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Highly selective and wash-free visualization of resistant bacteria with a relebactam-derived fluorogenic probe†

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Reported herein is a relebactam-derived fluorogenic reagent for covalent labeling of serine β-lactamases (SBLs), which are the major causes of bacterial resistance to β-lactam antibiotics. This highly selective imaging reagent generates over 300-fold stronger near-infrared fluorescence signals upon covalently bonding to SBLs, allowing wash-free visualization of live antimicrobial-resistant bacteria.

Bacterial resistance to β-lactam antibiotics, which massively undermines the efficacy of this class of most widely used therapeutic agents for infectious diseases, has been a major threat to public health globally.[1] The production of β-lactamases (blas), a class of β-lactam-hydrolyzing enzymes, by Gram-negative and positive bacteria plays a major role in allowing bacteria to survive β-lactam antibiotic therapy.[2]

So far, over 1800 β-lactamases have been identified.[3] On the basis of a hydrolysis mechanism, these enzymes can be grouped as serine β-lactamase (SBLs) and metallo β-lactamases (MBLs, Ambler class B), and SBLs (including Ambler class A, class C, and class D) are the most commonly encountered types of β-lactamases in pathogenic bacteria.[4] For Gram-negative bacteria, β-lactamases are mostly present in the periplasmic space, and β-lactamases of Gram-positive bacteria are either bound to the cytoplasmic membrane or excreted.[2a]

Early identification of resistant bacteria is crucial for therapy of infectious diseases and the prevention of the spreading of antibiotic resistance. The presence of β-lactamases, on the other hand, offers a great opportunity for rapid detection of resistant pathogenic bacteria,[5] or even selective release of therapeutic agents.[6]

Fluorescent substrates of β-lactamases are capable of detecting enzymatic activities with high sensitivity and have emerged as powerful tools in the screening of antibiotic resistance.[7] Currently, the majority of these sensors employ substituted cephalosporin or carbapenem as the enzymatic recognition motif; hydrolysis of the β-lactam ring leads to release of the fluorophore and thus turns on a fluorescent signal.

This strategy, though efficient, is somewhat limited by the diffusion of the activated fluorophore from resistant bacteria. To address this challenge, Xing's lab and our lab have independently applied the chemistry of quinone methide to develop self-immobilizing probes for the detection of β-lactamase activities.[8] Furthermore, lipids were also introduced to a β-lactamase probe to facilitate the localization of activated fluorophores on the surface of resistant bacteria.[9] Recently, Rao's group reported a dual targeting fluorogenic probe for the highly specific detection of the activities of *Mycobacterium tuberculosis* (*Mtb*) β-lactamase (BlaC), in which nitrophenyl was incorporated to anchor on the surface of the microbe.[10] Herein, with relebactam as an enzymatic recognition moiety, we wish to report a highly selective fluorogenic probe for the wash-free visualization of single live SBL-expressing bacteria.

Relebactam, an analogue of avibactam, is a non-β-lactam inhibitor of β-lactamases with broad inhibition spectrum; it can readily reduce the activity of SBLs, including class A, class C, and class D β-lactamases, and thus restores the activity of β-lactam antibiotics.[11] Similar to avibactam, relebactam is believed to inhibit the activities of β-lactamase by forming a covalent bond with the enzyme.[12] More importantly, relebactam has a free amino group far away from the active site; chemical modification of this amino group may generate little interference on its ability to form a covalent bond with SBLs. We envisaged a relebactam-derived fluorescent sensor might be capable of capturing SBLs and thus selectively labeling resistant bacilli (Fig. 1).

To test the feasibility of our hypothesis, we commenced our investigation by the synthesis of a fluorescein-tethering relebactam probe (RLB-1). As shown in Scheme S1 (ESI†), the synthesis of RLB-1 was achieved in two steps from commercially available relebactam: a simple condensation reaction followed by a CuAAC click reaction.[13]

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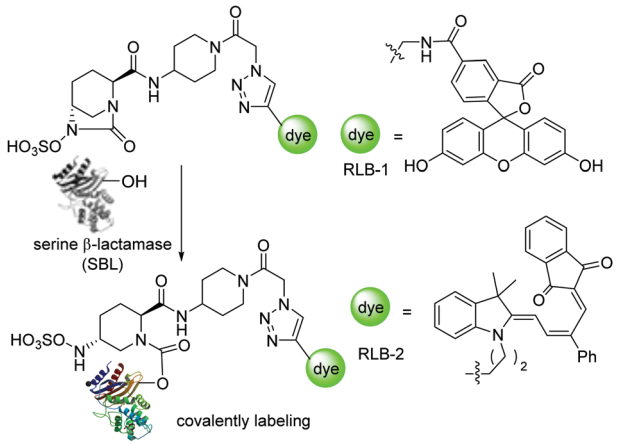

Fig. 1 Design of relebactam-based covalent labeling reagents for serine β-lactamase.

With RLB-1 in hand, we first examined whether this fluorescein-tethering relebactam maintained its high inhibition efficiency against SBLs. TEM-1 bla (class A β-lactamase) is among the most common β-lactamases found in Gram-negative bacteria. To investigate the inhibition activity of RLB-1, we used recombinant TEM-1 bla as a model enzyme and fluorogenic CDC-1[7e,14] as a substrate. As shown in Fig. 2a, the addition of as low as 1 μM of RLB-1 led to over 99% inhibition of TEM-1 bla activity, suggesting that the introduction of fluorophore to relebactam had an insignificant effect on its inhibition activity to SBLs.

Encouraged by these results, we further investigated the labeling ability of RLB-1 to SBLs. We incubated TEM-1 bla with RLB-1 and then ran polyacrylamide gel electrophoresis (PAGE) analysis. As shown in in-gel fluorescence imaging (Fig. 2b and c), we observed strong green fluorescence from the RLB-1-incubated TEM-1 bla. The addition of avibactam, an efficient SBL inhibitor, dramatically reduced the fluorescence signal on the RLB-1-treated TEM-1 bla.

To further reveal the labeling specificity of RLB-1 to TEM-1 bla, we mixed RLB-1 and TEM-1 bla along with a large quantity of lysate of *E. coli*. In-gel fluorescence analysis indicated that only TEM-1 bla was marked with strong green fluorescence whereas all of the other proteins remained dark, which further confirms the high labeling specificity of RLB-1 to TEM-1 bla. And the fluorescent labeling of TEM-1 bla was a dose-dependent process; higher concentration of RLB-1 led to stronger fluorescence on the protein until it was saturated (Fig. 2d and e).

Moreover, to validate the formation of a covalent bond between TEM-1 bla and RLB-1, we analyzed the RLB-1-incubated TEM-1 bla with mass spectrometry (ESI). As illustrated in Fig. 2f, TEM-1 bla alone showed a molecular ion peak at 29983.8 whereas the RLB-1-treated sample peaked at 30828.0; the mass difference between these two samples is 844.2, which matches perfectly the molecular weight of RLB-1. These results unambiguously demonstrated the formation of a covalent bond between this small ligand and the enzyme.

Fluorescent sensors that are able to significantly increase fluorescence intensity upon covalently bonding to target proteins are particularly attractive due to the ability to reduce the background signal and thus enhance the detection sensitivity. With this mind, we replaced the fluorescein of RLB-1 with a switchable near-infrared dye (P-Mero4)[15] to get a near-infrared labeling reagent RLB-2.

We next assessed the labeling efficiency of RLB-2 to TEM-1 bla by in-gel fluorescence analysis as described above, and it turned out that this molecule could also selectively capture TEM-1 bla in the presence of a large amount of *E. coli* lysate, meaning the replacement of fluorescent dye had little interference on the protein labeling (Fig. 3a and b).

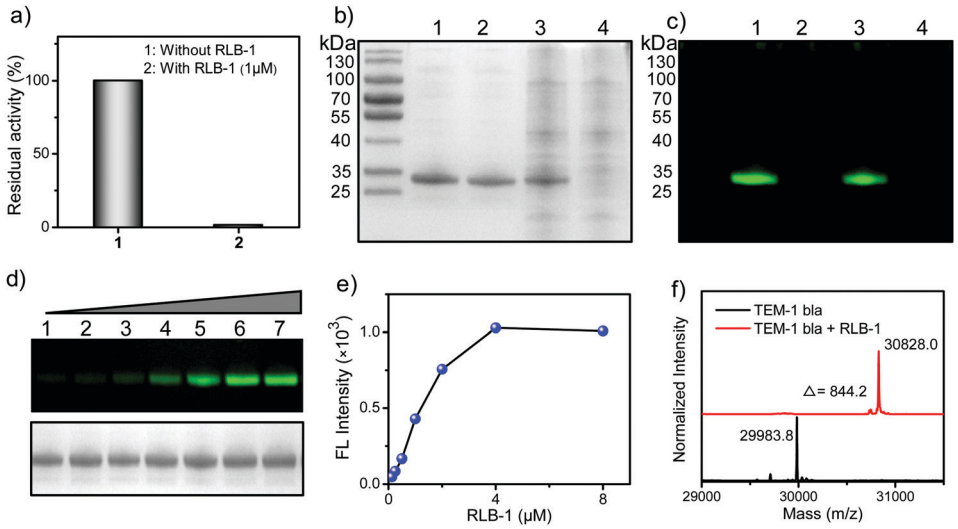

Fig. 2 Covalent labeling of recombinant TEM-1 bla with RLB-1. (a) Inhibition of TEM-1 bla activity by RLB-1, monitored by fluorogenic substrate CDC-1. (b) Coomassie blue staining and fluorescent images (c) of TEM-1 bla upon treatment with RLB-1. 1: TEM-1 bla + RLB-1; 2: TEM-1 bla + RLB-1 + avibactam; 3: TEM-1 bla + RLB-1 + lysate of *E. coli*; 4: lysate of *E. coli* + RLB-1, $\lambda_{ex}/\lambda_{em}$ = 365/535 nm; (d) fluorescent (top) and Coomassie blue staining (bottom) images of TEM-1 bla after incubation with a variety of RLB-1 concentrations (0.125, 0.25, 0.5, 1, 2, 4, and 8 μM). (e) Plot of integrated fluorescence intensity on (d) *versus* concentration of RLB-1. (f) Mass spectrum of TEM-1 bla before and after incubation with RLB-1.

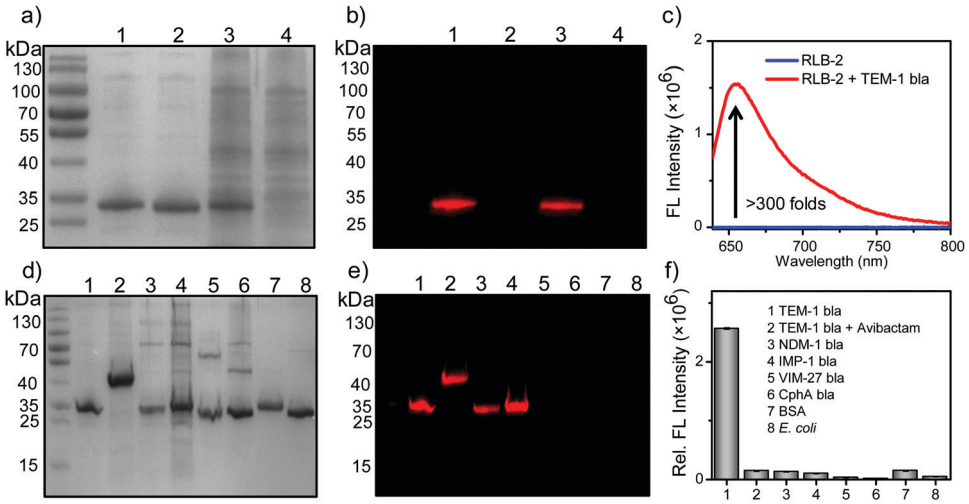

Fig. 3 Labeling and fluorescence response of RLB-2 to SBLs. (a) Coomassie blue staining and (b) fluorescent images of TEM-1 bla upon treatment with RLB-2. 1: TEM-1 bla + RLB-2; 2: TEM-1 bla + RLB-2 + avibactam; 3: TEM-1 bla + RLB-2 + lysate of *E. coli*; 4: lysate of *E. coli* + RLB-2. $\lambda_{ex}/\lambda_{em}$ = 685/720 nm. (c) Fluorescence spectrum of RLB-2 before and after incubation with TEM-1 bla. (d) Coomassie blue staining and (e) fluorescent images of a range of β-lactamases upon treatment with RLB-2. 1: TEM-1 bla; 2: AmpC bla; 3: KPC-2 bla; 4: OXA-1 bla; 5: NDM-1 bla; 6: IMP-1 bla; 7: VIM-27 bla; 8: CphA bla. (f) Fluorescence enhancement of RLB-2 after incubation of a variety of proteins (1 μM) or live bla-negative *E. coli* (1×10^8 cfu mL^{-1}).

Relebactam has been known as an efficient inhibitor for a wide range of SBLs. To investigate the labeling profile of the relebactam-derived RLB-2, we incubated RLB-2 with a number of β-lactamases, including TEM-1, KPC-2 (class A), AmpC (class C), and OXA-1 β-lactamase (class D), as well class B β-lactamases (NDM-1, IMP-1, VIM-27, and CphA). In-gel fluorescence imaging (Fig. 3d and e) disclosed that all of the SBLs, including class A, C, and D β-lactamases, were readily labeled by RLB-2, emitting a strong NIR fluorescence signal. And, in sharp contrast, all of the tested MBLs (*i.e.*, class B β-lactamases) remained non-fluorescent, highlighting the specificity of RLB-2 to SBLs.

Unlike fluorescein, P-Mero4 is a fluorogenic fluorophore upon interaction with protein. To examine the fluorogenic ability of RLB-2 to β-lactamases, we recorded the fluorescence spectrum of RLB-2 before and after incubation of TEM-1 bla. As shown in Fig. 3c, RLB-2 in phosphate buffered saline (PBS, pH 7.4) alone was basically non-fluorescent while the addition of TEM-1 bla led to a dramatic enhancement of the fluorescence intensity at 655 nm; the fluorescence turn-on ratio was more than 300 folds, which is consistent with previous reports.[15] As expected, the addition of avibactam, SBL inhibitor, to TEM-1 bla before incubation with RLB-2 blocked the covalent interaction of RLB-2 with TEM-1 bla, significantly reducing the ratio of fluorescence enhancement (Fig. 3f). Moreover, the incubation with MBLs (*e.g.*, NDM-1, IMP-1, VIM-27, and CphA), as well as an equal amount of bovine serum albumin (BSA), with RLB-2 only led to an insignificant increase of fluorescence, suggesting that the turn-on of fluorescent signal was mainly induced by the formation of a covalent bond between RLB-2 and protein, instead of non-specific binding. More importantly, RLB-2 was also inert to live β-lactamase-negative *E. coli*, resulting in little fluorescence enhancement.

SBLs are among the major causes for pathogenic bacteria to resist the presence of β-lactam antibiotics. For instance, the expression of chromosomal AmpC in *Enterobacter cloacae* (*E. cloacae*) accounts for the bacterial resistance to β-lactam antibiotics. Having demonstrated the covalent labeling of RLB-2 to SBLs, we wondered whether this reagent could be used in the fluorescent labeling of live resistant bacteria. We incubated AmpC-expressing *E. cloacae* (ATCC BAA-1143) with RLB-2 and then applied fluorescence microscope imaging. RLB-2 itself is hardly fluorescent and it only turns fluorescent after covalently bonding to SBLs, which allowed us to image individual live resistant bacteria without washing away unbound probe. As depicted in Fig. 4, upon incubation with RLB-2, strong fluorescence was observed from the AmpC-expressing *E. cloacae* even without washing. As a control, the pretreatment of bacteria with SBL inhibitor, avibactam, resulted in nearly no fluorescent signal on bacteria. Furthermore, under identical circumstances, the β-lactamase-negative *Escherichia coli* (*E. coli*) remained non-fluorescent. These results have demonstrated that the relebactam-derived RLB-2 has the ability to capture and visualize SBL-expressing bacteria.

In summary, this study reports an unprecedented relebactam-derived probe for covalent labeling of all serine β-lactamases, including class A, C, and D β-lactamases. This imaging reagent exhibited excellent selectivity over other proteins found in susceptible bacteria. And the use of near-infrared P-Mero4 as a fluorescent reporter led to a fluorogenic probe; the fluorescence turn-on ratio can be over 300 folds upon covalent binding to serine β-lactamases. The applicability of this reagent has been illustrated by the wash-free imaging of serine β-lactamase-expressing live bacteria. Moreover, this study has demonstrated that relebactam may be used as a highly selective ligand to target serine β-lactamase-expressing bacteria, which may find great value in targeting drug delivery against infectious diseases caused by a resistant microbe.

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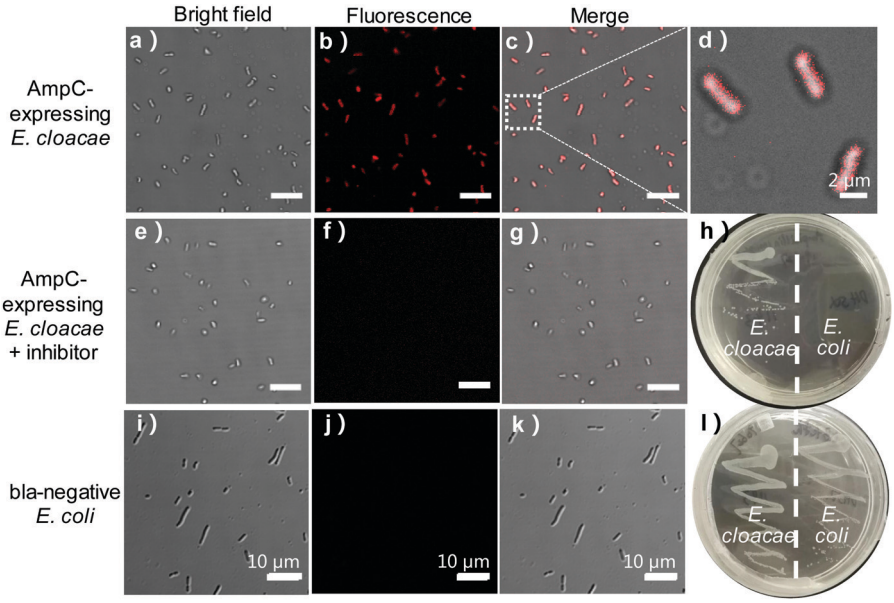

Fig. 4 Wash-free fluorescence microscope images of AmpC-expressing *Enterobacter cloacae* with RLB-2. *Enterobacter cloacae* (ATCC BAA-1143) or bla-negative *E. coli* (DH5α) was incubated with RLB-2 (1 μM) in the absence or presence of avibactam (50 μM) for 3 h before imaging (a–g and i–k). (h) and (l) Images of resistant *E. cloacae* (left) and susceptible *E. coli* (right) in LB plates with (h) or without (l) 100 μg mL^{-1} of Ampicillin.

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Conflicts of interest

There are no conflicts to declare.

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