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LL-37_Renalexin hybrid peptide exhibits antimicrobial activity at lower MICs than its counterpart single peptides

Julius Kwesi Narh¹, Nestor G. Casillas-Vega², and Xristo Zarate^{1, *}

¹ Facultad de Ciencias Quimicas, Universidad Autonoma de Nuevo Leon, Avenida Universidad s/n, Ciudad Universitaria, San Nicolas de los Garza, NL, 66455, Mexico

² Departamento de Patologia Clinica, Hospital Universitario Dr. Jose Eleuterio Gonzalez, Universidad Autonoma de Nuevo Leon, Monterrey, NL, 64460, Mexico

* Corresponding author: Email: xristo.zaratekl@uanl.edu.mx Tel: 8183294000 ext. 3444

(A)

CATATG

ATGCACCACCACCACCACCACGAGACCATGAGCGAGGCCAGCCCCAGGTGATCAGCG
CCACCGGCGTGGTGAAGGGCATCGACCTGGAGAGCAAGAAGATCACCATCCACCACGA
CCCCATCGCCGCCGTGAACTGGCCCCGAGATGACCATGAGGTTACCATACCCCCCAGA
CCAAGATGAGCGAGATCAAGACCGGCGACAAGGTGGCCTTCAACTTCGTGCAGCAGGG
CAACCTGAGCCTGCTGCAGGACATCAAGGTGAGCCAGGTACCGACGACGACGATAAAC
TGCTGGGTGATTTCTTTTCGTAAAAGCAAAGAGAAAATCGGCAAAGAGTTCAAGCGCATC
GTGCAGCGTATCAAGGACTTCCTGCGTAATCTGGTTCCGCGTACGGAAGCGGTAGCTT
TTAGGTGGTCTGATTAAGATCGTGCCGGCAATGATTTGTGCTGTGACCAAAAAGTGC
TAACTCGAG

(B)

CATATG

TCAGGACATACAGCTCACGTAGATGAAGCAGTCAAACACGCTGAAGAAGCGGTGGCCC
ATGGCAAGGAGGGCCACACCGATCAACTGCTGGAACACGCGAAGGAGTCGTTGACCCA
TGCGAAAGCGGCGTCTGAAGCGGGTGGAAACACCCACGTTGGTCACGGCATTAAAGCAT
CTCGAGGATGCAATCAAGCACGGCGAAGAGGGTCATGTTGGCGTTGCTACCAAGCACG
CGCAGGAGGCCATTGAACATTTGCGCGCATCCGAGCACAAAAGCCATGGTACCGACGA
CGACGATAAACTGCTGGGTGATTTCTTTTCGTAAAAGCAAAGAGAAAATCGGCAAAGAG
TTCAAGCGCATCGTGCAGCGTATCAAGGACTTCCTGCGTAATCTGGTTCCGCGTACGGA
AAGCGGTAGCTTTTTAGGTGGTCTGATTAAGATCGTGCCGGCAATGATTTGTGCTGTGAC
CAAAAAGTGC **TAACTCGAG**

Figure S1: DNA nucleotide sequence of cDNA (DNA insert). (A) cDNA nucleotide sequence encoding for CusF3H+_LL-37_Renalexin fusion protein. (B) cDNA nucleotide sequence encoding or SmbP_LL-37_Renalexin fusion protein.

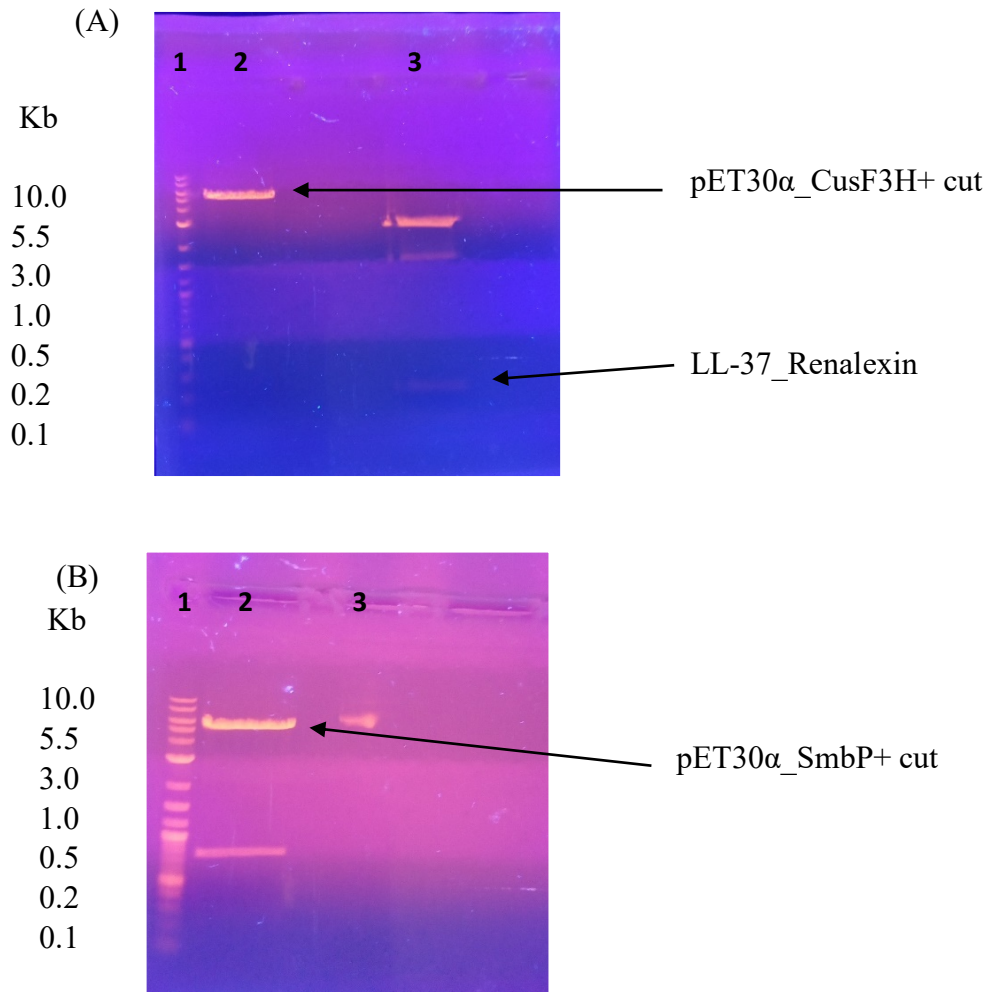


Figure S2: Agarose gel analysis of restriction enzyme digestion of plasmid DNA. (A) 1% Agarose gel analysis of restriction digestion: Lane1; DNA ladder, Lane 2: pET30 α _CusF3H+ cut, Lane3; pUC57_SmbP_LL-37_Renalexin cut. (B): 1% Agarose gel analysis of restriction digestion: Lane1; DNA ladder, Lane2; pET30 α _SmbP_GFP cut, Lane 3; pET30 α _SmbP_GFP uncut.

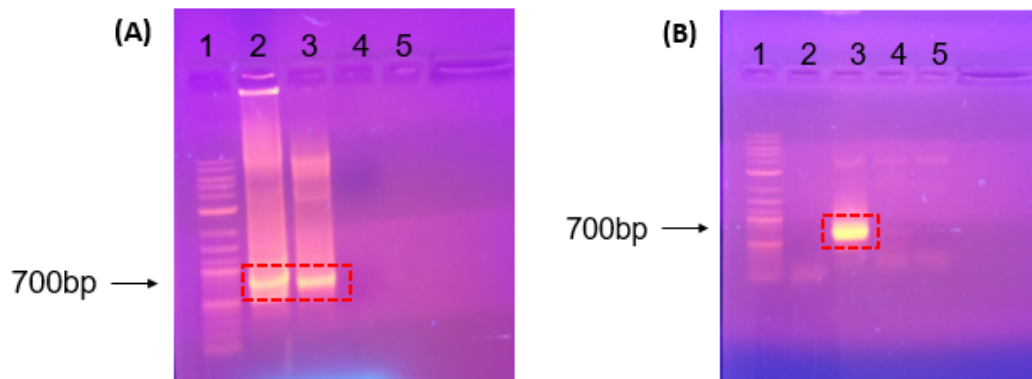


Figure S3: PCR analysis of synthetic DNA inserts in the designed recombinant plasmid constructs (A): 1% Agarose gel analysis of CusF3H+_LL-37_Renalexin DNA amplicons: Lane 1; DNA ladder, Lane 2, 3 & 4; PCR amplicons, Lane 5: Negative control (No DNA sample). Amplicons in the red rectangular label indicate the target amplifications. (B): 1% Agarose gel analysis of SmbP_LL-37_Renalexin DNA amplicons: Lane 1; DNA ladder, Lane 2; Negative control (No DNA sample), Lane: 3,4 & 5; PCR amplicons. Amplicons in the red rectangular label indicate the target amplifications.

(A)

# Length: 480				
# Identity: 480/480 (100%)				
# Similarity: 480/480 (100%)				
# Gaps: 0/480 (0.0%)				
# Score: 1662				
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#				
#=====				
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EMBOSS_001	85	CACCACCACCATCATCATGAAACCATGAGCGAAGCACACCACAGGTTAT		134
EMBOSS_001	60	TAGCGCCACTGGCGTGGTAAAGGGTATCGATCTGGAAGCAAAAAATCA		109
EMBOSS_001	135	TAGCGCCACTGGCGTGGTAAAGGGTATCGATCTGGAAGCAAAAAATCA		184
EMBOSS_001	110	CCATCCATCACGATCCGATTGCTGCCGTGAACTGGCCGGAGATGACCATG		159
EMBOSS_001	185	CCATCCATCACGATCCGATTGCTGCCGTGAACTGGCCGGAGATGACCATG		234
EMBOSS_001	160	CGCTTTACCATCACCCCGCAGACGAAATGAGTGAAATTAAACCGGCGA		209
EMBOSS_001	235	CGCTTTACCATCACCCCGCAGACGAAATGAGTGAAATTAAACCGGCGA		284
EMBOSS_001	210	CAAAGTGGCGTTTAAATTTGTCCAGCAGGGCAACCTTTCTTTATTACAGG		259
EMBOSS_001	285	CAAAGTGGCGTTTAAATTTGTCCAGCAGGGCAACCTTTCTTTATTACAGG		334
EMBOSS_001	260	ATATTAAAGTCAGCCAGGGTACCGACGAAAGGATATACTGCTGATGGAT		309
EMBOSS_001	335	ATATTAAAGAAAGCCAGGGTGCCGACCGGCAGGATCTACAACCGCTTGGT		384
EMBOSS_001	310	ATATTAAAGAAAGCCAGGGTGCCGACCGGCAGGATCTACAACCGCTTGGT		359

(B)

# Length: 492				
# Identity: 492/492 (100%)				
# Similarity: 492/492 (100%)				
# Gaps: 0/492 (0.0%)				
# Score: 1831				
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#				
#=====				
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EMBOSS_001	31	CATATGTGAGGACATACAGCTCACGTGGACGAAGCAGTGAAGCACGCTGA		80
EMBOSS_001	51	AGAAGCGGTGGCCCATGGCAAAGAGGCCATACCGATCAATTACTGGAAC		100
EMBOSS_001	81	GGAAAGCGGTGGCCCATGGTAAAGAGGCCATACCGATCAATTACTGGAAC		130
EMBOSS_001	101	ATGCGAAGGAATCTCTGACACATGCGAAAGCTGCGACTGAAGCAGGTGGA		150
EMBOSS_001	131	ATGCGAAGGAATCTCTGACACATGCGAAAGCTGCGACTGAAGCAGGTGGA		180
EMBOSS_001	151	AACACGCATGTTGGTCACGGAATCAAACATCTCGAAGATGCAATCAAGCA		200
EMBOSS_001	181	AACACGCATGTTGGTCACGGAATCAAACATCTCGAAGATGCAATCAAGCA		230
EMBOSS_001	201	CGGCGAAGAGGGTCATGTCGGTGTGCTACCAAGCATGCGCAAGAGGCTA		250
EMBOSS_001	231	CGGCGAAGAGGGTCATGTCGGTGTGCTACCAAGCATGCGCAAGAGGCTA		280
EMBOSS_001	251	TCGAGCATTTGCGTGCAATCCGAACATAAATGGCACGGTACCGTCGACGTT		300
EMBOSS_001	281	TCGAGCATTTGCGTGCAATCCGAACATAAATGGCACGGTACCGTGGACGTT		330
EMBOSS_001	301	GAGCTAAACTGCTCGGAAATGTTCAATCGTAAACGCCTACACAAGCTTGG		347
EMBOSS_001	331	GAGCTAAACTGCTCGGAAATGTTCAATCGTAAACGCCTACACGAGCTTGG		377

Figure S4: DNA nucleotide sequence alignment using the Needleman–Wunsch algorithm for pairwise analysis between the synthetic cDNA and designed plasmid DNA insert (amplicon). (A) Sequence nucleotide alignment for CusF3H+_LL-37_Renalexin DNA amplicon. (B) Sequence nucleotide alignment for SmbP_LL-37_Renalexin DNA amplicon.

Table S1: Protein (recombinant fusion peptides) band purity using densitometry in ImageJ.

Densitometry in ImageJ							
ID	Area	Mean	Min	Max	Max – Min	% Purity	
Lysate	446080	122.752	69	167	98		
Elution 1	446080	145.113	66	170	104	84.72367	
Elution 2	446080	145.912	66	165	99	80.65042	
Elution 3	446080	148.103	66	177	111	90.42623	
Elution 4	446080	149.279	64	182	118	96.12878	
Elution 5	446080	151.18	63	175	112	91.24088	
Elution 6	472320	150.977	66	180	114	92.87018	
Elution 7	472320	154.113	66	179	113	92.05553	

Determination of recombinant fusion peptide concentration using Bradford analysis with Bovine Serum Albumin (BSA) as standard protein

Table S2: CusF3H+_LL-37_Renalexin, expressed in *E. coli* BL21(DE3), quantification.

BSA (mg/ml)	Absorbance (595nm) A	B	C	average	ave-blank
1.3	1.224	1.252	1.327	1.267667	0.648333
1	1.189	1.232	1.134	1.185	0.565667
0.7	1.084	1.062	1.045	1.063667	0.444333
0.5	0.963	0.99	0.936	0.963	0.343667
0.3	0.811	0.795	0.762	0.789333	0.17
0.1	0.694	0.669	0.65	0.671	0.051667
Sample	0.767	0.776	0.74	0.761	0.141667
Blank	0.644	0.612	0.602	0.619333	

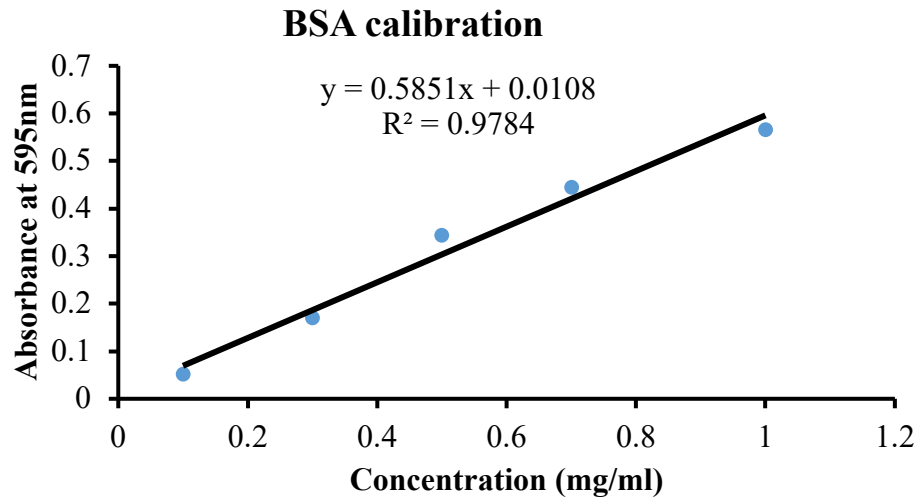


Figure S5: Calibration curve for CusF3H+_LL-37_Renalexin (with disulfide bond) quantification.

Concentration: $(F9 - 0.0108) / 0.5851 = 0.223665 \text{ mg/ml}$ X total elution fractions (14) = **3.136 mg/L**
F9 = 0.141667.

Table S3: SmbP_LL-37_Renalexin, expressed in *E. coli* BL21(DE3), quantification.

BSA (mg/ml)	Absorbance (595nm) A	Abs B	Abs C	Average Abs	Av Abs – blk
1	0.989	1.042	1.057	1.029333	0.450666333
0.7	0.969	0.92	0.91	0.933	0.354333
0.5	0.86	0.836	0.839	0.845	0.266333
0.3	0.698	0.676	0.687	0.687	0.108333
0.2	0.697	0.756	0.687	0.713333	0.134666333
0.1	0.716	0.615	0.614	0.648333	0.069666333
Sample	0.654	0.662	0.639	0.651667	0.072999667
Blank	0.546	0.589	0.601	0.578667	

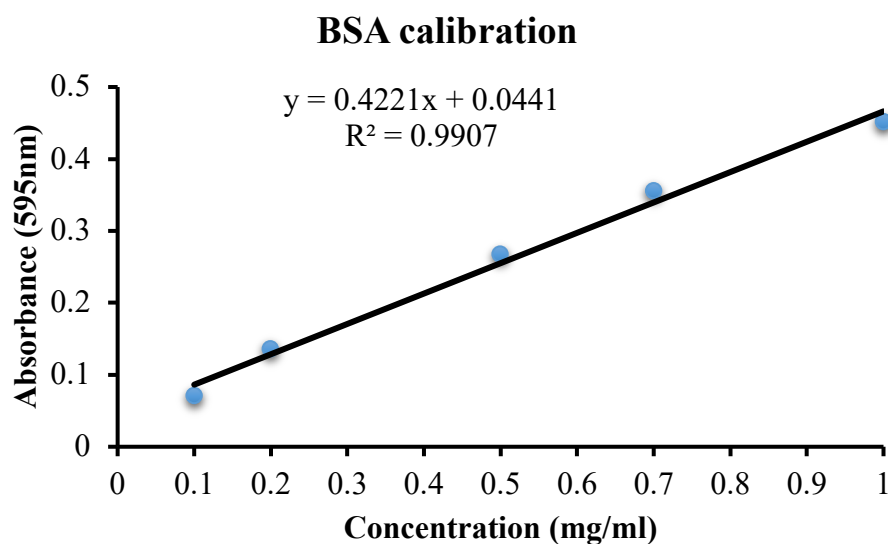


Figure S6: Calibration curve for SmbP_LL-37_Renalexin (without disulfide bond) quantification.

Concentration= $(F9 - 0.04221) / 0.4221 = 0.1 \text{ mg/ml}$ X total elutions (15) = **1.5 mg/L**

F9= 0.07299967.

Concentration after enterokinase cleavage

Table S4: LL-37_Renalexin (expressed in *E. coli* SHuffle T7) cleaved, quantification using NanoDrop at (A_{280nm})

BSA (mg/ml)	A	B	C	Ave Abs
1.3	0.97	0.997	1.001	0.989333
1	0.75	0.749	0.747	0.748667
0.7	0.487	0.512	0.507	0.502
0.5	0.341	0.359	0.356	0.352
0.3	0.22	0.193	0.201	0.204667
0.1	0.084	0.061	0.103	0.082667
Sample	0.105	0.0956	0.0958	0.0988
Blank	0	0	0	0

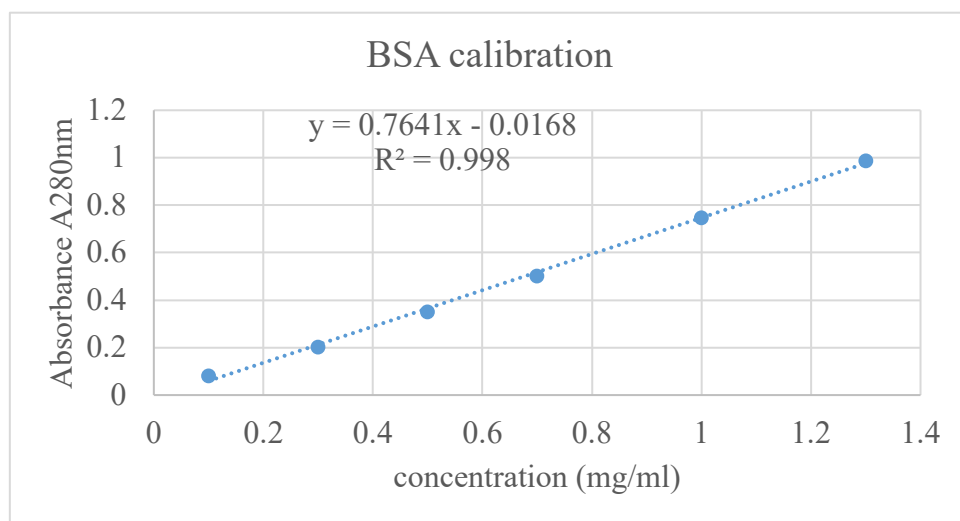


Figure S7: Calibration curve for LL-37_Renalexin (with disulfide bond) cleaved.

Concentration= $(F9 + 0.0168) / 0.7641 = 0.151 \text{ mg/ml}$ X total elutions (14) = **2.1 mg/L**

F9= 0.0988

Table S5: LL-37_Renalexin, expressed in *E. coli* BL21(DE3), cleaved, quantification using NanoDrop at (A280nm).

BSA	A	B	C	Average
1.3	0.956	0.955	0.952	0.954333
1	0.712	0.701	0.717	0.71
0.7	0.464	0.494	0.495	0.484333
0.5	0.333	0.34	0.342	0.338333
0.3	0.175	0.228	0.183	0.195333
0.1	0.072	0.073	0.086	0.077
Sample	0.02	0.019	0.013	0.017333

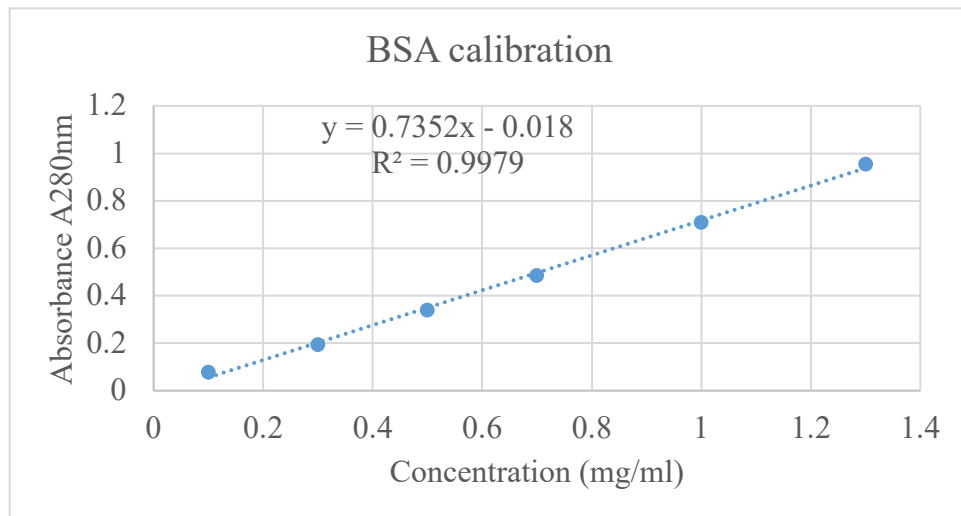


Figure S8: Calibration curve for LL-37_Renalexin (without disulfide bond) cleaved.

Concentration= $(F9+0.018)/0.7352 = 0.0467\text{mg/ml}$ X total elutions (15) = **0.7mg/L**

F9= 0.01733

Antimicrobial activity (Dose_response) assay

Table S6: LL-37_Renalexin (expressed in *E. coli* SHuffle T7) against *Staphylococcus aureus*

Peptide concentration (μM)	CFU/plate					
	Rep1	Rep2	Rep3	average	Reduction	% Reduction
0	81	70	64	72	0	0
0.5	51	65	60	59	13	13
1	49	60	57	55	17	19
2.5	45	52	52	50	22	26
5	44	38	46	43	29	37
10	35	28	40	34	38	50
15	25	14	22	20	52	71
20	17	14	9	13	59	81
25	12	11	9	11	61	84

30	9	11	7	9	63	87
33	6	9	7	7	65	90

Table S7: LL-37_Renalexin (expressed in *E. coli* SHuffle T7) against *Escherichia coli*

Conc (μM)	CFU/Plate					
	Rep1	Rep2	Rep3	Average	Reduction	% Reduction
0	101	126	113	113	0	0
0.5	97	89	94	93	20	18
1	84	80	79	81	32	29
2.5	82	72	80	78	35	31
5	69	61	70	67	47	41
10	54	60	58	57	56	49
15	52	50	47	50	64	56
20	37	40	31	36	77	68
25	32	25	21	26	87	77
30	24	15	19	19	94	83
33	13	14	17	15	99	87

Table S8: LL-37_Renalexin, expressed in *E. coli* BL21(DE3), against *Staphylococcus aureus*

Peptide concentration (μM)	CFU/plate					
	Rep1	Rep2	Rep3	Average	Reduction	% Reduction
0.5	71	69	81	74	0	0
1	69	68	74	70	3	5
1.5	64	67	71	67	6	9
3	63	62	70	65	9	12
6	47	54	50	50	23	32
8	41	35	39	38	35	48
10	27	35	33	32	42	57
12	23	26	30	26	47	64
15	19	25	20	21	52	71
20	13	11	17	14	60	81
25	12	10	16	13	61	83

Table S9: LL-37_Renalexin, expressed in *E. coli* BL21(DE3), against *Escherichia coli*

Peptide concentration (μ M)	CFU/plate					
	Rep1	Rep2	Rep3	Average	Reduction	% Reduction
0	102	98	111	104	0	0
0.5	86	90	80	85	18	18
1	80	88	81	83	21	20
1.5	80	70	78	76	28	27
3	74	68	70	71	33	32
6	71	67	62	67	37	36
8	70	66	60	65	38	37
10	65	64	60	63	41	39
12	61	59	58	59	44	43
15	56	54	51	54	50	48
20	49	46	43	46	58	56
25	35	29	31	32	72	69

Antimicrobial activity (Time-killing kinetic assay)**Table S10:** LL-37_Renalexin (expressed in *E. coli* SHuffle T7) against *Staphylococcus aureus*

Time (h)	CFU/ml								
	LL-37_Renalexin (33 μ M)			Kanamycin (33 μ M)			Control		
0	41	45	37	43	37	40	47	39	43
0.3	45	47	43	28	22	25	49	51	50
0.6	56	50	62	18	22	20	65	59	62
1	49	45	51	15	11	13	67	61	64
1.3	57	55	59	11	7	9	83	75	79
1.6	40	37	43	5	9	7	84	80	82
2	31	25	37	5	3	4	88	84	86
2.3	26	29	23	3	3	3	90	94	92
2.6	21	19	23	1	5	3	92	96	94
3	11	10	12	2	4	3	96	98	97

Table S11: LL-37_Renalexin (expressed in *E. coli* SHuffle T7) against *Escherichia coli*

Time (h)	LL-37_Renalexin (33µM)			Kanamycin (33µM)			Control		
0	57	55	57	55	51	50	61	57	51
0.3	58	49	59	29	31	34	68	59	66
0.6	63	62	59	19	21	23	79	83	69
1	65	61	55	11	14	15	85	78	81
1.3	49	50	40	7	9	7	87	97	89
1.6	25	21	27	4	7	5	117	109	122
2	15	17	14	3	2	2	119	121	130
2.3	12	10	11	1	2	0	132	140	143
2.6	9	7	11	0	0	0	145	147	150
3	4	5	6	0	0	0	150	151	149

ANOVA (With total possible CFU/ml)**Table S12:** LL-37_Renalexin (expressed in *E. coli* SHuffle T7) against *Staphylococcus aureus*

Average of CFU/ml (3Reps)		
Control	LL-37_Renalexin (33µM)	Kanamycin (33µM)
43	41	40
50	45	25
62	56	20
64	49	13
79	57	9
82	40	7
86	31	4
92	26	3
94	21	3
97	11	3

Table S13: LL-37_Renalexin (with disulfide bond) against *Escherichia coli*

Average CFU/ml (3Reps)		
Control	LL-37_Renalexin (33µM)	Kanamycin (33µM)
61	57	55

68	58	29
79	63	19
85	65	11
87	49	7
117	25	4
119	15	3
132	12	1
145	9	0
150	4	0

ToxIBTL

a generic webserver for peptide toxicity prediction.

Result			
Results			
Sequence No.	Sequence label	result	score
1	0	non-toxic	3.7139784e-05
Back			

Figure S10: In-silico cellular toxicity against normal human cells prediction of the hybrid peptide (LL-37_Renlexin) in the ToxIBTL server. The amino acid sequence of the hybrid peptide was submitted to the server in FASTA format. The predicted toxicity score was 3.7139784e-05 (0.0000371), which was far below the threshold of 0.5, indicating a zero-peptide toxic effect on healthy human cells.

Table S14: LL-37_Renalexin expressed in *E. coli* SHuffle T7(DE3) against MRSA

Conc (uM)	Colony Forming Units		
	Rep 1	Rep 2	Rep 3
0	101	119	108
0.5	87	89	78
1	65	70	76
2.5	58	63	69
5	68	57	59
10	49	56	50
15	46	51	45
20	42	33	38
25	19	29	32
30	13	12	14
35	11	13	10

Table S15: LL-37_Renalexin expressed in *E. coli* SHuffle T7(DE3) against *K. pneumoniae*

Conc (uM)	Colony Forming Units		
	Rep 1	Rep 2	Rep 3
0	101	120	108
0.5	90	98	91
1	80	82	79
2.5	77	69	74
5	66	65	70
10	54	61	59
15	50	52	48
20	43	41	44
25	33	19	25
30	24	10	15
35	16	10	11