# Identification of novel peptide binders targeting TPP riboswitch in *E. coli*

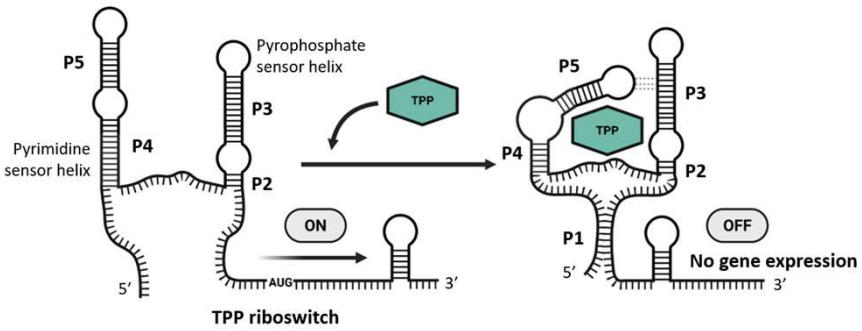
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## Abstract

Antimicrobial resistance (AMR) strains has been directly emerged by the improper use of antibiotics. Gram-negative pathogens, including *Pseudomonas aeruginosa*, *Klebsiella*, and *Escherichia coli*, are four-times more likely to cause Healthcare-associated Infections (HAI) compare to Gram-positive pathogens. Development of antibiotics for Gram-negative bacteria is difficult because their additional membrane, name as LPS, restricts the intracellular penetration of antibiotics. The presence of efflux pumps within bacteria makes it harder to develop the antibiotics.[1] The previous studies of antibiotics have mainly focused on bacterial proteins such as efflux pumps, ribosomal subunits, and RNA polymerases. Targeting the bacterial proteins has the disadvantage that easily proceeds bacterial mutation. Constantly, targeting RNA structure for novel antimicrobial therapy might be one of the promising strategies to overcome the limitation of current treatments. Among these, riboswitches, highly conserved tertiary RNA structures located in the untranslated regions (UTRs) of mRNA, regulate approximately 4% of the bacterial genome by sensing specific ligands.[2] Riboswitches have the unique tertiary structure that comprises the aptamer region and expression platform. When the specific ligand binds the aptamer region of riboswitch, expression platform regulates the gene of bacterial metabolism through structure change. The thiamine pyrophosphate (TPP) riboswitch is particularly significant as it is widely conserved in pathogenic bacteria and strictly regulates the synthesis of TPP, the active form of thiamine. The regulation of TPP supplement through TPP riboswitch plays a key role in bacterial survival. In this study, novel peptide binders targeting TPP riboswitch of *E. coli* were investigated by a modified fluorescent indicator displacement with the peptide library. Identified peptide binders demonstrated significant binding affinities as well as potent antibacterial activities. Therefore, peptide binders suggest a potent candidate of antibiotics via the regulation of TPP riboswitch in *E. coli*.

## Introduction




The **TPP riboswitch** forms tertiary interactions with its specific ligand, TPP, and is divided into the pyrimidine sensor helix and pyrophosphate sensor helix according to the ligand-binding sites. Upon TPP binding to the bulges of the two sensor helices, the loop of the pyrimidine sensor helix forms hydrogen bonds, resulting in clamp closure. Simultaneously, hydrogen bonds form between the terminal regions, leading to repression of gene expression.

## Results

### 1. Screening hit binders against TPP riboswitch

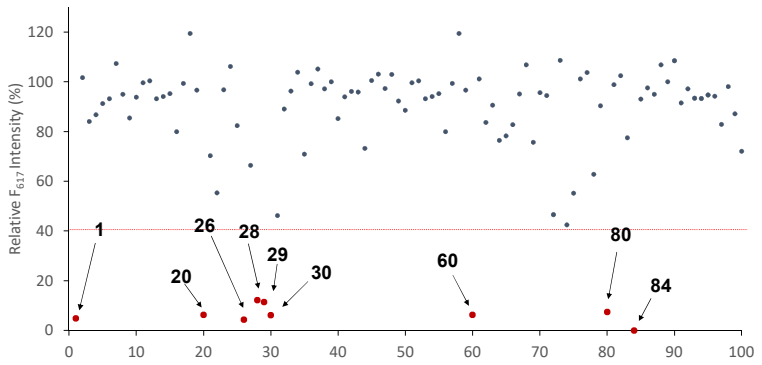
- **A Modified Fluorescent Indicator Displacement (FID) Assay**




Peptide binders targeting the TPP riboswitch were identified by utilizing the competitive binding between ligands and fluorophores via the FID principle.

**100 peptides**

Selection criteria : < 60% inhibition of relative fluorescence intensity

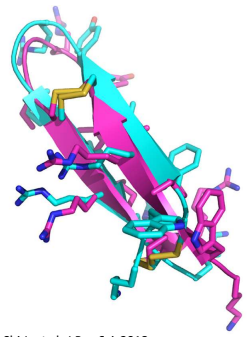



**9 hit peptides**

### 2. Peptide sequences of peptide binders

| | Sequences |
|---|---|
| CTPep-1 | RSVCRQIKICRRRGGCYYKCTNRPY |
| CTPep-20 | RKIKKRRKKWR |
| CTPep-26 | FKCRRWQWRMKKLG |
| CTPep-28 | FKCRRWQWRMKKLGAPSITCVRRAF |
| CTPep-29 | CRRWQWRMKKLGAPSITCV |
| CTPep-30 | CFQWQRNMRKVRGPPVSCI |
| CTPep-60 | RLCRIVVIRVCR |
| CTPep-80 | KWCFRVCYRGICYRRCRG |
| CTPep-84 | RGGRLCYCRRRFCVCVGR |

### 3. Structural Feature of Tachyplesin-1 (CTPep-80)



**CTPep-80** KWCFRVCYRGICYRRCRG

Shi J. et al. *J Pep Sci.* 2018

- CTPep-80 is naturally formed a **β-sheet structure** containing two cysteine bridges.
- Disulfide-bridged form of CTPep-80 binds to the bacterial membrane and forms pores to induce cellular leakage.
- The linear form of CTPep-80 also exhibited **similar antimicrobial activity** compared to the intra disulfide-bridged form of CTPep-80.

### 4. Antimicrobial activities of CTPep-80

| Number | | $MIC_{50}$ of *E. coli* ATCC 25922 (μM) | | |
|---|---|---|---|---|
| | | Muller-Hinton Broth (MH) | LB | M9 glucose |
| **CTPep-80** | Intra S-S bridge | 2.863 | 2.529 | 2.452 |
| | Linear* | 5.262 | 2.691 | 8.354 |
| Colistin (Control) | | 2.277 | 0.485 | 1.173 |

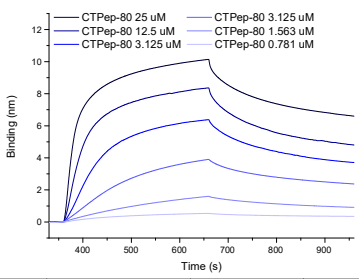
*Treated with 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP).

Abbreviations : Abs. : Absorbance, PS : Polystyrene, TC : Tissue-culture surface treatment

### 5. Binding Affinity between CTPep-80 and TPP riboswitch

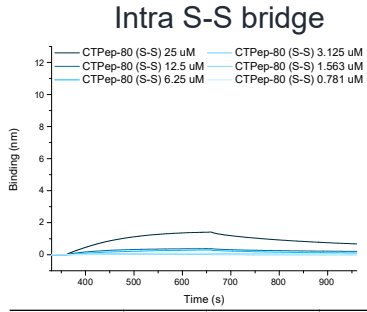
- **Bio-layer Interferometry (BLI) Assay**

Linear




| KD (μM) | 1.741 | kd (1/s) | $2.461 \times 10^{-3}$ |
|---|---|---|---|
| ka (1/Ms) | $1.413 \times 10^{3}$ | R-square | 0.994 |

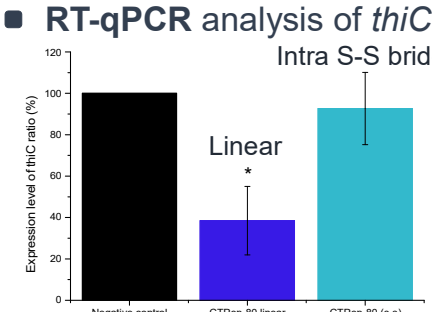
Intra S-S bridge




| KD (μM) | 42.35 | kd (1/s) | $3.705 \times 10^{-3}$ |
|---|---|---|---|
| ka (1/Ms) | 87.47 | R-square | 0.998 |

### 6. mRNA Expression of *thiC* in *E. coli*

- **RT-qPCR** analysis of *thiC* mRNA with CTPep-80 for 3 hours




*p-value < 0.005 vs. negative control

- Structure-dependent effects of peptide binders on **mRNA expression** of *thiC* in *E. coli*

## Conclusion

- Among the 9 hit peptide binders, CTPep-80 exhibited antimicrobial activity regardless of its structures, with intra disulfide bridges or not.
- The higher binding affinity of the linear form CTPep-80 was investigated than the intra disulfide-bridged form.
- The downstream gene *thiC*, regulated by the TPP riboswitch, was also downregulated by the linear form of CTPep-80.

**Therefore, the linear form of CTPep-80 suggest a potent candidate of antibiotics via the regulation of TPP riboswitch in *E. coli*.**

[1]D. Terefinko, M. Caban, A. Motyka, et al. *Chem. Eng. J.* **2023**, 452, 139415.
[2]A. Hübenthal, V. Panchal, R. Brenk, M. Mack. *J. Bacteriol.* **2025**, 207.