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Selective inhibition of resistant bacterial pathogens using a β-lactamase-activatable antimicrobial peptide with significantly reduced cytotoxicity

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ABSTRACT

The expression of β-lactamase, particularly metallo-β-lactamase (MBL) in bacteria has caused significant resistance to clinically important β-lactam antibiotics, including life-saving carbapenems. Antimicrobial peptides (AMPs) have emerged as promising therapeutic agents to combat antibiotic resistance. However, the cytotoxic AMPs has been one of the major concerns for their applications in clinical practice. Herein, we report a novel cephalosporin-caged AMP, which shows significantly reduced cytotoxicity, hemolytic activity, and antibacterial activity but turns highly active against bacteria upon specific hydrolysis by the antimicrobial resistance-causative β-lactamase. Further investigations demonstrate this β-lactamase-activatable AMP selectively inactivates resistant bacterial pathogens over susceptible bacteria. This strategy should be applicable to other AMPs as a potential solution for the treatment of infectious diseases caused by β-lactamase-expressing pathogenic bacteria.

Bacterial resistance to antibiotics, particularly β-lactam antibiotics, the most important class of therapeutic agents for infectious diseases, has emerged and spread rapidly all over the world, which poses a severe threat to public health [1–3]. The major mechanism for pathogenic bacilli to survive in the presence of β-lactam antibiotics is the production of a β-lactam-hydrolyzing enzyme, named as β-lactamase (bla) [4,5]. Particularly, recent studies indicated metallo-β-lactamases (MBLs, or class B bla) [6], such as New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM), and Imipenemase (IMP), are capable of degrading β-lactams with high efficiency and thus render broad-spectrum resistance to nearly all β-lactam antibiotics, including carbapenems, "the antibiotics of last resort" [7]. Moreover, some of these MBLs are plasmid-encoded enzymes, which are transmissible between different bacterial strains. The selection pressure caused by β-lactam antibiotics results in further reduction of susceptible bacterial strains and expansion of resistant strains, leading to the increase of community antimicrobial resistance.

The rapidly increasing resistance to β-lactam antibiotics has severely weakened the efficacy of these life-saving molecules. Though combination of β-lactams with synthetic β-lactamase inhibitors have been used to inactivate resistant pathogens [8–10], novel therapeutic agents to combat resistant bacteria are still of high demand. Antimicrobial peptides (AMPs), or host defense peptides, have emerged as a new class of compelling antimicrobial agents [11–14]. These antibacterial compounds typically share several similar properties, such as cationicity, hydrophobicity, and amphiphilicity [12,15,16]. Most of AMPs kill bacteria through non-selective physical disruption of outer and/or cytoplasmic membrane of bacteria. However, the high cationicity, hydrophobicity, and amphiphilicity of AMPs might also give rise to increased toxicity to normal mammalian cells. One of the major challenges for the application of AMPs in clinical practice is to balance antibacterial activities and cytotoxicity to mammalian cells [17–20].

Bacterial production of β-lactamase leads to severe antibiotic resistance, which, on the other side, also offers a unique opportunity to specifically detect [21–26] or even inhibit [27–29] resistant microbes. Moreover, Devocelle and co-workers attempted to conjugate β-lactam with AMP as antibiotic prodrug to target re-

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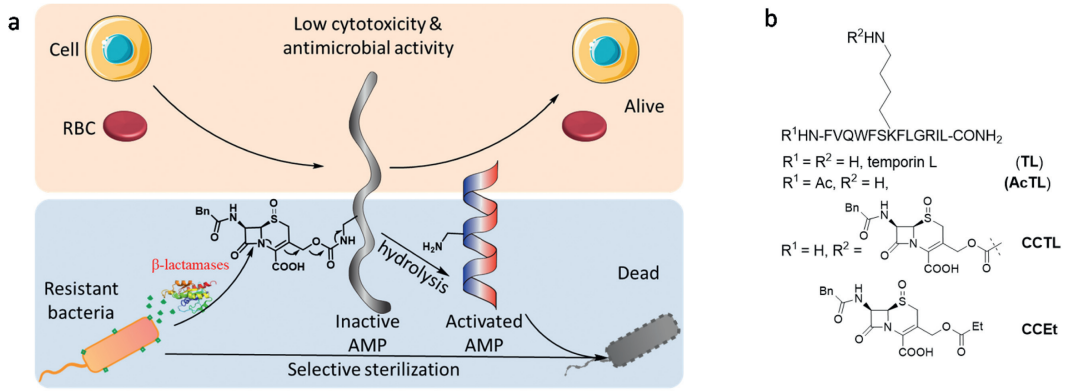


Fig. 1. (a) Schematic diagram of β-lactamase-activatable antimicrobial peptide (AMP) for the selective sterilization of resistant bacteria. (b) Chemical structures of AMPs and related compounds in this study.

sistant bacteria, but resulted in only marginal selectivity against β-lactamase-positive and -negative bacteria [30]. Herein, we report a novel β-lactamase-activatable AMP with high specificity against β-lactamase-producing resistant bacterial pathogens over susceptible microbes. This antibacterial activity of the AMP is initially masked by a cephalosporin group and shows massively reduced toxicity to mammalian cells but it turns highly active against bacteria upon specific hydrolysis by the β-lactamase produced by bacterial pathogens and thus selectively inactivates β-lactamase-producing resistant bacteria.

The bactericidal activity and cytotoxicity of AMPs depends on the charge, amphipathicity, hydrophobicity, and structural propensities. We conceived the introduction of bla-activatable caging group on one or two key side chains of AMP could switch these properties and thus reduce membrane damage of AMP to mammalian cells. Once the caging group is selectively removed by β-lactamase, the major causes for antibiotic resistance, free AMP is released and kills resistant bacteria with high specificity (Fig. 1a). In this study, we employed oxidized cephalosporin as the bla-responsive unit. The selection of oxidized form of cephalosporin over the native form of cephalosporin is mainly based on the following considerations: (1) the oxidized form of cephalosporin is more stable in physiological conditions than the native form of cephalosporin [21,31]; (2) the oxidized cephalosporin might have reduced antibacterial activity against cephalosporin-susceptible bacteria compared to the native form of cephalosporin; (3) the oxidation of cephalosporin effectively blocks the migration of double bond between Δ_2 and Δ_3 isomers in the presence of base and thus simplifies the synthesis of cephalosporin-peptide conjugate [21].

Temporin L (**TL**, FVQWFSKFLGRIL-NH_2, Fig. 1b) is a type of AMP found in the skin of European red frog *Rana temporaria* [32,33]. Previous studies have revealed the bactericidal activity of **TL** is associated with its positive charge and the α-helix structure [34]; the caging of the ε-amine residue of lysine might disrupt the α-helix structure [35,36] and thus leads to massive reduction of bactericidal activity. To test our β-lactamase-activatable AMP strategy, we used **TL** as a model AMP in this study.

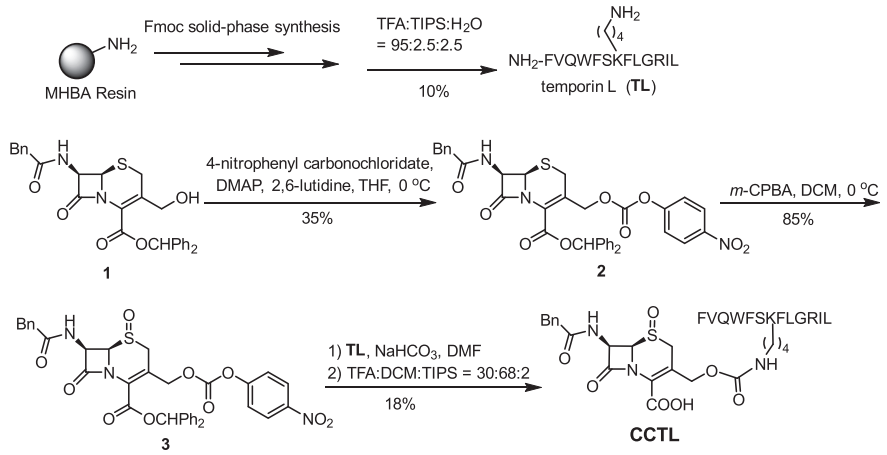
TL and its N-terminal acetylated analogue (**AcTL**) were readily prepared by Fmoc solid-phase peptide synthesis (SPPS) [33]. As depicted in Scheme 1, the cephalosporin-caged **TL** (**CCTL**, Fig. 1b) was easily synthesized by the conjugation of pre-activated cephalosporin **3** and protecting group-free **TL** in the presence of sodium bicarbonate followed by a trifluoroacetic acid (TFA)-mediated deprotection. It is worth noting that the N-terminal amine of **TL** is significantly less reactive compared to the ε-amine; selective protection of the N-terminal amine is not necessary for this reaction. Additionally, leaving group-bearing cephalosporin **CCEt** (Fig. 1b) was also prepared as a control compound.

The antimicrobial activity of peptide is closely related to its folding in solution. With **CCTL** in hand, we first investigated whether the caging of the ε-amino group of lysine on **TL** had any impact on the folding of peptide. We thus performed a circular dichroism (CD) study on both peptides, free **TL** and caged **TL** (**CCTL**). As exhibited in Fig. 2a, the CD spectra of **TL** in a DSPE solution showed a positive band at around 190 nm and two negative bands at 209 and 222 nm, respectively, confirming the formation of α-helix structure in solution. In stark contrast, the CD spectra of **CCTL** displayed a much weaker absorption under identical conditions, suggesting this compound might not form a α-helix structure in solution or at a much lower degree. These results demonstrate the introduction of a single caging group on the ε-amino group of **TL** could dramatically affect the original secondary structure of peptide in solution.

New Delhi metallo-β-lactamase 1 (NDM-1) hydrolyzes all types of β-lactam-based antibiotics with high efficiency. We incubated **CCTL** with this enzyme in PBS (pH 7.4) and analyzed by HPLC. As shown in Fig. 2b, the treatment of **CCTL** with NDM-1 led to disappearance of **CCTL**, leaving uncaged **TL** as the major product. Additionally, time-course hydrolysis of **CCTL** in the presence of NDM-1 was also monitored by HPLC (Fig. 2c and Fig. S1 in Supporting information), which indicates over 80% of **CCTL** has been hydrolyzed by NDM-1 within 0.5 h.

Moreover, other β-lactamases, such as KPC-2 (class A bla), VIM-1 (class B bla), AmpC (class C bla) and OXA-1 (class D bla), were also tested with **CCTL** and all resulted in the release of free **TL** as the major product (Fig. 2d). In contrast, the incubation of **CCTL** with other proteins, for instance, catalytic alkaline phosphatase (ALP), lipase or non-catalytic bovine serum albumin (BSA), gave rise to no free **TL** at all, demonstrating the high specificity of **CCTL** to β-lactamases over other proteins.

One of the major concerns for the application of AMPs in clinical practice is the toxicity to mammalian cells. To assess the biocompatibility of **CCTL**, we performed a live/dead staining assay using Calcein AM and propidium iodide (PI). As exhibited in Fig. 3a, upon incubation with 30 μmol/L of **CCTL** for 1 h, the Calcein AM-treated Human Embryonic Kidney 293 (HEK 293) cells emitted strong green fluorescence as the **CCTL**-free cells while the PI-treated cells were fluorescently silent, suggesting **CCTL** is compatible with HEK 293 cells. In sharp contrast, under identical circumstances, the **TL**-incubated HEK 293 cells generated strong red fluorescence but with basically no green fluorescence emission, indicating there were hardly any living cells left. Additionally, MTT assay was also performed to probe the cytotoxicity of



Scheme 1. Preparation of **CCTL**.

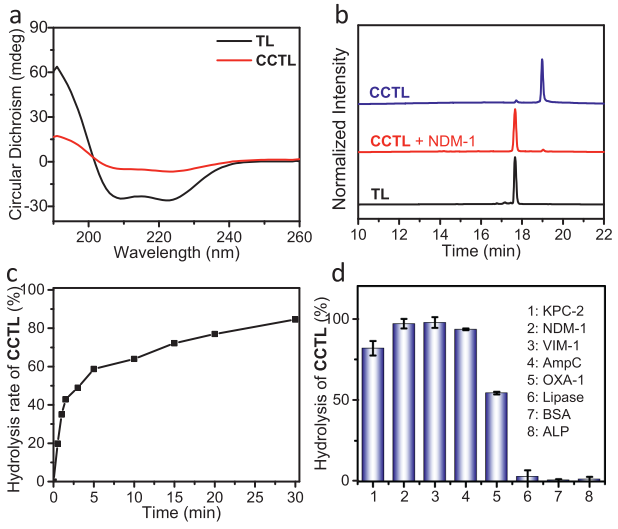


Fig. 2. *In vitro* characterization of cephalosporin-caged AMP **CCTL**. (a) CD spectra of **TL** and **CCTL** in a DSPE solution at r.t. (b) HPLC traces of **CCTL** before and after incubation with NDM-1 (20 nmol/L) at 37 °C for 2 h. (c) Hydrolysis of **CCTL** incubated with NDM-1 (3 nmol/L) at 37 °C in a period of 0.5 h. (d) HPLC-analyzed hydrolysis of **CCTL** upon incubation with a variety of proteins for 2 h at 37 °C. 1: KPC-2 (10 nmol/L); 2: NDM-1 (10 nmol/L); 3: VIM-1 (10 nmol/L); 4: AmpC (10 nmol/L); 5: OXA-1 (10 nmol/L); 6: lipase (1 U/mL); 7: BSA (100 nmol/L); 8: ALP (1 U/mL). DSPE: 1,2-distearoyl-*sn*–glycero-3-phosphoethanolamine. Data are the average of two experiments. Error bars are ± s.d.

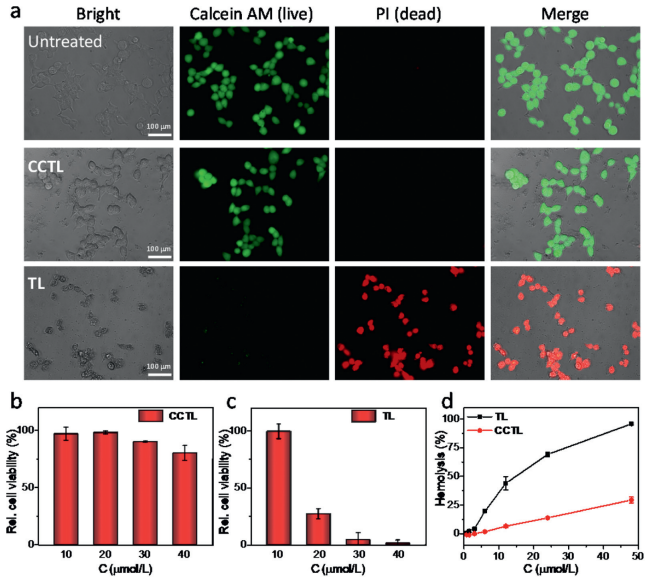


Fig. 3. *In vitro* assessment of biocompatibility of **CCTL** and **TL**. (a) Microscopic fluorescence images of **CCTL** or **TL** (30 μmol/L)-treated HEK293 cells after staining with Calcein-AM (green: live) and PI (red: dead). Relative viability of HEK 293 cells after incubation with (b) **CCTL** and (c) **TL** for 24 h. (d) Hemolysis test of **CCTL** and **TL** on human red blood cells (hRBCs). Data are the average of three experiments. Error bars are ± s.d.

CCTL, which gave comparable results as the live/dead staining assay: **CCTL**, showed significantly less impact on HEK293 cells compared to free **TL** (Figs. 3b and c). Additionally, the hemolysis test of **TL** and **CCTL** on human red blood cells (hRBCs) revealed the caging of **TL** with cephalosporin significantly reduced the hemolytic activity (Fig. 3d).

We then moved to study the antibacterial activity of this compound. We first measured the minimum inhibitory concentration (MIC) of **CCTL**, as well as **TL**, against susceptible *Escherichia coli* (*E. coli*, Gram-negative bacteria). As illustrated in Table 1, **TL** inhibited *E. coli* with a MIC of 6 μmol/L [33]. However, the caged **TL**, **CCTL**, hardly inhibited the growth of *E. coli* (MIC > 48 μmol/L), manifesting the caging of ε-amino group on **TL** reduces its antimicrobial ability significantly. The control compound, cephalosporin **CCEt**, was ineffective to suppress the growth of *E. coli* (MIC > 48 μmol/L). Moreover, the MIC of N-terminal acetylated **TL**, **AcTL**, against *E. coli* were also tested and it turned out the MIC of **AcTL** was over 4-fold higher than that of **TL** (> 24 μmol/L *vs.* 6 μmol/L).

Table 1
Antibacterial activity of caged AMPs against *E. coli* (μmol/L).

Compounds	β-lactamase negative *E. coli*		NDM-1-expressing *E. coli*[a]	
	MIC	MBC	MIC	MBC
TL	6	6	6	6
AcTL	>24	>24	>24	>24
CCTL	>48	>48	12	12
CCTL+NDM-1	12	12	6	6
CCEt	>48	>48	>48	>48
Meropenem	0.08	0.08	>130	>130

MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration.
[a] *E. coli* transformed with NDM-1-encoded plasmid.

We next determined the minimum bactericidal concentrations (MBCs) of these compounds against the same bacterial strain and comparable results were obtained: caged **CCTL** resulted in significantly higher MBC against susceptible *E. coli* compared to free **TL** (> 48 μmol/L *vs.* 6 μmol/L).

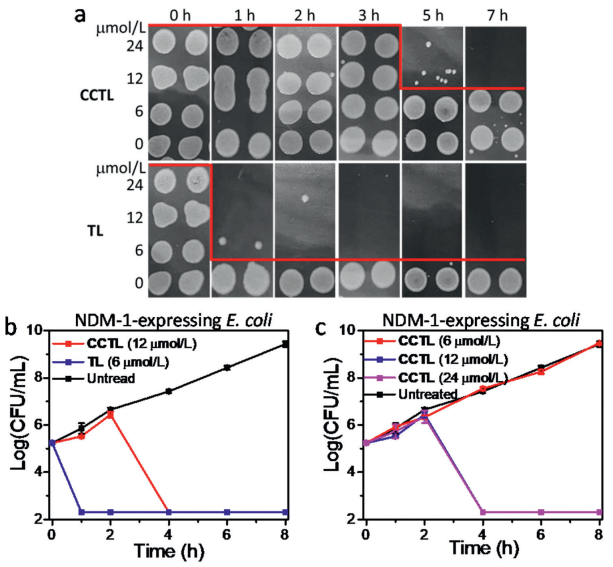


Fig. 4. Time-kill kinetics of **CCTL** against NDM-1 expressing *E. coli*. (a) Colony images of NDM-1 expressing *E. coli* upon incubation with serial concentrations of **CCTL** or **TL** in Mueller–Hinton Broth (MHB) for indicated time points. (b) Time-kill curves of **CCTL** or **TL** and (c) time-kill curves for **CCTL** at indicated concentrations against NDM-1 expressing *E. coli*. Data are the average of three experiments. Error bars are ± s.d.

On the other hand, the addition of NDM-1 effectively resumed the antibacterial activity of **CCTL** against susceptible *E. coli*: both the MIC and MBC of **CCTL** dropped to 12 μmol/L from > 48 μmol/L in the presence of NDM-1 (3 nmol/L). These results clearly demonstrate antimicrobial resistance-causative-β-lactamase could effectively revive the antibacterial activity of caged AMPs.

Encouraged by these results, we conducted further investigation to reveal the antibacterial activity of **CCTL** against β-lactamase-producing resistant microbes. We employed NDM-1-transformed *E. coli* [25] as a model resistant bacterium. Notably, the expression of NDM-1 in bacteria dramatically increases its resistance towards meropenem (MIC: 0.08 μmol/L *vs.* > 130 μmol/L). The measurement of MIC and MBC of **CCTL** revealed that this compound, despite low activity against susceptible *E. coli*, inactivates NDM-1-producing bacteria effectively with the MIC and MBC close to those of free **TL**. Given the fact that a large number of β-lactamases are plasmid-encoded enzymes, the selective inhibition of bla-expressing bacteria over β-lactam-susceptible bacteria might be of help to generate a selection pressure to reverse bacterial resistance to β-lactam antibiotics, and thus maintain the efficacy of the most widely used antibiotics.

To gain further information on the bactericidal kinetics of **CCTL**, we incubated NDM-1-expressing *E. coli* with serial concentrations of **CCTL** and investigated its sterilization activity by placing an aliquot of diluted sample on a LB Agar plate at different time points. As shown in Fig. 4, cephalosporin-caged **CCTL**, unlike its free analogue **TL**, which displayed its bactericidal activity immediately, killed resistant bacteria in a delayed manner. The delay of antibacterial activity for **CCTL** obviously is due to the time needed for the β-lactamase-mediated hydrolysis of the caged peptide.

Besides resistant model bacteria, we further examined the bactericidal kinetics of **CCTL** with clinically occurring resistant microbes. Three strains of clinically important β-lactamase-expressing pathogenic bacteria were selected for this test, including, KPC-2-expressing *E. coli*, NDM-1-expressing *K. pneumoniae*, and multi-drug resistant *A. baumannii*. All of these bacterial pathogens are resistant to most β-lactam antibiotics, particularly carbapenems. As illustrated in Fig. S3 (Supporting information), 12 μmol/L or 24 μmol/L of **CCTL** selectively inhibited KPC-2-expressing *E. coli* within 2 h albeit with little impact on the growth of β-lactamase-negative *E. coli* (ATCC 25,922). As expected, the addition of avibactam, an FDA-approved inhibitor for KPC-2, blocked the antibacterial activity of **CCTL** against KPC-encoded *E. coli*, demonstrating the dependence of bactericidal activity of **CCTL** on the bacterial production of β-lactamases. As a control, free **TL** killed both resistant and susceptible bacteria. What's more, the test of **CCTL** with NDM-1-expressing *K. pneumoniae* and multi-drug resistant *A. baumannii* resulted in similar outcomes (Fig. S3 in Supporting information): **CCTL** effectively killed resistant bacteria in a delayed manner.

The MICs of **CCTL** against KPC-2-expressing *E. coli*, NDM-1-expressing *K. pneumoniae,* multi-drug resistant *A. baumannii* and AmpC-expressing *E. cloacae* have been determined. As shown in Table S1 (Supporting information), **CCTL** could inhibit the growth of KPC-2-expressing *E. coli* and AmpC-expressing *E. cloacae,* but with less efficiency compared to **TL**. This might associate with the relatively low expression of β-lactamase by these resistant bacteria.

To assess the stability of **TL** and **CCTL** in serum, we determined the MICs of these compounds against bla-expressing bacteria in the presence of 10% fetal bovine serum (FBS). As illustrated in Table S2 (Supporting information), the addition of FBS decreased the antibacterial activity of **TL** and **CCTL**. This might result from their relatively low stability in serum, which might be improved by structural modifications and the replacement of unnatural amino acids or D-amino acids.

In summary, we have developed a novel cephalosporin-caged antimicrobial peptide (AMP) for the selective inhibition of resistant bacterial pathogens. As a proof of concept, we have demonstrated the introduction of cephalosporin to AMP significantly disrupts the α-helix structure of AMP in aqueous solution and thus massively reduces the cytotoxicity and hemolytic activity, as well as antibacterial activity against β-lactam-susceptible bacteria. The antibacterial activity of the cephalosporin-caged AMP is selectively unmasked by the antibiotic resistance-causative β-lactamase and thus inactivates resistant bacteria with high specificity. Further optimization of the structure of antimicrobial peptide to enhance its antibacterial activity and a more thorough biological study on this cephalosporin-conjugated peptide, including the stability in serum, are still under investigation. Moreover, this strategy should be extendable to other AMPs, providing a potential solution for the treatment of infectious diseases caused by β-lactamase-producing pathogens. Moreover, the selective inhibition of β-lactamase-expressing microbes by cephalosporin-caged AMPs might potentially reverse the selection pressure caused by the wide application of β-lactam antibiotics and thus reduce antimicrobial resistance.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.107847.

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