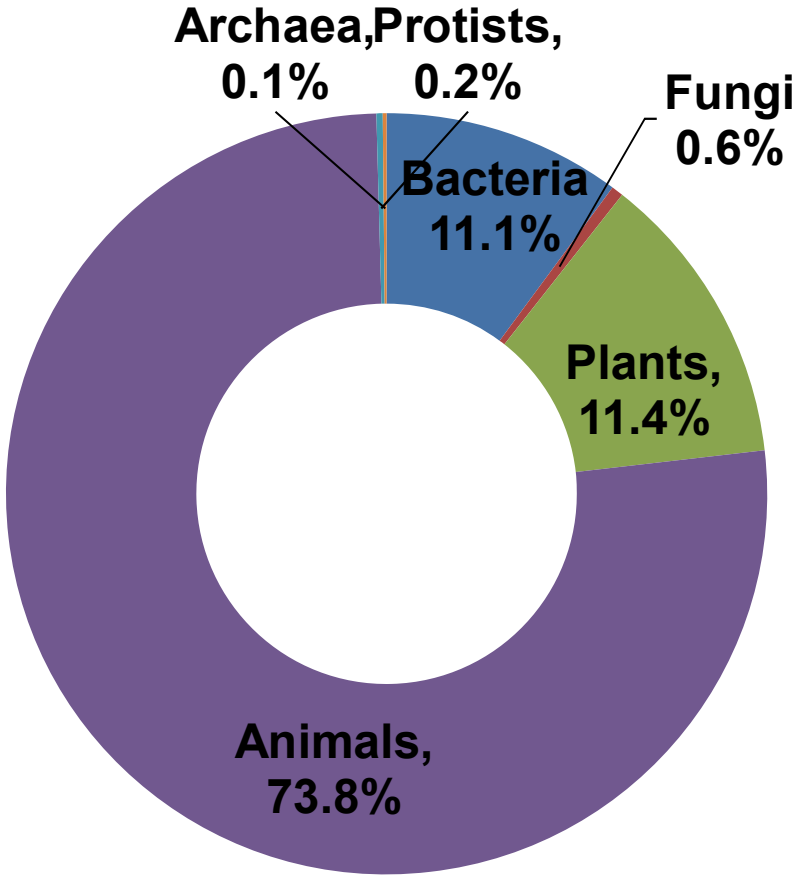
Natural Occurring Antimicrobial Peptides



http://aps.unmc.edu/AP (Nov2018)

AMPs from the six kingdoms

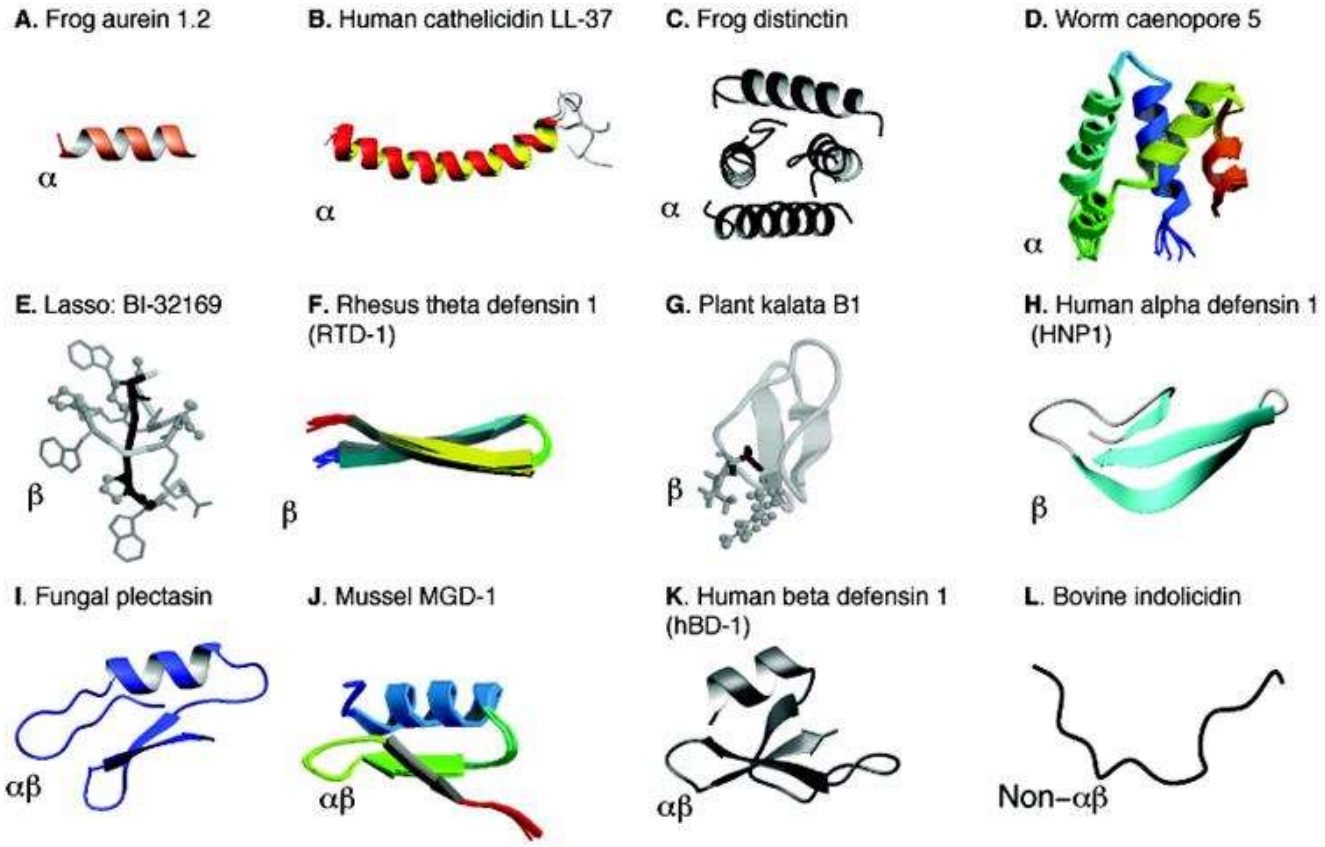
Kingdom	Count
bacteria	336
Archaea	4
Protists	8
Fungi	18
Plants	344
Animals	2236



Eukaryota: 2606 (86%)

Total: 3027 (Oct 2018)

Unified classification of 3D structures: α, β, αβ, and non-αβ



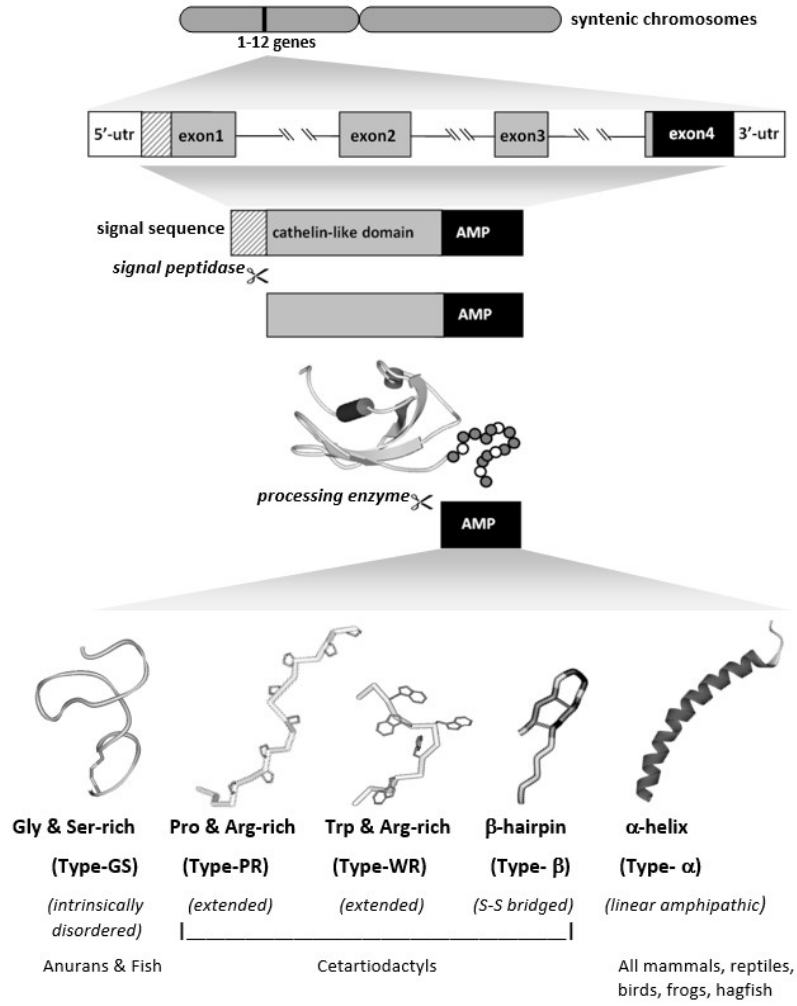
Wang, G. (ed.) 2010. *Antimicrobial Peptides: Discovery, Design and Novel Therapeutic Strategies, CABI, England*.

Select human AMPs

Lysozyme (1922) in saliva, tears, and intestine;
Alpha-defensins HNP-1 (1985) in neutrophils and bone marrow;
Histatins (1988) in saliva;
RNase 2 (1990) in eosinophils;
Beta-defensin HBD-1 (1995) in kidney, skin, saliva;
Cathelicidin LL-37 (1995) skin and neutrophils;
Granulysin (1998) in cytolytic T cells and NK cells;
Ubiquicidin (1999) in macrophages;
Thrombocidin-1 (2000) in human blood platelets;
Dermcidin (2001) in skin and sweat

Wang G (2014) *Pharmaceuticals* 7, 545-594.

Cathelicidins: biosynthesis and cleavage



N-terminus:
The cathelin domain is highly conserved and can be used to predict cathelicidins in the genome.

C-terminus:
The mature antimicrobial peptide is extremely variable in terms of sequence and structure.

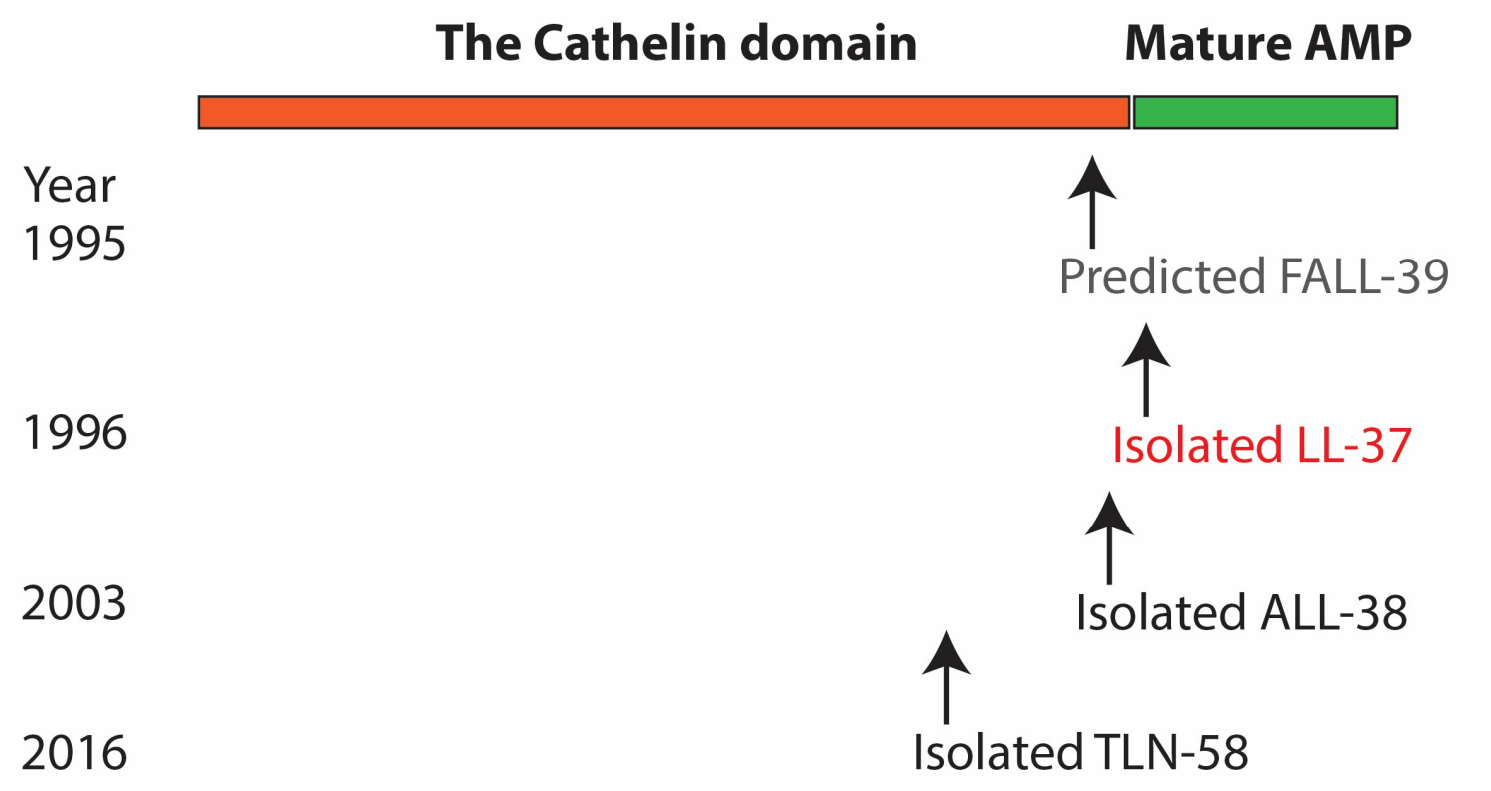
Tossi et al. (2017). In "*Antimicrobial Peptides*" (Wang G, ed.), Chapter 2

The only human cathelicidin: a helical peptide

The human genome project was started in 1990 and completed 2003.

There are multiple copies of genes in horse, sheep and cattle, but only one cathelicidin gene in humans.

Cathelicidin: one gene, multiple peptides



Refs: 1) Agerberth et al., 1995; 2) Gudmundsson GH et al., 1996; 3) Sorensen OE et al, 2003; 4) Murakami et al., 2016 (lesion vesicle of palmoplantar pustulosis in the skin).

Human cathelicidin LL-37 and its relationship with disease

Patients with morbus Kostmann and atopic dermatitis have a low level of cathelicidin (Putsep et al., 2002).

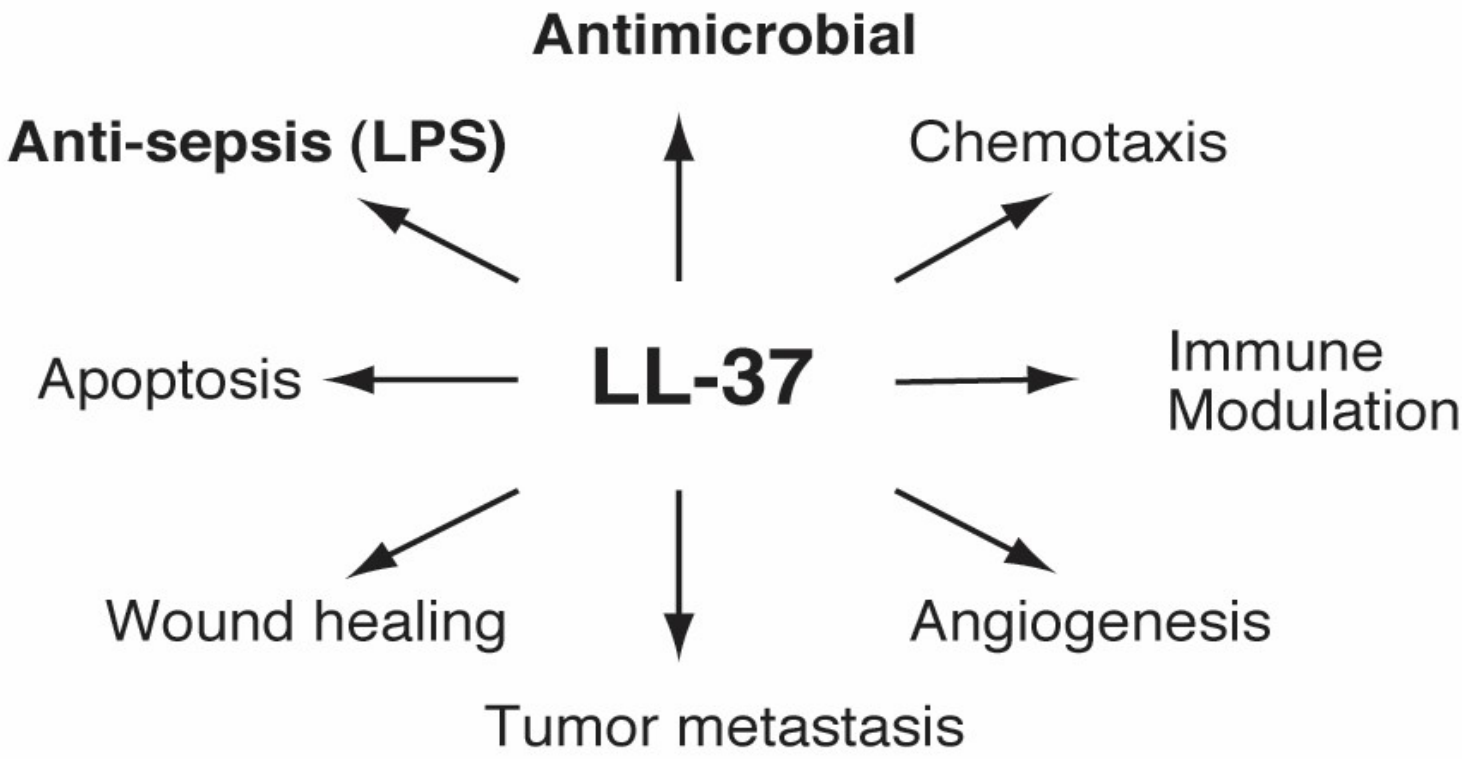
Gene KO mice increased infection and overexpression reduced infection (Nizet et al., 2001; Lee et al. 2005).

Binding to LPS (endotoxin) protects rats from **sepsis** (Cirioni et al., 2006).

LL-37 is reduced in **cystic fibrosis** due to interactions with DNA and filamentous F-actin (Bucki et al. 2007).

LL-37 is overexpressed in breast, ovarian and lung **cancer**s (Wu, Wang, Coffelt et al. 2010).

Multiple functions of LL-37: an innate immune peptide



Wang et al. (2014) *Biochim. Biophys. Acta* 1838: 2160-2172.

There is a great interest in developing LL-37 into therapeutic molecules

Part II: How to identify peptide leads?

Antimicrobial peptides (AMPs)

LL-37-based peptide library

Peptide library design

Commonly designed libraries:

1) Overlapping library (seq scanning);
2) Alanine scanning;
3) Positional library;
4) Truncation;
5) Random library;
6) Scrambled library (seq is important).

LL-37 peptides

1. 37 amino acids (long and costly);
2. Decide on the peptide length (20, 22, 24mer?);
3. Scan the sequence from the N-terminus to the C-terminus;
4. Make peptides;
5. Quality check;
6. Antimicrobial assays
7. Cytotoxicity assays
8. Most selective and potent peptide.

LPS-neutralizing activity

Peptide	Sequence	IC50 (uM)
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	0.29
LL-22	LLGDFFRKSKEKIGKEFKRIVQ	>3
.		
.		
.		
IG-24(P60)	IGKEFKRIVQRIKDFLRNLVPRTE	0.48
P60.4	IGKEFKRIV**E**RIK**R**FLR**E**LV**RPLR**	0.55

The most promising peptide is P60.4, a 24 amino acid peptide with similar efficacy as LL-37 in terms of LPS and LTA neutralization and lower pro-inflammatory activity.

Nell MJ et al. (2006) *Peptides.* 2006 Apr;27(4):649-60.

SAAP-148 is topically effective

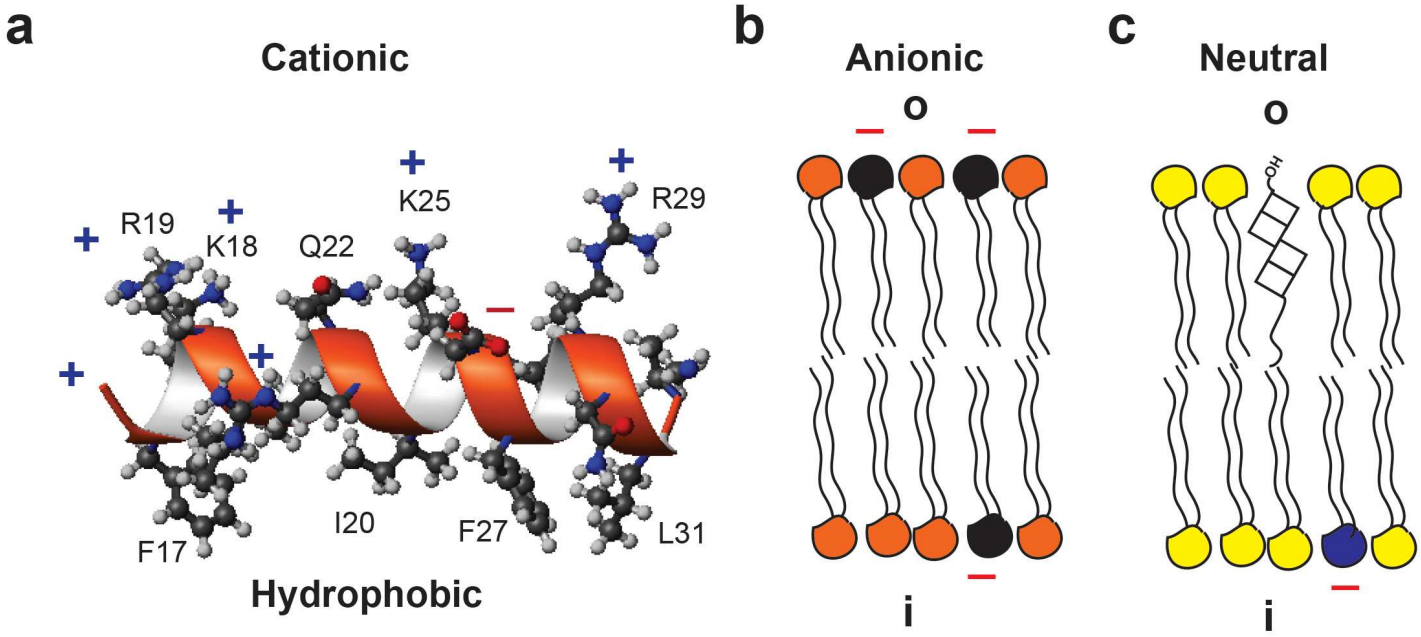
Peptide	Sequence	LC99.9 (μM)	PBS 50% plasma
LL-37	L L G D F F R K S K E K I G K E F K R I V Q R I K D F L R N L V P R T E S	1.6 (1.6–6.4)	>204.8
P139	L K K L W K R V F R I W K R I F R Y L K R P V R	1.6 (0.8–1.6)	51.2
P140	L R R L W K R L V R I I K R I Y R Q L K R P V R	1.6	38.4 (25.6–51.2)
P141	L R R L Y K R V F R L L K R W W R Y L K R P V R	1.6 (0.8–1.6)	38.4 (25.6–51.2)
P142	L R R L W K R L V K I L K R W F R Y L R R P V R	1.6 (0.8–1.6)	51.2 (51.2–102.4)
P143	L R R L Y K R V V K L W K R L F R Q L R R P V R	1.6 (1.6–3.2)	51.2 (51.2–102.4)
P144	L K K L Y K R V A K I W K R W I R Y L K K P V R	1.6	38.4 (25.6–51.2)
P145 (SAAP-145)	L K R L Y K R L A K L I K R L Y R Y L K K P V R	1.6 (0.8–1.6)	12.8 (12.8–25.6)
P146	L K K L Y K R L F K I L K R I L R Y L R K P V R	1.2 (0.8–1.6)	51.2 (25.6–51.2)
P147	L K K L W K R L A R L L K R F I R Q L R R P V R	1.6	51.2 (25.6–51.2)
P148 (SAAP-148)	L K R V W K R V F K L L K R Y W R Q L K K P V R	1.6	12.8 (12.8–25.6)

1. SAAP-148 formulated in an ointment is safe in an animal model (a 3.75% (w/w) hypromellose gel base);
2. SAAP-148 ointments are highly effective against (biofilm)-associated skin infections.

de Breij A et al. (2018). *Sci Transl Med.* Jan 10;10(423). pii: eaan4044.

Structure-based design

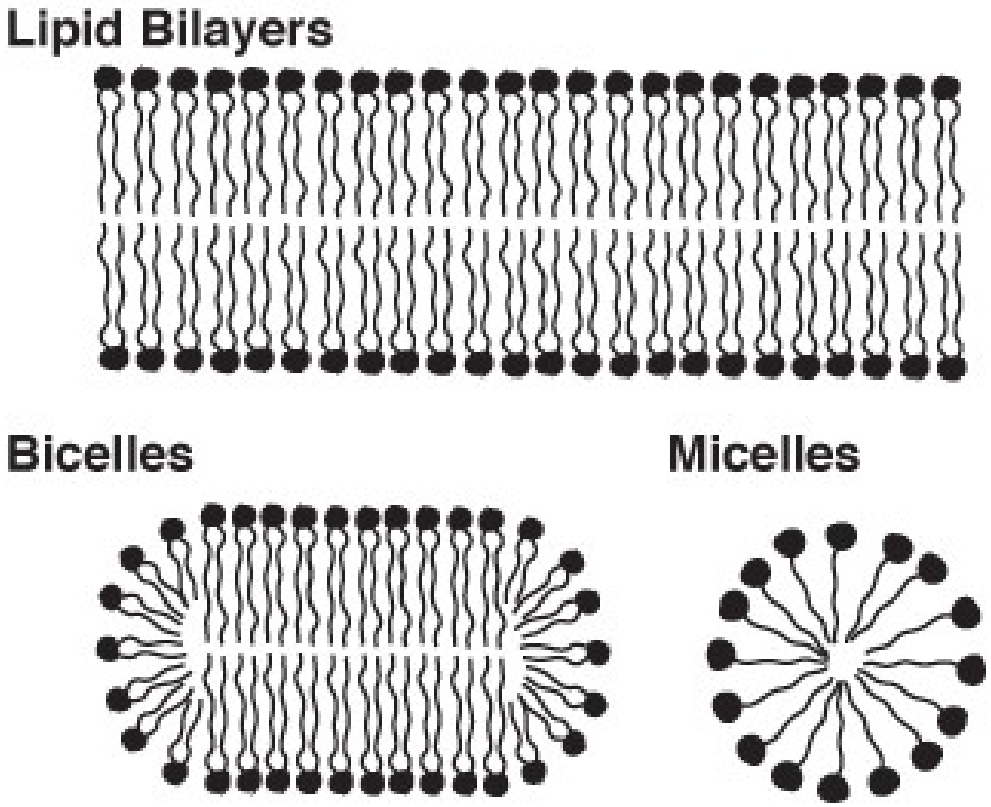
Physical basis of peptide selectivity



The amphipathic helix of cationic AMPs (a) is ideal to interact with anionic bacterial membranes (b), but not zwitterionic human cell membranes (c).

Mishra, B., Reiling, S., Zarena, D., Wang, G. (2017). Host defense antimicrobial peptide as antibiotics: design and application strategies. *Curr. Opin. Chem. Biol. 38*, 87-96.

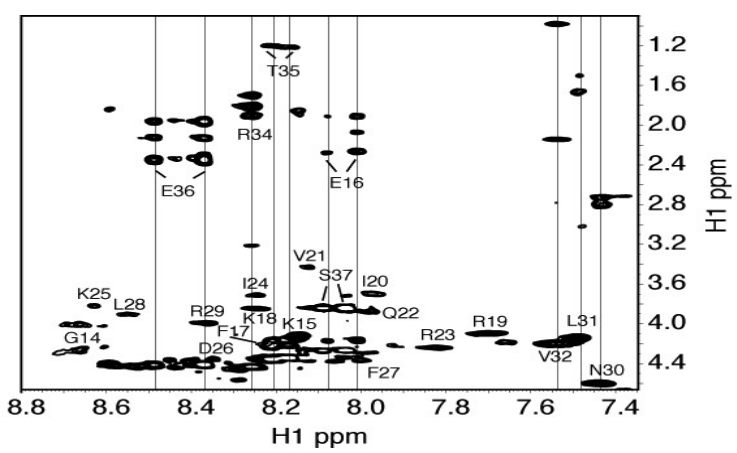
Membrane-mimetic Models

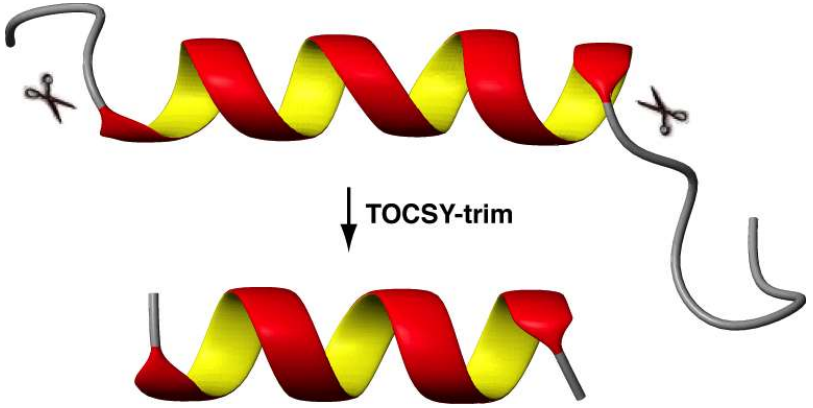


The smaller the particles, the high resolution the solution NMR spectra.

Wang G. (2010). In "*Antimicrobial Peptides*" (Wang G, ed.), Chapter 9.

Identification of the Core Antibacterial and Anticancer Region in Human LL-37 by NMR





Micelle-bound state: strong peaks suggest no binding or weak binding (e.g., tails), weak peaks suggest stronger binding to micelles (e.g. the core region).

LLGDFFRKSKEKIGKE**FKRIVQRIKDFLRNLV**PRTES (major antimicrobial region)

The GF-17 model: G + **FKRIVQRIKDFLRNLV (FK-16)**

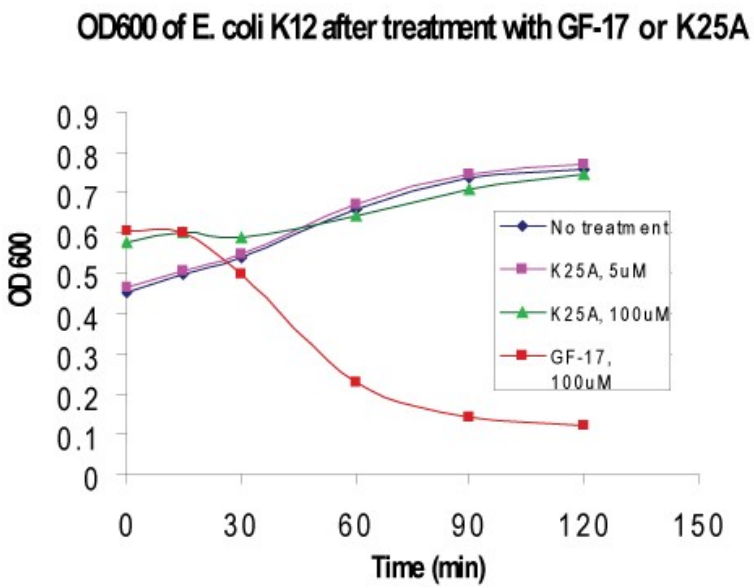
Li et al. 2006. *J Am Chem Soc*. May 3;128(17):5776-85.

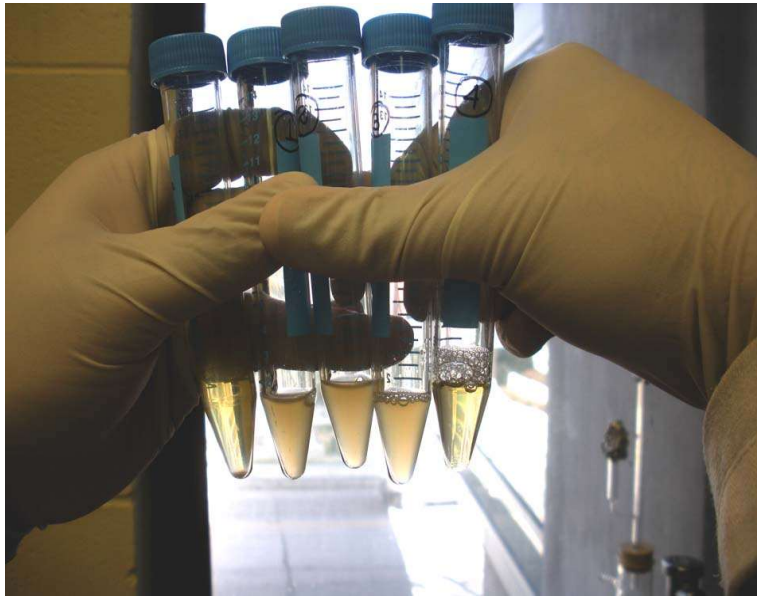
Alanine scan of GF-17: *Importance of R23 and K25*

Peptide	Sequence	*E. coli* K12	*S. aureus* UAMS-1	MRSA USA300
GF-17	GFKRIVQRIKDFLRNLV-NH_2	7.5	7.5	7.5
K18A	GF**A**RIVQRIKDFLRNLV-NH_2	15	7.5	7.5
R19A	GFK**A**IVQRIKDFLRNLV-NH_2	15	7.5	7.5
R23A	GFKRIVQ**A**IKDFLRNLV-NH_2	**60**	7.5	15
K25A	GFKRIVQRI**A**DFLRNLV-NH_2	**60**	15	7.5
R29A	GFKRIVQRIKDFL**A**NLV-NH_2	15	7.5	7.5

Wang, G. et al. (2012). *Antimicrob Agents Chemother*. 56: 845-56

GF-17 can lyse bacteria much more effectively than the K25A mutant

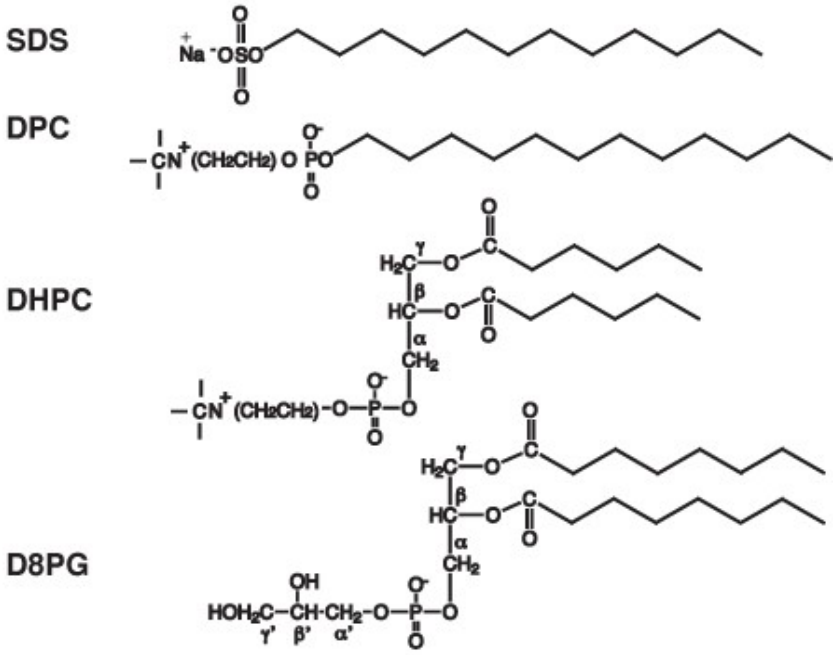


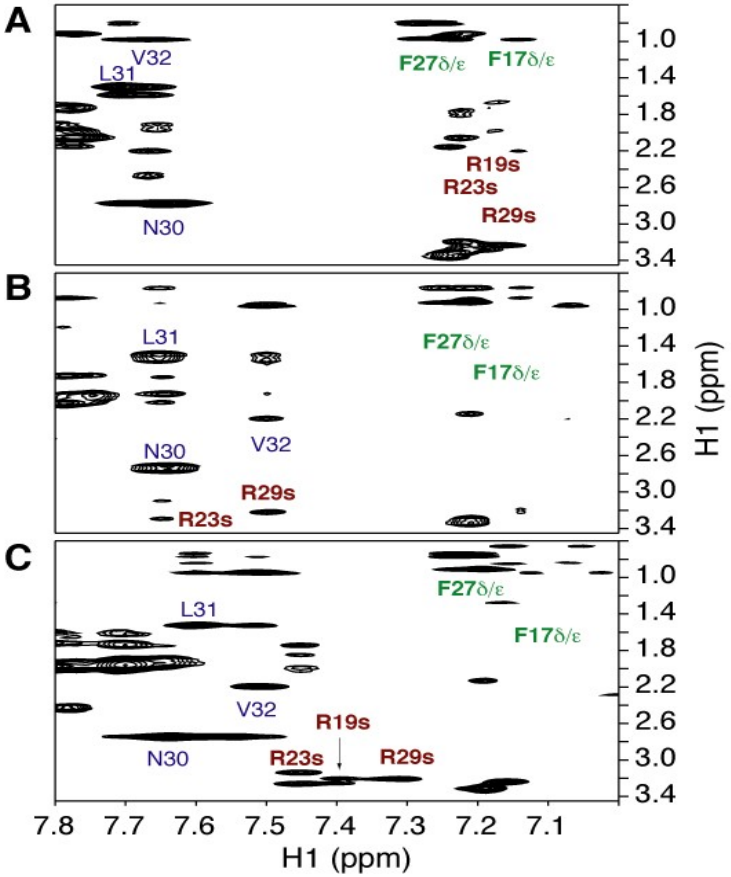


What is the physical basis of AMPs binding to bacterial membranes?

Credit: Biswajit and Tamara (Wang lab unpublished).

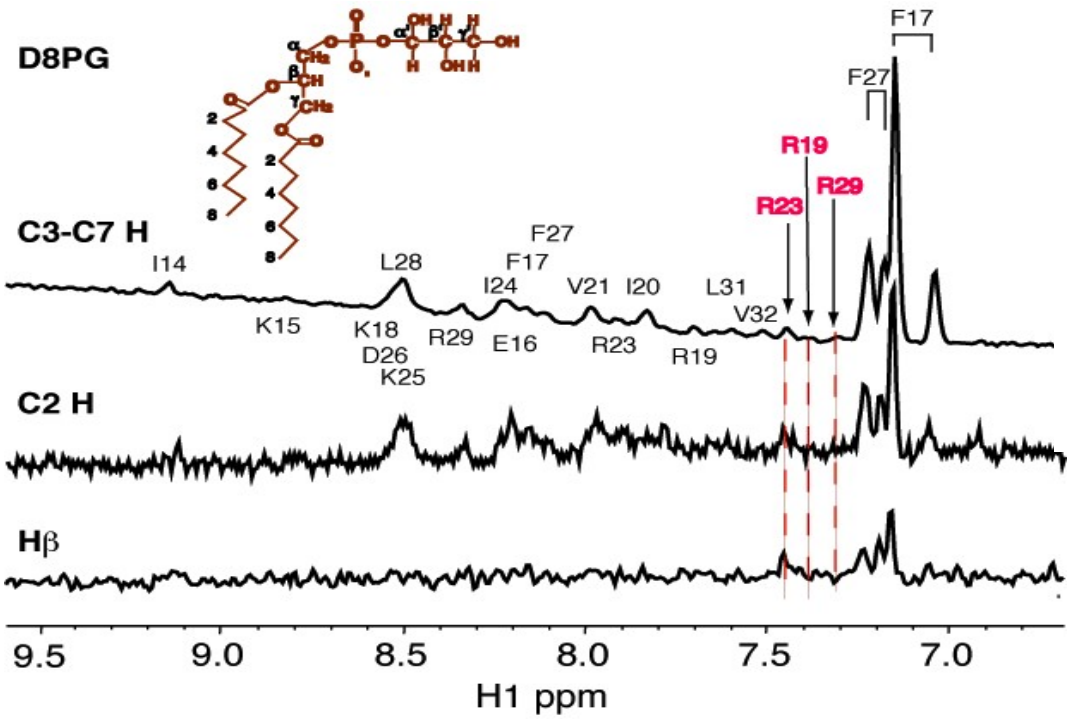
D8PG is a unique bacterial membrane-mimetic model for NMR studies

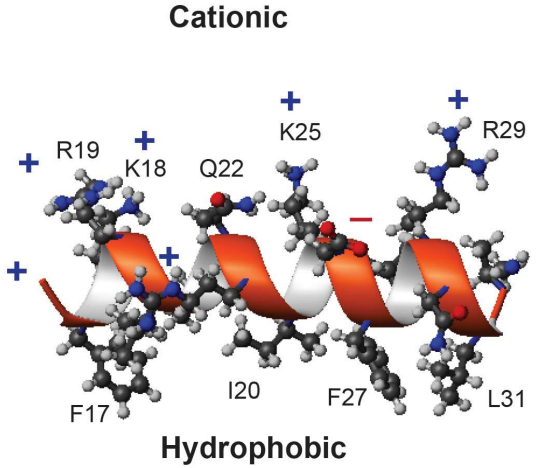




The sidechain NH signals of arginines overlap with the aromatic Phe protons in SDS micelles (A) and amide signals in DPC micelles (B). However, they are well resolved in D8PG (c).

Intermolecular Arg-D8PG Interactions by Solution NMR





This NMR study correlates nicely with the activity data of the single residue alanine variants.

The intensity of the peptide-lipid cross peaks is inversely proportional to the distance between the peptide and lipid protons: Aromatic protons of F17 and F27 > hydrophobic backbone amides > R23 sidechain > R19/R29

Wang, G. (2007) *Biochim Biophys Acta* 1768: 3271-3281

How to design selective, potent, and stable peptides?

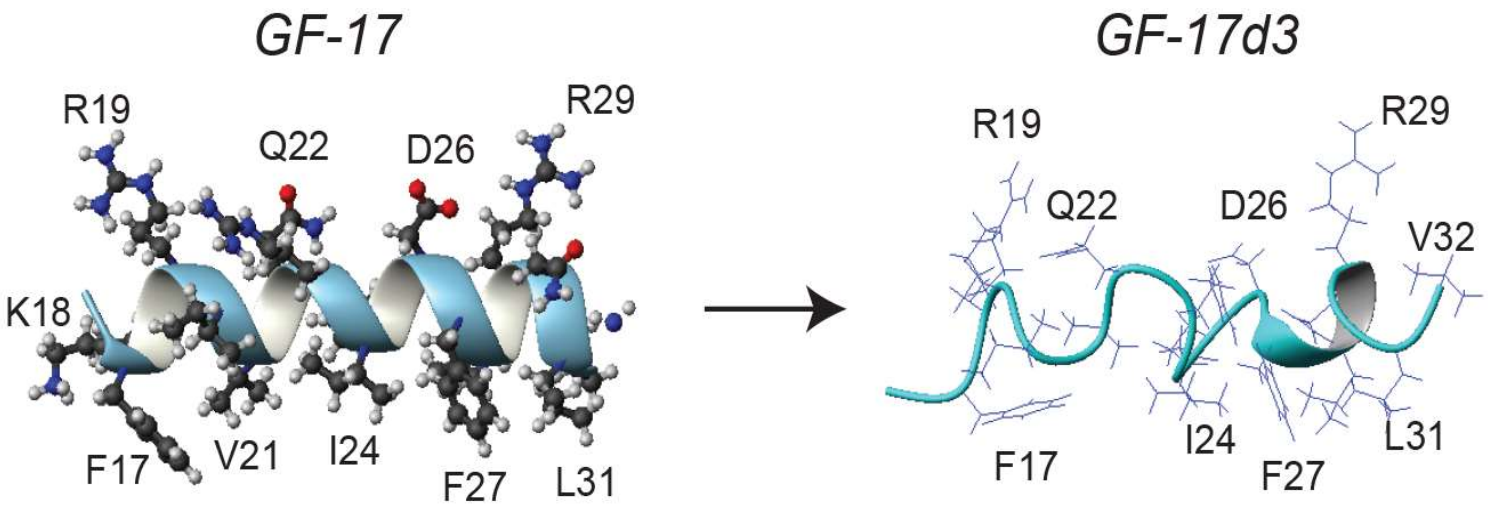
MRSA: Simultaneous activity and stability assays in 96-well plates

Name	Peptide amino acid sequence[a]	MIC (μM)	Stability[b]
FK-21	FKRIVQRIKDFLRNLVPRTE	160	-
GK-21	GKEFKRIVQRIKDFLRNLVPR	40	-
KI-22	KIGKEFKRIVQRIKDFLRNLVP	10	-
EK-20	EKIGKEFKRIVQRIKDFLRN	>160	-
KR-12	KRIVQRIKDFLR	>160	-
GF-17	GFKRIVQRIKDFLRNLV	2.5	-
GF-16	GFKRIVQRIKDFLRNL	10	-
BMAP-18	GRFKRFRKKFKKLFKKLS	>160	-
GF-17d3	GFKRIVQRIKDFLRNLV	>160	+[c]

GF-17d3 retained activity against E. coli in the presence of chymotrypsin, but lost activity against MRSA.

Wang G et al. (2014). *ACS Chem Biol* 9: 1997-2002

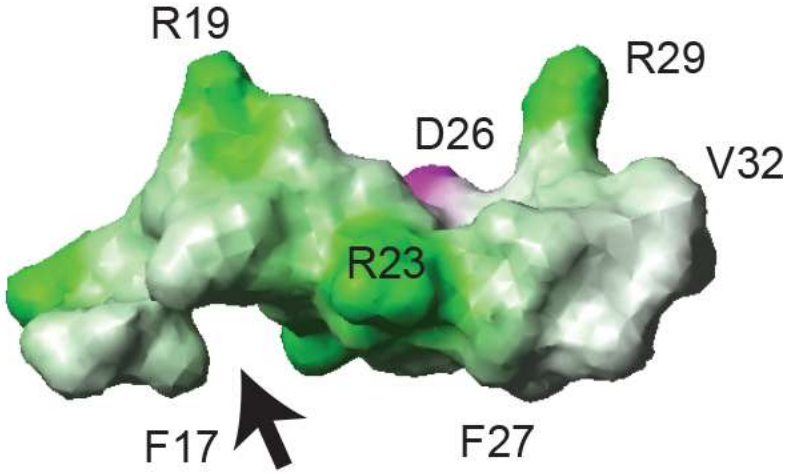
Structures of GF-17 (helical) and GF-17d3 (non-helical)

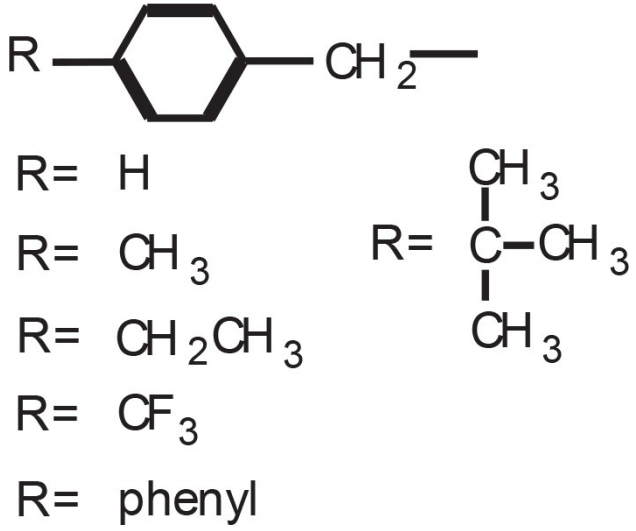


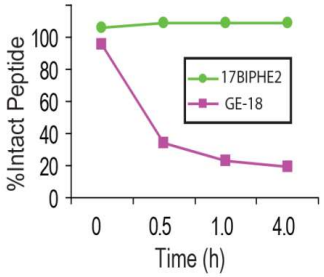
Protease-susceptible

Chymotrypsin-resistant

Structure-based design of antimicrobial agents







17BIPHE2 remains stable to Chymotrypsin (left).

Wang G et al. (2014) *ACS Chem. Biol.* 9: 1997-2002.

The ESKAPE Pathogens

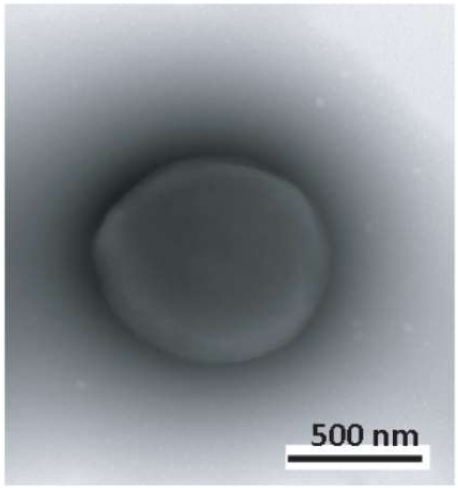
Enterococcus faecium (VRE);
Staphylococcus aureus (MRSA);
Klebsiella pneumonia (nightmare);
Acinetobacter baumannii;
Pseudomonas aeruginosa;
Enterobacter species.

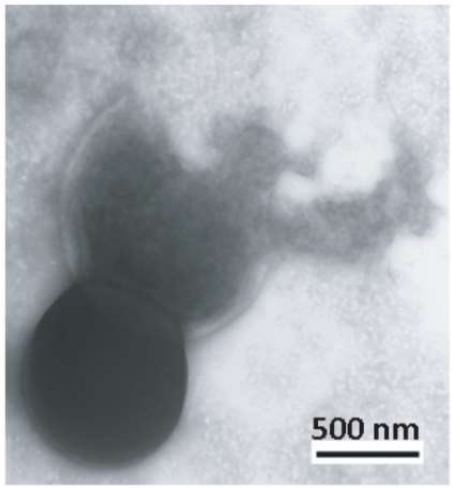
17BIPHE2 is effective against the ESKAPE pathogens

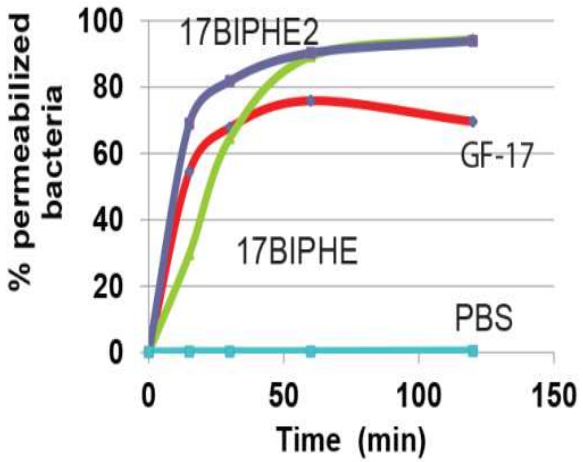
Peptide	MIC (μM)						HL_{50} (μM)[b]
	E. faecium	*S. aureus*	*K. pneumonia*	*A. baumannii*	*P. aeruginosa*	*E. cloacae*	
17F2	>100	>100	>100	6.2-12.5	100	25	>900
17mF-F	25-50	25	50	3.1-6.2	25	25	>900
17F-Naph	3.1	25	25	3.1	12.5	12.5	>900
17mF-Naph	3.1	6.2	12.5	3.1	6.2-12.5	6.2	500
17Naph-mF	3.1	6.2	12.5	3.1	6.2-12.5	6.2-12.5	950
17BIPHE	12.5	12.5	25	3.1	12.5	12.5	>900
17BIPHE2	3.1	3.1	3.1	3.1	6.2	3.1	225

Wang G et al. (2014). *ACS Chem Biol* 9: 1997-2002.

17BIPHE2 damages bacterial Membrane

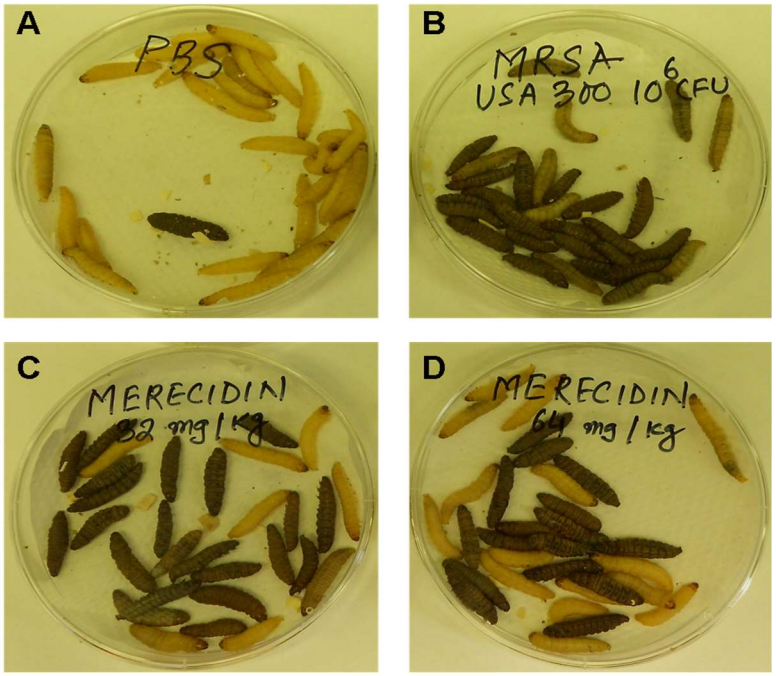




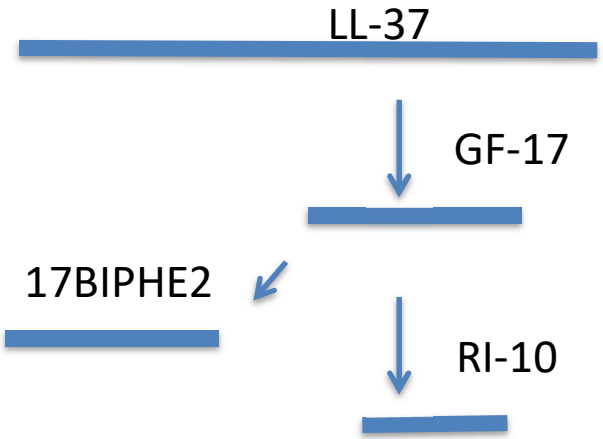


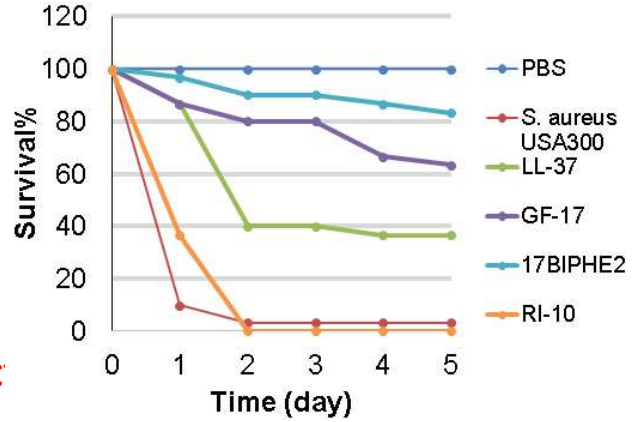
TEM: before and after peptide treatment.
Propidium iodide: membrane permeation is slightly more potent than GF-17

In vivo model I: the wax moths model illustrate advantages of peptide engineering



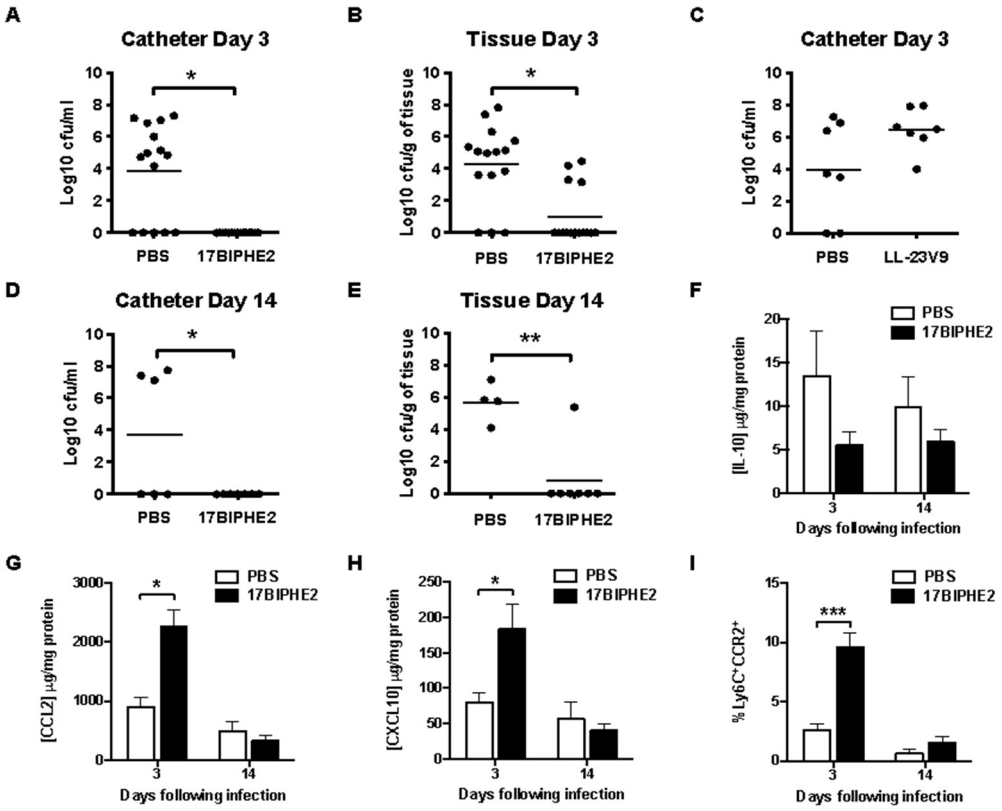
Galleria mellonella





The engineered peptide 17BIPHE2 is most effec in this model compared to LL-37 and its native fragments.

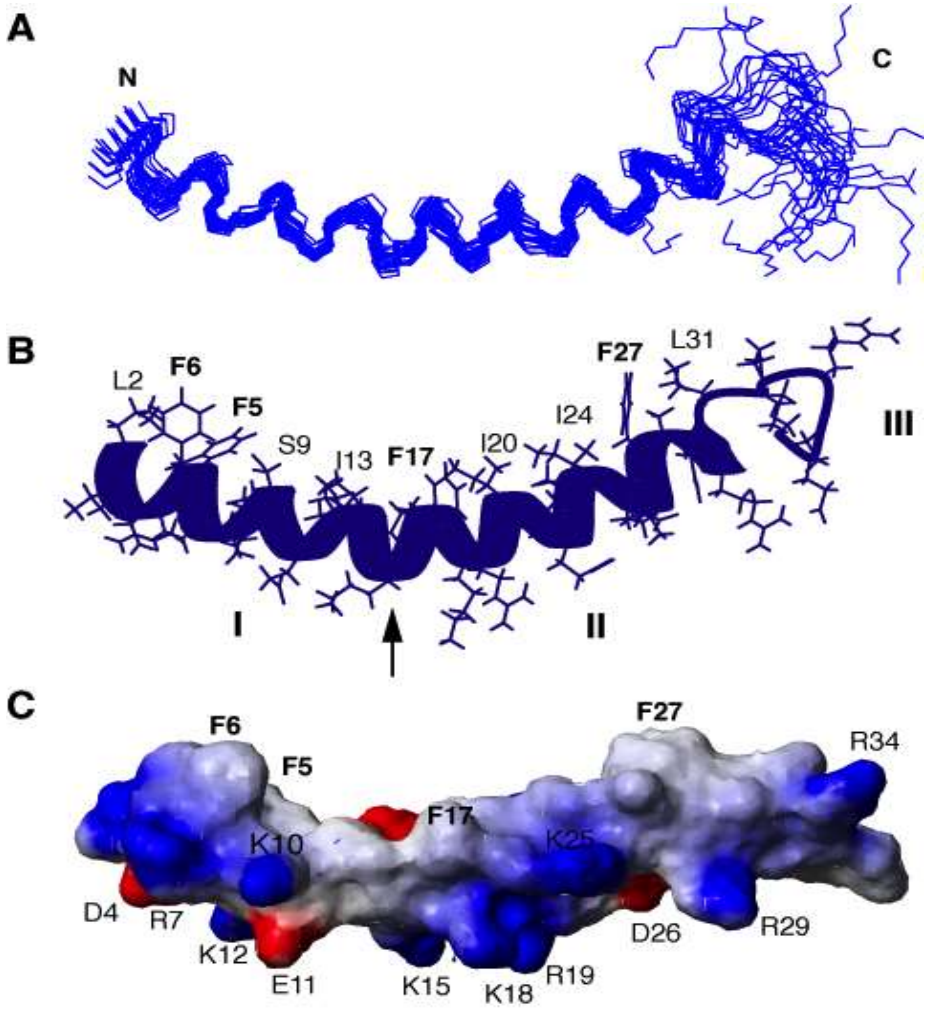
In vivo model II: a catheter *S. aureus* Biofilm model (by Tammy Kielian's lab)



While an inactive peptide did not work (c), 17BIPHE2 was effective in reducing MRSA CFU in the catheters (A & D) and surrounding tissues (B & E) at both days 3 and 14. In addition, the peptide was able to induce MCP-1 at day 3 (G) that recruited monocytes (I) to further clear the infection.

Wang G et al. (**2014**) *ACS Chem. Biol.* 9: 1997-2002

The NMR structure of LL-37 bound to SDS micelles

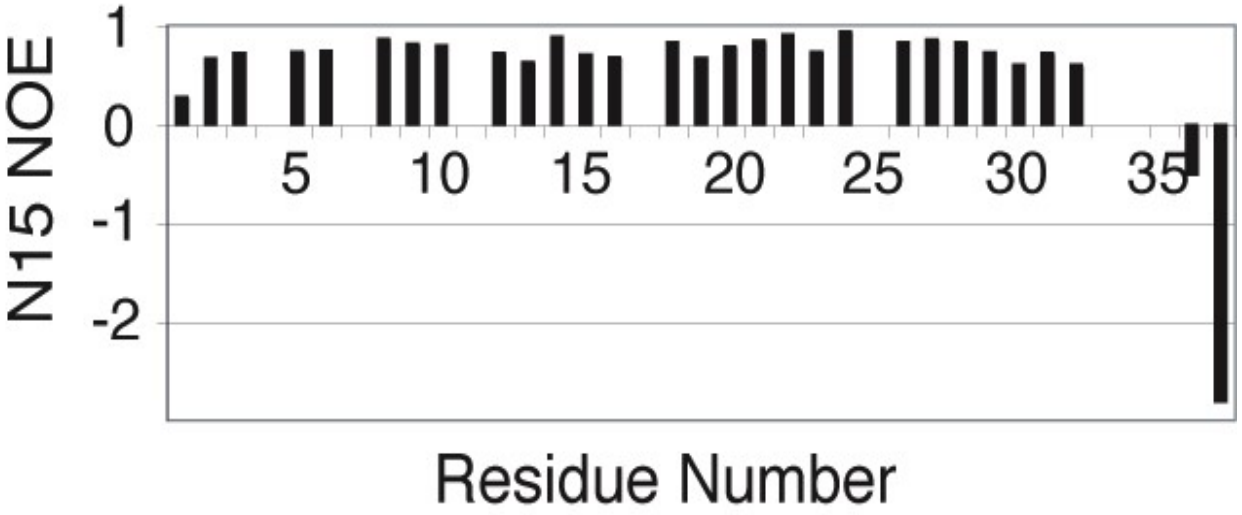


A. Superimposed Backbone;
B. Ribbon diagram;
C. Potential surface.

3D NMR studies revealed a helical structure for human LL-37 covering residues 2-31, while the tail portion is disordered.

Wang, G. (2008) *J Biol Chem* 283: 32637.

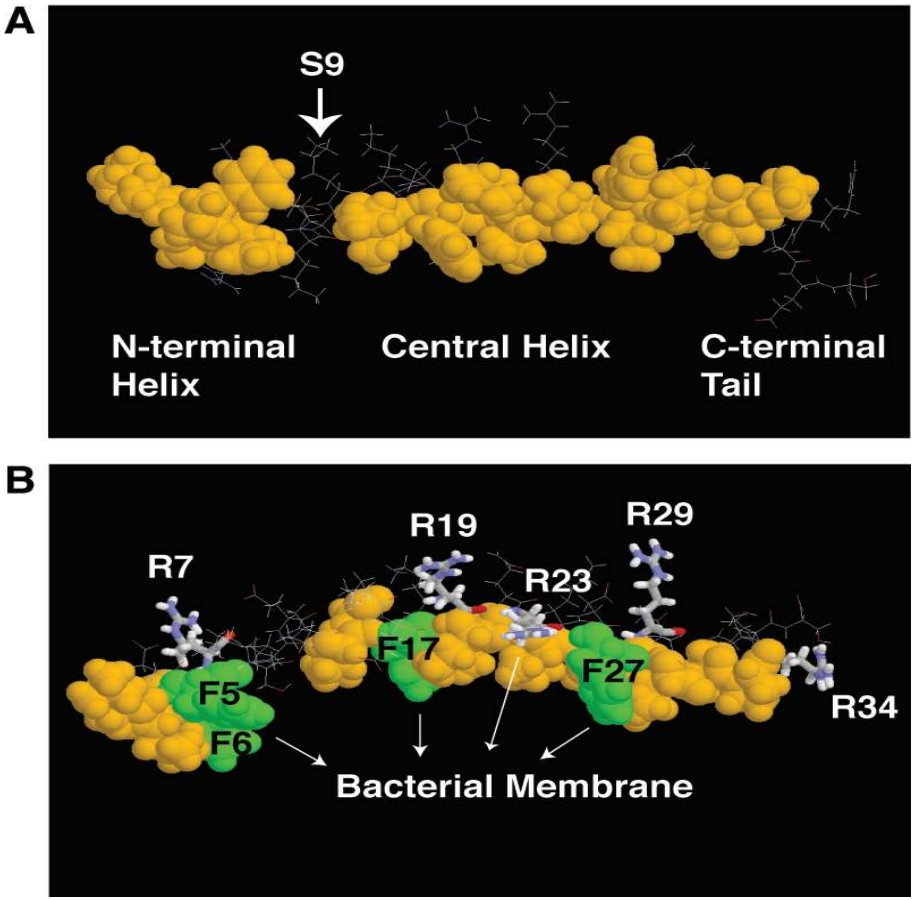
NMR Dynamics: Depicting the Motional Picture of Micelle-bound Human LL-37



This figure indicates that residues 2-32 are ordered, while the C-terminal tail of LL-37 is mobile. This picture is fully consistent with the 3D structure of LL-37 determined independently without using this backbone dynamics information.

Wang, G. (2008) *J Biol Chem* 283: 32637.

Structural light on antibacterial, antibiofilm, and antiviral activity of LL-37



Structural validation 1: Peptide dynamics. **Validation 2**: structure bound to anionic phosphatidylglycerol is the same. **Validation 3**: structure bound to LPS also indicates a disordered C-terminal tail.

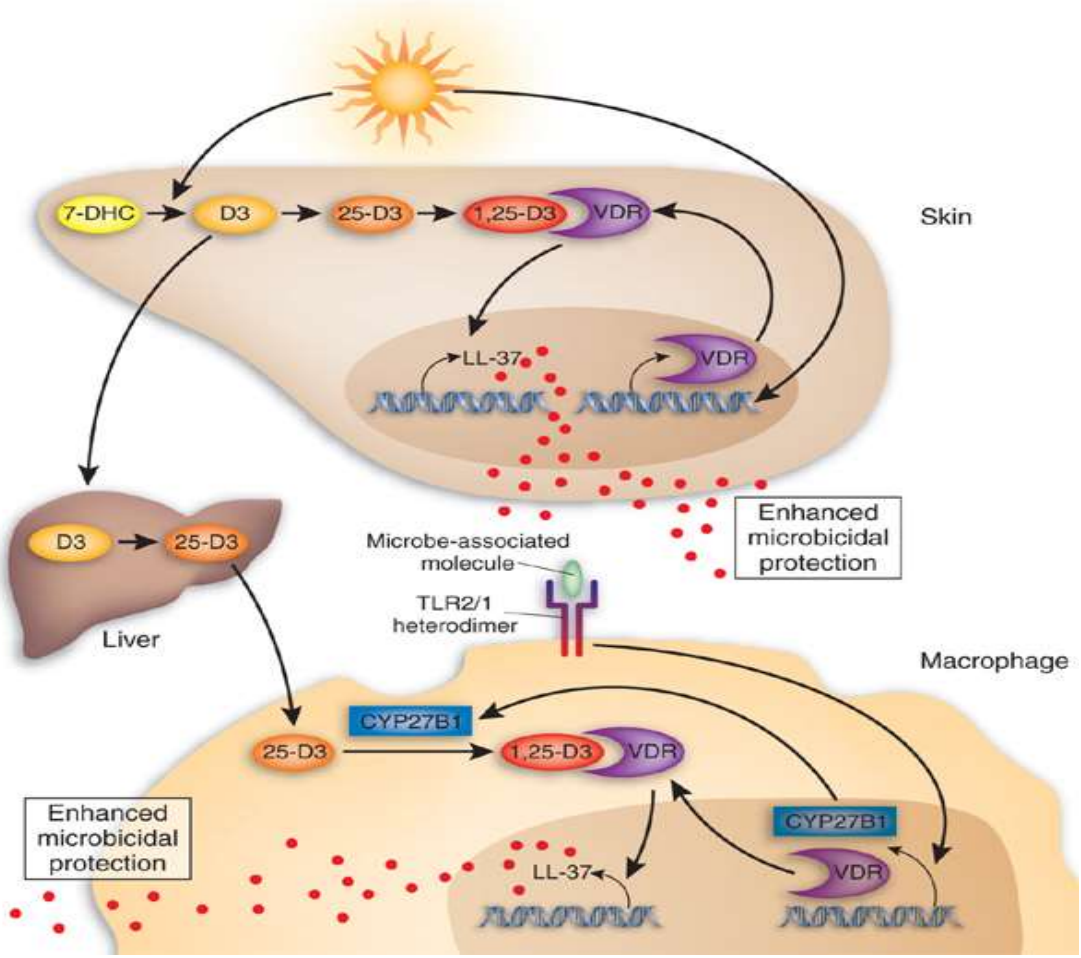
Wang et al. (2014) *Biochim. Biophys. Acta* 1838: 2160-2172.

Sequence-dependent activity:
Templates for peptide engineering

Peptide	Amino acid sequence	LL-37 region	Activity
KR-12	KRIVQRIKDFLR	18-29	E. coli
FK-13	FKRIVQRIKDFLR	17-29	HIV
GF-17	FKRIVQRIKDFLRNLV	17-32	MRSA/biofilms/cancer
GI-20	GIKEFKRIVQRIKDFLRNLV	13-32	Viruses/immune modulation[1]
RK-25	RKSKEKIGKEFKRIVQRIKDFLRNL	7-31	Biofilm

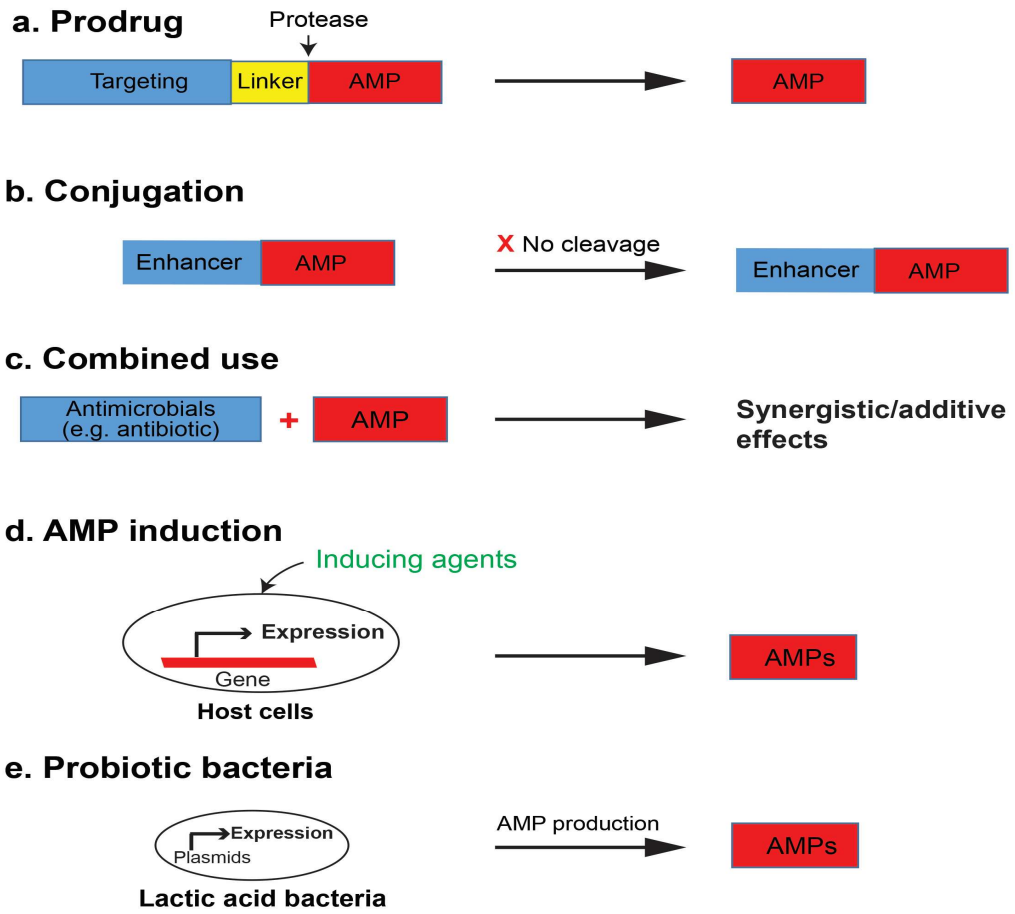
Wang G et al. (2014). *Biochim Biophys Acta*. Sep;1838(9):2160-72.

Light therapy for TB, Vitamin D and LL-37



Liu P et al. (2006) **Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response.** Science 311: 1770-3.

Advanced application strategies



Mishra, B., Reiling, S., Zarena, D., Wang, G. 2017. Host defense antimicrobial peptide as antibiotics: design and application strategies. *Curr. Opin. Chem. Biol. 38*, 87-96.

Summary

1. Over 3000 natural antimicrobial peptides have been identified and registered in the antimicrobial peptide database (http://aps.unmc.edu/AP). Importantly, some AMPs are already in use.
2. There is a great interest in developing the therapeutic use of human cathelicidin LL-37.
3. Both library screen and structure-based design are in use. They can be combined.
4. LL-37 derived peptides can kill the ESKAPE pathogens, disrupt biofilms, and show topical efficacy in animal models.
5. The engineered peptide 17BIPHE2 is superior to LL-37 and its native fragments in protecting the wax moths.

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