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Rapid point-of-care detection of the tuberculosis pathogen using a BlaC-specific fluorogenic probe

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Abstract

Early diagnosis of tuberculosis can dramatically reduce both its transmission and the associated death rate. The extremely slow growth rate of the causative pathogen, *Mycobacterium tuberculosis* (*Mtb*), however, makes this challenging at the point of care, particularly in resource-limited settings. Here we report the use of BlaC (an enzyme naturally expressed/secreted by tubercle bacilli) as a marker and the design of BlaC-specific fluorogenic substrates as probes for *Mtb* detection. These probes showed an enhancement by 100–200 times in fluorescence emission on BlaC activation and a greater than 1,000-fold selectivity for BlaC over TEM-1 β -lactamase, an important factor in reducing false-positive diagnoses. Insight into the BlaC specificity was revealed by successful co-crystallization of the probe/enzyme mutant complex. A refined green fluorescent probe (CDG-OMe) enabled the successful detection of live pathogen in less than ten minutes, even in unprocessed human sputum. This system offers the opportunity for the rapid, accurate detection of very low numbers of *Mtb* for the clinical diagnosis of tuberculosis in sputum and other specimens.

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Tuberculosis is one of the most deadly diseases that kills over one million people each year and infects one-third of the world's population¹. The disease is spread by infection with *Mycobacterium tuberculosis* (*Mtb*). Owing to its airborne transmission, early diagnosis is critical to the prevention and control of TB. Standard diagnostic methods, acid-fast smear from sputum, often do not become positive until after transmission occurs, which allows the spread of the disease. Culture-based techniques are more sensitive, but take weeks to obtain results because of the extremely slow growth rate of *Mtb*. Recently, nucleic acid-based diagnostic strategies were shown to have a higher sensitivity than that of sputum smear microscopy^{2–4}. With a semi-automated DNA amplification system (Cepheid GeneXpert), it is thought that TB can be diagnosed with 98% reliability⁵. However, its high cost⁶ and requirement for a high technical competence to carry out the test prevent it from being widely available in resource-limited settings. In addition, the sensitivity of this test has not reached that of the gold standard culture, and it cannot evaluate bacterial viability, a critical aspect of evaluating therapeutic outcome. TB eradication efforts would be facilitated greatly by methods that can detect tubercle bacilli in a sensitive, rapid, specific and quantitative manner *in vitro* at a low cost, particularly in resource-limited settings where TB is the most prevalent.

Tubercle bacilli naturally express BlaC, an enzyme that belongs to the class A β -lactamase family^{7,8}. Extended spectrum class A β -lactamases are capable of hydrolysing all classes of β -lactam substrates, including cephalosporins. The mechanism of cephalosporin hydrolysis by β -lactamases yields hydrolysed β -lactam and, more importantly, may be concomitant with the loss of a 3' leaving group^{9–12}. Based on this mechanism, a number of fluorogenic and bioluminogenic probes were developed for the detection of β -lactamase activity *in vitro*, in living cells and even in whole animals^{13–17}. We developed cephalosporin-based fluorogenic substrates that enable the sensitive detection of *Mtb* and bacillus Calmette–Guérin (BCG) *in vitro* and in living mice¹⁸. Previous probes lack specificity for BlaC in *Mtb*; the common TEM-1 β -lactamase (TEM-1 Bla) in gram-negative bacteria can also generate fluorescence, which reduces its utility for TB diagnosis. Earlier probes are generally large and display slow hydrolytic kinetics for BlaC. Here we report a rational design of a series of fluorescent probes based on chemically modified cephalosporins by taking advantage of the unique flexibility of the BlaC substrate-specificity loop. Enzymatic kinetic, structural analyses and whole-cell assays confirmed their high specificity and sensitivity for BlaC over its close class A homologue TEM-1 Bla as well as over β -lactamases produced by *Pseudomonas*, *Staphylococcus* and the environmental mycobacterium *M. smegmatis*. Successful detection and imaging of BCG directly in unprocessed patient sputum at levels of less than 100 bacilli are demonstrated, even with a simple, inexpensive imaging system of a cellular phone. This new BlaC-specific probe has potential for the clinical diagnosis of *Mtb* in patient sputum and other diagnostic specimens. Structural insights obtained from the BlaC acyl intermediates in this work should facilitate the development of more BlaC-specific probes for *Mtb* detection and imaging, and of BlaC-specific inhibitors to be used in anti-*Mtb* therapeutic regimens.

Results

Design of BlaC-specific fluorogenic probes

The substrate specificity of class A β -lactamases is conferred by Ambler residues (163–178) that comprise the omega loop, or substrate-specificity loop, and is partially dependent on the ability of the catalytic base, E166, to assume the optimal conformation for hydrolytic water coordination and deacylation^{19,20}. To design a cephalosporin substrate specific for BlaC to achieve the specific detection of *Mtb*, we exploited the unique flexibility of the BlaC substrate-specificity loop by introducing bulky substitutions on the lactam. Such a cephalosporin may be accommodated by the BlaC substrate-specificity loop with the coordination of E166 still optimal for efficient deacylation.

We designed a series of fluorescent probes with substitutions on the side chain (R^1) of the 7-amino group or the 7-position of the lactam ring (R^2) (Fig. 1). Each probe contains the alkylated umbelliferone at the 3'-position and initially fluoresces little when excited at 400 nm. We hypothesized that these substituted substrates could be hydrolysed readily by BlaC and release free fluorophore to turn on the fluorescence, but their hydrolysis by other β -lactamases, such as TEM-1 Bla, would proceed with much slower kinetics. These blue fluorescent probes were synthesized^{21,22} as outlined in Supplementary Fig. S1 for **CDC-1** and **CDC-OMe**, and unambiguously characterized (see the Supplementary Information).

Enzymatic kinetics for BlaC and TEM-1 Bla

We first recorded the fluorescent spectra of **CDC-1**, **CDC-3**, **CDC-OMe** and **CDC-OEt** before and after BlaC treatment to examine their responses to BlaC. With excitation at 400 nm, all the probes exhibited over a 100-fold increase in the fluorescence intensity at 455 nm after incubation with BlaC (Fig. 2a), but their reaction rates varied significantly (Fig. 2b and Supplementary Fig. S2). Under the same conditions, the hydrolytic rate by BlaC decreased in the order **CDC-1** > **CDC-OMe** > **CDC-OEt**, which appears to correlate with the size of the R^2 group. **CDC-OMe** showed more than a 30-fold enhancement of fluorescence intensity in less than 30 minutes. The larger substitution (ethoxy versus methoxy) provided **CDC-OEt** with only a slight fluorescence enhancement within 30 minutes. In comparison, the same concentration of TEM-1 Bla (50 nM) gave no change in the fluorescence intensity within 30 minutes with **CDC-OMe**, but produced rapid fluorescence with **CDC-1** and **CDC-3**. These results demonstrate that **CDC-OMe** can preferentially detect BlaC over TEM-1 Bla.

We next examined the response of **CDC-OMe** to varying concentrations of BlaC and TEM-1 Bla (in the range 1 pM to 100 nM) to investigate its specificity (Fig. 2c). BlaC (1 nM, 100 μ l) induced a 130% increase in the fluorescence intensity within one hour ($F/F_0 = 2.3$), but a 100-fold higher TEM-1 Bla concentration (100 nM) produced only a 30% fluorescence enhancement ($F/F_0 = 1.3$). In contrast, TEM-1 Bla generated a stronger signal with **CDC-1** than with BlaC (Fig. 2d).

The kinetic parameters of fluorescent probes for both BlaC and TEM-1 Bla, including the catalytic constant k_{cat} and the Michaelis constant K_m , were obtained from Lineweaver–Burk

plots (Supplementary Fig. S3) and are summarized in Table 1. Consistent with the above observations, the kinetic efficiency ($k_{\text{cat}}/K_{\text{m}}$) of CDC-OMe for BlaC is $2.1 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$, over 1,000 times higher than that for TEM-1 Bla ($16 \text{ s}^{-1} \text{ M}^{-1}$). When an ethoxy group is introduced at the 7-position (**CDC-OEt**), no hydrolysis by TEM-1 Bla was detected, and the value of $k_{\text{cat}}/K_{\text{m}}$ by BlaC decreased substantially to $1.9 \times 10^2 \text{ s}^{-1} \text{ M}^{-1}$.

Additional structural modifications were explored for their effects on the enzymatic kinetics of BlaC and TEM-1 Bla. Probes with a large substitution group on the 7-amine position (R^1), such as **CDC-3**, displayed less catalytic efficiency for BlaC ($8.7 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$) than for TEM-1 Bla ($1.3 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$). However, substitution by phenylacetyl or acetyl groups (for example, **CDC-1** and **CDC-4**) at this position resulted in only a slight difference in the catalytic efficiency between BlaC and TEM-1 Bla (**CDC-1**, 2.1×10^5 versus $3.6 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$; **CDC-4**, 6.8×10^4 versus $7.5 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$, respectively). Oxidation of the sulfur into sulf-oxide (**CDC-2**) caused a decrease in the kinetic efficiency for both BlaC and TEM-1 Bla.

These kinetic analyses confirm **CDC-OMe** as a BlaC-specific fluorogenic probe with more than a 1,000-fold higher catalytic efficiency for BlaC than for TEM-1 Bla.

Analysis of the crystal structure

X-ray crystallographic structures of the acyl intermediate complex structures of **CDC-OMe** and **CDC-1** with BlaC were obtained to probe the structural origin of the observed specificity of **CDC-OMe** for BlaC (Table 1). The substrate-specificity loops of TEM-1 Bla and BlaC share a 67% sequence identity over 20 residues (160–180). Conserved salt bridges in both enzymes formed by D172–R178 (E171–R178 in TEM-1 Bla) and D176–R178 stabilize the loop conformers of the unoccupied active sites (Fig. 3a)^{23,24}.

The largest phenotypic difference between the two enzymes results from residue 164, which is an arginine in TEM-1 Bla and an alanine in BlaC. R164 plays a central role in the molecular dynamics of the substrate-specificity loop in TEM-1 Bla. The R164 guanidinium side-chain nitrogens ($\text{N}\epsilon$, NH and NH_2) stabilize a triad of carboxylates from the D176, D179 and E171 side chains with electrostatic interaction distances of 3.99 Å, 2.75 Å and 2.87 Å, respectively²⁴, by forming three additional salt bridges (Fig. 3b)^{23,24}.

A164 mutation results in the loss of these stabilizing salt bridges in BlaC, which ultimately increases the flexibility of the BlaC substrate-specificity loop relative to that of TEM-1 Bla. The increased flexibility is underscored by B-factors for residues that surround E166 (164–168), which are 108% of the mean B-factor in BlaC and 87% of the mean B-factor in TEM-1 Bla (refs 23,24). The structural plasticity of these residues allows the catalytic base E166 to sample multiple conformations and increases the capability of BlaC to hydrolyse substrates in the acyl intermediate state.

The acyl intermediate complex structures of BlaC–**CDC-1** and BlaC–**CDC-OMe** exist in similar orientations, and share conserved electrostatic interactions with substrate-recognition residues T235, T237, S130 and S70 (Fig. 4). The phenyl groups in both **CDC-1** and **CDC-OMe** are disordered as a result of the lack of active-site contacts and facing open to the

solvent. In contrast, **CDC-1** makes a unique hydrogen bond (2.76 Å) with the backbone carbonyl oxygen of T237. The methoxy group of **CDC-OMe** forms an electrostatic interaction with the side-chain amino group of K73 (3.29 Å). The orientation of the **CDC-OMe** 7'-phenylacetamino substituent is rotated approximately 90° relative to the **CDC-1** acyl intermediate (Fig. 4c). The **CDC-OMe**–K73 interaction positions the methoxy group directly in the path between the acyl bond and catalytic residue E166, which may interfere with the hydrolytic water coordination for deacylation and lead to a decreased turnover number for **CDC-OMe** (Table 1). Replacement of the methoxy group with a bulkier ethoxy group (**CDC-OEt**) causes a more serious occlusion, which leads to a further 40-fold reduction in turnover number (Table 1). However, these unique interactions in the **CDC-OMe** acyl intermediate accommodated by the BlaC flexible substrate-specificity loop confer the specificity of **CDC-OMe** for BlaC (Fig. 3c,d).

Detection and imaging of live mycobacteria

We first sought to evaluate the feasibility of **CDC-OMe** for the detection of BlaC expressed by *Escherichia coli*. **CDC-OMe** was incubated with the same number of *E. coli* that expressed no β -lactamase, TEM-1 Bla or BlaC (Supplementary Fig. S5) for two hours and a strong fluorescence was observed only with *E. coli* that expressed BlaC. As a control, the fluorescence for **CDC-1** was present in *E. coli* that expressed either TEM-1 Bla or BlaC. This result demonstrates that **CDC-OMe** can detect BlaC specifically in intact *E. coli*. However, the sensitivity for detecting BlaC expressed by the *Mtb* var. *bovis* strain BCG is low because of the high background of BCG at the excitation and emission wavelengths of umbelliferone. Therefore, we replaced umbelliferone with the green fluorescent dye Tokyo Green (Fig. 5a). Tokyo Green allows a stable single-site attachment at its phenolic position²⁵, but the direct coupling product with cephalosporin at the 3'-position, similar to that in **CDC** probes, displayed a 21-fold less stability (with a spontaneous hydrolysis rate of $7 \times 10^{-6} \text{ s}^{-1}$ in PBS) than that of **CDC-OMe**. A benzyl ether linker was thus introduced between the 3'-position of the lactam and Tokyo Green to increase its stability (Fig. 5a). The syntheses of green fluorogenic substrates **CDG-1** and **CDG-OMe** are outlined in Supplementary Fig. S6.

On treatment with BlaC, the fluorescence emission of **CDG-OMe** at 520 nm increased by up to 218-fold (Fig. 5b). In addition to the gain in the stability of the probe obtained from the additional linker, the specificity of **CDG-OMe** for BlaC improved, with an 11-fold increase in $k_{\text{cat}}/K_{\text{m}}$ to $2.3 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$ in PBS (Table 1). Even at a more than 1,000-fold higher concentration, the fluorescence signal of **CDG-OMe** generated by TEM-1 Bla (400 nM) was just 30% of that generated by BlaC (400 pM) in 300 minutes, which confirms its high selectivity for BlaC (Fig. 5c). Less than 1 fmol of BlaC was readily detectable with **CDG-OMe** after eight hours of incubation (Fig. 5d). When TEM-1 Bla- and BlaC-expressing *E. coli* were incubated with **CDG-1** and **CDG-OMe**, **CDG-1** displayed no selectivity, but **CDG-OMe** showed excellent selectivity for BlaC over TEM-1 Bla (Supplementary Fig. S7).

We further tested the sensitivity and specificity of **CDG-OMe** for detecting bacteria present in raw unprocessed sputum samples obtained from cystic fibrosis patients. BCG or other

bacteria that expressed β -lactamase, including *E. coli*, methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa* strain PA01 and *M. smegmatis*, were incubated with **CDG-OMe** in human sputum. We could detect *Mtb* readily in sputum down to ten colony-forming units (c.f.u.) ($P < 0.05$), which demonstrates the high sensitivity of this system for evaluating clinical samples (Fig. 6a). Fluorescence emission generated by 10 c.f.u. of BCG in sputum is significantly ($P < 0.05$) higher than fluorescence from the negative control and 10^5 c.f.u. of *E. coli*, MRSA, PA01 and *M. smegmatis* (Fig. 6b).

To investigate the applicability of this system in low-resource settings, we built a handmade box equipped with a simple LED light source, excitation filter and emission filter, and took a picture with a cell phone through a hole in the box (Supplementary Fig. S8). As shown in Fig. 6c, 10 c.f.u. of BCG in 200 μ l of human sputum was detected readily within ten minutes of incubation with the probe, and the use of luminance mapping allowed facile optimization of image visualization. In summary, **CDG-OMe** demonstrates excellent sensitivity and specificity for detecting *Mtb* var. *bovis* strain BCG in clinical specimens, even using low-cost detection systems.

Discussion

One of the critical needs for improving the diagnosis of TB is to identify accurate biomarkers for active *Mtb* (ref. 26). Recently, the trehalose mycolyltransferase enzymes were utilized to incorporate unnatural trehalose analogues into *Mtb* for TB detection²⁷. As it requires cell culturing for the probe incorporation, the process can take a long time because of the slow growth rate of *Mtb*. BlaC has the potential to serve here and has many advantages as a diagnostic marker. All TB complex bacteria examined displayed a strong signal dependent on β -lactamase, including BCG, TB laboratory strains and TB clinical isolates, with a total of more than 20 different strains. In addition, complete genome sequencing of more than 54 *Mtb* strains from numerous geographical regions, multidrug resistant and extensively drug-resistant strains demonstrates that BlaC is highly conserved in all *Mtb* clinical isolates, with only two non-synonymous single-nucleotide changes, but they still produce an active enzyme²⁸. These observations are consistent with reports in the literature that indicate 100% (18 examined for *Mtb*, nine for *M. bovis*, five for *M. africanum*) of the strains within the TB complex and *M. kansasii* (17 examined) produce β -lactamase and are positive for enzymatic activity²⁹. As BlaC is an enzyme, its activity promises great sensitivity for the detection of tubercle bacilli, as we have shown with fluorogenic substrates. However, previously available probes lack specificity for BlaC (ref. 18), which allows activation by other β -lactamases, such as TEM-1 Bla, commonly found in gram-negative bacteria^{30,31}, which reduces their accuracy for TB diagnosis.

Extensive research is devoted to β -lactamases because of their role in rendering bacteria resistant to lactam antibiotics. However, most previous studies focused on designing molecules that are poor β -lactamase substrates^{32,33}, in contrast to the goal of this study, to design substrate probes that have rapid kinetics and are selective for BlaC. We took advantage of the unique substrate-specificity loop of BlaC and explored its chemistry to design BlaC-specific fluorogenic substrates. The replacement of R164 in TEM-1 Bla by Ala results in the loss of stabilizing salt bridges in BlaC and thus an increase in the flexibility of

the BlaC substrate-specificity loop. Therefore, it is more probable that the active site of BlaC accommodates chemical modifications on the lactam structure. Based on this structural insight, we explored substitutions at the 7-C position of the lactam ring. The introduced methoxy group is accommodated well in the pocket of BlaC, as revealed by the acyl intermediate structural complex. In this study, we examined the effect of modifications only on the 7-C position of the lactam ring, and it remains possible that other positions on the lactam structure may be modified to generate specificity for BlaC.

CDC-OMe showed excellent selectivity for BlaC over TEM-1 Bla, but low catalytic efficiency for BlaC ($k_{\text{cat}}/K_{\text{m}} = 2.1 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$), which requires long co-incubation times for a positive result. **CDG-OMe** has a much improved kinetic efficiency (an 11-fold increase of $k_{\text{cat}}/K_{\text{m}}$, $2.3 \times 10^5 \text{ s}^{-1} \text{ M}^{-1}$) and still maintains high stability and selectivity for BlaC over TEM-1 Bla. The use of green fluorophore further enhanced its sensitivity for detecting live mycobacteria in solution.

Using a simple handmade device and a cell phone, we detected as low as 10 c.f.u. of BCG in human sputum, which not only demonstrates excellent sensitivity, but also suggests high specificity, because the cystic fibrosis patients from whom these samples were obtained commonly have chronic infection of *S. aureus*, *Haemophilus influenza* and *P. aeruginosa*³⁴, which can express β -lactamase activity. Indeed, we found high levels of ampicillin-resistant bacteria in our sputum samples (Supplementary Fig. S9), but their presence did not impact selectivity or sensitivity.

In summary, we have developed a series of fluorogenic probes specific for *Mtb* by taking advantage of the uniquely flexible substrate-specificity loop of the BlaC enzyme expressed by tubercle bacilli. These probes are chemically modified cephalosporin lactams with a 7 α -methoxy substitution. The acyl intermediate complexes of the E166A BlaC mutant and the probes were co-crystallized successfully for X-ray structure determination to reveal structural insights into the observed specificity of the probes for BlaC. A green fluorescent probe, **CDG-OMe**, enabled the successful detection and imaging, with high specificity, of live pathogen present at very low levels in patient sputum. With such a good sensitivity and specificity, it is highly promising that **CDG-OMe** could be developed easily into a rapid, low-cost TB diagnostic tool to meet the needs of people who live in remote, resource-limited areas. The success of this strategy may greatly contribute to decreasing *Mtb*-associated death rates and transmission.

Methods

Synthetic procedures and chemical characterizations of all the probes are given in the Supplementary Section. Fluorescence spectra were collected on a Fluoromax-3 spectrofluorometer (Jobin Yvon). Kinetic experiments were conducted in a M1000 microplate reader (TECAN, Research Triangle Park, North Carolina).

Cloning, expression and purification of BlaC

Wild-type BlaC was cloned from *Mtb* H37Rv genomic DNA as described previously²³. The deacylation deficient E166A mutant was generated using the Quickchange site-directed

mutagenesis kit (Stratagene no. 200519). Mutant and wild-type protein expression and purification were performed as described previously²³.

Enzymatic kinetics

To a series of different concentrations of the probe (20, 40, 60, 80, 100, 120 and 150 μM) in $1 \times \text{PBS}$ (pH 7.4) in a 96-well plate (black and flat bottomed) was added TEM-1 Bla or BlaC. PBS was added to adjust the total volume to 100 μl . The fluorescence intensity at 454 nm was measured immediately in a microplate reader (excitation wavelength of 400 nm) over a period of 20-minutes at 22 °C. The values of the kinetic parameters (K_m and k_{cat}) were determined from the double-reciprocal plot of the hydrolysis rate versus the substrate concentration (Lineweaver–Burk plot). To determine the spontaneous hydrolysis rate of the probes, the fluorescence intensity was monitored over five days without the addition of enzyme. The rate was calculated from the plot of $\ln([S]_0/([S]_0 - [P]))$ versus time (Supplementary Fig. S4).

Crystallization

Crystals of wild-type and E166A BlaC were grown using the hanging-drop vapour-diffusion method³⁵. BlaC was concentrated to 10 mg ml^{-1} and equilibrated overnight at 4 °C with the mother liquor (2.0 M $\text{NH}_4\text{H}_2\text{PO}_4$, 0.1 M Tris buffer, pH 8.0) in a 1:1 ratio (protein:mother liquor). The solution was centrifuged for ten minutes at 13,000 revolutions per minute to remove insoluble precipitate. Hanging drops were set up and equilibrated against 1 ml of mother liquor. Microseeding with horsehair was sufficient to produce large diffraction-quality crystals. Crystals were transferred to a stabilization solution that contained 30% glycerol in the mother liquor and subsequently soaked with substrates for 2–4 hours. The concentration of substrate was increased slowly by transferring the crystals to successive drops to prevent cracking. Derivatized crystals were flash frozen in liquid nitrogen.

Data collection and processing

X-ray diffraction data were collected on beam lines 19ID and 23ID at the Advanced Photon Source, Argonne National Laboratory, Argonne, Illinois. Data sets were reduced using HKL3000 (ref. 36). Data were collected at a wavelength of 0.97 Å and a temperature of 120K.

Structure determination

Initial phases were obtained by molecular replacement using the CCP4 suite³⁸ and 2GDN as a search model²³. Each data set was refined against the resulting model, and iterative cycles of model building and refinement were performed with Coot 0.6.1 (ref. 39) and PHENIX (ref. 40). Protein Data Bank codes for BlaC–CDC-1 and BlaC–CDC-OMe acyl intermediate crystal structures are 3VFH and 3VFF, respectively.

Detection of mycobacteria in sputum

Mtb var. *bovis* strain BCG was cultured in 7H9 medium with a 10% oleic acid albumin dextrose complex (OADC) and 0.25% Tween-80 until it reached the log phase (optical density at 600 nm (OD_{600}) of 0.5–1). *E. coli*, MRSA, *P. aeruginosa* strain PA01 and *M.*

smegmatis were cultured in Luria–Bertani medium until $OD_{600} = 0.5$ – 1 . After measuring the bacterial OD_{600} , 10^7 c.f.u. of each bacterial strain was added into Eppendorf tubes. Bacteria were centrifuged, the supernatant removed and resuspended into the same medium (7H9 medium with 10% OADC) to normalize the autofluorescence from different media. A series of tenfold dilutions of BCG were made. Bacteria were then incubated at $37\text{ }^{\circ}\text{C}$ to allow β -lactamase production for 0.3–0.5 bacterial generations, using a 20 hour calculated generation time for BCG, a two hour calculated generation time for *M. smegmatis* and a 20 minute calculated generation time for *Pseudomonas*, *Staphylococcus* and *E. coli*. Then $10\text{ }\mu\text{l}$ of bacteria, $40\text{ }\mu\text{l}$ of sputum and $50\text{ }\mu\text{l}$ of **CDG-OMe** ($5\text{ }\mu\text{M}$) were mixed in a 96-well plate, incubated at room temperature for 160 minutes and the fluorescence read with a spectrometer (Mithras LB 940, Berthold Technologies, Oakridge, Tennessee) at 490 nm excitation and 535 nm emission.

For cellular phone imaging, the handmade imaging box consisted of a light-tight box, true cyan colour light-emitting diode (LED) with a peak emission at 505–510 nm, an excitation filter ($490\pm 10\text{ nm}$) between the LED and sample, and an emission filter (530 nm, bandpass 43 nm) between the sample and an imaging hole in the box (Supplementary Fig. S8). BCG ($10\text{ }\mu\text{l}$), sputum ($90\text{ }\mu\text{l}$) and **CDG-OMe** ($10\text{ }\mu\text{M}$, $100\text{ }\mu\text{l}$) were mixed, and after ten minutes samples were photographed with an Apple iPhone 4S through a hole in the box. The photograph was then analysed for luminance (cd m^{-2}) using iPhotoLux v.1.0 (Maxime Bombrun, ISIMA, France), a free iPhone application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Dye C, et al. Measuring tuberculosis burden, trends, and the impact of control programmes. *Lancet Infect Dis.* 2008; 8:233–243. [PubMed: 18201929]
2. Dinnes J, et al. A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. *Health Technol Assess.* 2007; 11:1–196. [PubMed: 17266837]
3. Ling DI, Flores LL, Riley LW, Pai M. Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta-regression. *PLoS One.* 2008; 3:e1536. [PubMed: 18253484]
4. Greco S, Girardi E, Navarra A, Saltini C. Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. *Thorax.* 2006; 61:783–790. [PubMed: 16738037]
5. Boehme CC, et al. Rapid molecular detection of tuberculosis and rifampin resistance. *N Engl J Med.* 2010; 363:1005–1015. [PubMed: 20825313]
6. Hughes R, Wonderling D, Li B, Higgins B. The cost effectiveness of nucleic acid amplification techniques for the diagnosis of tuberculosis. *Respir Med.* 2012; 106:300–307. [PubMed: 22137190]

7. Flores AR, Parsons LM, Pavelka MS Jr. Genetic analysis of the betalactamases of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* and susceptibility to beta-lactam antibiotics. *Microbiology*. 2005; 151:521–532. [PubMed: 15699201]
8. Hugonnet JE, Tremblay LW, Boshoff HI, Barry CE II, Blanchard JS. Meropenem–clavulanate is effective against extensively drug-resistant. *Mycobacterium tuberculosis* Science. 2009; 323:1215–1218.
9. Boyd DB, Lunn WH. Electronic structures of cephalosporins and penicillins. 9. Departure of a leaving group in cephalosporins. *J Med Chem*. 1979; 22:778–784. [PubMed: 448675]
10. Faraci WS, Pratt RF. Elimination of a good leaving group from the 3' -position of a cephalosporin need not be concerted with beta-lactam ring-opening – TEM-2 beta-lactamase-catalyzed hydrolysis of pyridine-2-azo-4'-(N',N'-dimethylaniline) cephalosporin (PADAC) and of cephaloridine. *J Am Chem Soc*. 1984; 106:1489–1490.
11. Faraci WS, Pratt RF. Mechanism of inhibition of the PC1 beta-lactamase of *Staphylococcus aureus* by cephalosporins: importance of the 3'-leaving group. *Biochemistry*. 1985; 24:903–910. [PubMed: 3873255]
12. Pratt RF, Faraci WS. Direct observation by proton NMR of cephalosporoate intermediates in aqueous solution during the hydrazinolysis and beta-lactamase-catalyzed hydrolysis of cephalosporins with 3' leaving groups: kinetics and equilibria of the 3' elimination reaction. *J Am Chem Soc*. 1986; 108:5328–5333.
13. Zlokarnik G, et al. Quantitation of transcription and clonal selection of single living cells with beta-lactamase as reporter. *Science*. 1998; 279:84–88. [PubMed: 9417030]
14. Gao W, Xing B, Tsien RY, Rao J. Novel fluorogenic substrates for imaging beta-lactamase gene expression. *J Am Chem Soc*. 2003; 125:11146–11147. [PubMed: 16220906]
15. Xing B, Khanamiryan A, Rao J. Cell-permeable near-infrared fluorogenic substrates for imaging beta-lactamase activity. *J Am Chem Soc*. 2005; 127:4158–4159. [PubMed: 15783183]
16. Yao H, So MK, Rao J. A bioluminogenic substrate for in vivo imaging of beta-lactamase activity. *Angew Chem Int Ed*. 2007; 46:7031–7034.
17. Rukavishnikov A, Gee KR, Johnson I, Corry S. Fluorogenic cephalosporin substrates for beta-lactamase TEM-1. *Anal Biochem*. 2011; 419:9–16. [PubMed: 21867672]
18. Kong Y, et al. Imaging tuberculosis with endogenous beta-lactamase reporter enzyme fluorescence in live mice. *Proc Natl Acad Sci USA*. 2010; 107:12239–12244. [PubMed: 20566877]
19. Banerjee S, Pieper U, Kapadia G, Pannell LK, Herzberg O. Role of the Omega-loop in the activity, substrate specificity, and structure of class A beta-lactamase. *Biochemistry*. 1998; 37:3286–3296. [PubMed: 9521648]
20. Knox JR. Extended-spectrum and inhibitor-resistant TEM-type beta-lactamases – mutations, specificity, and 3-dimensional structure. *Antimicrob Agents Chemother*. 1995; 39:2593–2601. [PubMed: 8592985]
21. Albrecht HA, et al. Cephalosporin 3'-quinolone esters with a dual mode of action. *J Med Chem*. 1990; 33:77–86. [PubMed: 2153215]
22. Baldwin JE, Urban FJ, Cooper RDG, Jose FL. Direct 6-methoxylation of penicillin derivatives – convenient pathway to substituted beta-lactam antibiotics. *J Am Chem Soc*. 1973; 95:2401–2403. [PubMed: 4709241]
23. Wang F, Cassidy C, Sacchettini JC. Crystal structure and activity studies of the *Mycobacterium tuberculosis* beta-lactamase reveal its critical role in resistance to beta-lactam antibiotics. *Antimicrob Agents Chemother*. 2006; 50:2762–2771. [PubMed: 16870770]
24. Minasov G, Wang XJ, Shoichet BK. An ultrahigh resolution structure of TEM-1 beta-lactamase suggests a role for Glu166 as the general base in acylation. *J Am Chem Soc*. 2002; 124:5333–5340. [PubMed: 11996574]
25. Urano Y, et al. Evolution of fluorescein as a platform for finely tunable fluorescence probes. *J Am Chem Soc*. 2005; 127:4888–4894. [PubMed: 15796553]
26. McNerney R, Daley P. Towards a point-of-care test for active tuberculosis: obstacles and opportunities. *Nature Rev Microbiol*. 2011; 9:204–213. [PubMed: 21326275]
27. Backus KM, et al. Uptake of unnatural trehalose analogs as a reporter for *Mycobacterium tuberculosis*. *Nature Chem Biol*. 2011; 7:228–235. [PubMed: 21378984]

28. Ioerger TR, et al. The non-clonality of drug resistance in Beijing-genotype isolates of *Mycobacterium tuberculosis* from the Western Cape of South Africa. *BMC Genomics*. 2010; 11:670. [PubMed: 21110864]
29. Kwon HH, Tomioka H, Saito H. Distribution and characterization of beta-lactamases of mycobacteria and related organisms. *Tuber Lung Dis*. 1995; 76:141–148. [PubMed: 7780097]
30. Majiduddin FK, Materon IC, Palzkill TG. Molecular analysis of beta-lactamase structure and function. *Int J Med Microbiol*. 2002; 292:127–137. [PubMed: 12195735]
31. Petrosino J, Cantu C III, Palzkill T. β -Lactamases: protein evolution in real time. *Trends Microbiol*. 1998; 6:323–327. [PubMed: 9746943]
32. Hugonnet JE, Blanchard JS. Irreversible inhibition of the *Mycobacterium tuberculosis* beta-lactamase by clavulanate. *Biochemistry*. 2007; 46:11998–12004. [PubMed: 17915954]
33. Tremblay LW, Fan F, Blanchard JS. Biochemical and structural characterization of *Mycobacterium tuberculosis* beta-lactamase with the carbapenems ertapenem and doripenem. *Biochemistry*. 2010; 49:3766–3773. [PubMed: 20353175]
34. Mitchell, RS.; Kumar, V.; Robbins, SL.; Abbas, AK.; Fausto, N. Robbins Basic Pathology. Saunders/Elsevier; 2007.
35. McPherson, A. Preparation and Analysis of Protein Crystals. Waverly: 1982.
36. Minor W, Cymborowski M, Otwinowski Z, Chruszcz M. HKL-3000: the integration of data reduction and structure solution – from diffraction images to an initial model in minutes. *Acta Crystallogr D Biol Crystallogr*. 2006; 62:859–866. [PubMed: 16855301]
37. McCoy AJ, et al. Phaser crystallographic software. *J Appl Crystallogr*. 2007; 40:658–674. [PubMed: 19461840]
38. Bailey S. The CCP4 suite – programs for protein crystallography. *Acta Crystallogr D Biol Crystallogr*. 1994; 50:760–763. [PubMed: 15299374]
39. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr*. 2010; 66:486–501. [PubMed: 20383002]
40. Adams PD, et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr*. 2010; 66:213–221. [PubMed: 20124702]

CDC-1: $R^1 = \text{Bn}$, $R^2 = \text{H}$, $n = 0$

CDC-2: $R^1 = \text{Bn}$, $R^2 = \text{H}$, $n = 1$

CDC-3: $R^1 = \text{EtN}$, $R^2 = \text{H}$, $n = 0$

CDC-4: $R^1 = \text{Me}$, $R^2 = \text{H}$, $n = 0$

CDC-5: $R^1 = \text{Me}$, $R^2 = \text{OMe}$, $n = 0$

CDC-OMe: $R^1 = \text{Bn}$, $R^2 = \text{OMe}$, $n = 0$

CDC-OEt: $R^1 = \text{Bn}$, $R^2 = \text{OEt}$, $n = 0$

Figure 1.

General structures of blue fluorescent probes and their hydrolysis by β -lactamase (Bla), which triggers the release of umbelliferone and turns on fluorescence.

Figure 2. Kinetic comparison of CDC probes with β -lactamases

a, Fluorescent emission spectrum of CDC-OMe (1 μM in PBS) before and after treatment with BlaC (1 μM) (excitation, 400 nm). **b**, Time courses of fluorescent activation of CDC-1 and CDC-OMe with BlaC (filled circles) or TEM-1 Bla (open squares). **c,d**, Enhanced fluorescent intensity of CDC-OMe (**c**) and CDC-1 (**d**) (5 μM in PBS) by BlaC or TEM-1 Bla at the indicated concentrations for one hour. F/F_0 represents the turn-on ratio of fluorescence intensity by β -lactamase. Error bars are $\pm$ standard deviation (s.d.). a.u. = arbitrary units.

Figure 3. Comparison of BlaC and TEM-1 Bla active sites and substrate-specificity loops
a. Conserved salt bridges in both enzymes formed by D172–R178 and D176–R178 stabilize the loop conformers of the unoccupied active sites. **b.** TEM-1 Bla (1M40, white) R164 forms salt bridges with E171 (2.87 Å), D176 (3.99 Å) and D179 (2.75 Å). BlaC (2GDN, purple) A164 prevents these stabilizing interactions, which results in an increased flexibility of the loop and catalytic base E166. **c.** Superimposition of unoccupied TEM-1 Bla and BlaC active sites. **d.** Superimposition of unoccupied TEM-1 Bla (white) and the BlaC–CDC-OMe acyl intermediate complex (purple). The arrow indicates the movement of the substrate loop in BlaC on binding CDC-OMe.

Figure 4. Active-site details of the BlaC-CDC-OMe (top) and BlaC-CDC-1 (bottom) acyl intermediate complexes

a,b, Electrostatic surface potential (**a**) and cartoon diagrams (**b**) of acyl intermediates with $F_O - F_C$ electron-density maps displayed in grey for **CDC-OMe** (2.0σ) and **CDC-1** (1.5σ) prior to fitting each ligand for refinement protocols. Active-site residues are displayed as sticks. F_O = observed amplitude; F_C = calculated amplitude. **c,** Ligplot analysis of active-site interactions.

Figure 5. β-Lactamase selectivity of green fluorescent probes CDG-1 and CDG-OMe

a, BlaC hydrolyses green probes and turns on the fluorescence signal. **b**, Fluorescent emission spectrum of **CDG-OMe** (20 nM in MES) before and after treatment of BlaC (0.2 μM) (excitation, 490 nm). **c**, Time courses of fluorescence enhancement with **CDG-OMe** (8 μM in MES buffer) and various concentrations of β-lactamases. **d**, Enhanced fluorescence intensity of **CDG-OMe** (8 μM in MES, front) and **CDG-1** (8 μM in MES, back) by serially diluted solutions of BlaC or TEM-1 Bla for eight hours. Data were collected in 384-well plates with a total volume of 25 μl in each well. **e**, Magnified view of the rectangular region in (d). F represents the difference in fluorescence intensity with and without β-lactamase incubation. Data in (d) and (e) are the average of three replicate experiments. Error bars are $\pm$ s.d.

Figure 6. Sensitivity and specificity of CDG-OMe in raw, unprocessed human sputum
a, Detection of BCG in sputum with **CDG-OMe**. **b**, Specificity of **CDG-OMe** for detecting BCG in sputum. 10 c.f.u. of BCG or 10⁵ c.f.u. of the indicated β -lactamase-expressing bacteria were added to human sputum samples obtained from cystic fibrosis patients and incubated with **CDG-OMe** (2.5 μM) at room temperature for 160 minutes. Fluorescence was measured with excitation at 490 nm and emission at 535 nm. **P*, 0.05 as compared to 10 c.f.u. of BCG. **c**, Imaging of BCG in sputum with **CDG-OMe** using a cellular phone. BCG-spiked sputum samples were incubated with **CDG-OMe** (5 μM) at room temperature for ten minutes before they were imaged in a simple handmade light box: light from an off-the-shelf LED assembly (>US\$10.00) passed through a 490 nm excitation filter (bandpass 20 nm) was reflected through a 530 nm emission filter (bandpass 43 nm) and photographed with an iPhone 4S. The photograph was analysed with iPhotoLux v. 1.0. Error bars are $\pm$ s.d.

Table 1
Kinetic parameters of fluorescent probes for BlaC and TEM-1 Bla

Probe	BlaC			TEM-1 Bla			Spontaneous hydrolysis rate ($\times 10^{-7} \text{ s}^{-1}$)
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{ M}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \text{ M}^{-1}$)	
1 CDC-1	63 $\pm$ 6	13 $\pm$ 0.5	2.1×10^5	135 $\pm$ 16	48 $\pm$ 3.8	3.6×10^5	2.4 $\pm$ 0.3
2 CDC-2	136 $\pm$ 44	0.5 $\pm$ 0.1	3.7×10^3	454 $\pm$ 37	7 $\pm$ 0.7	1.5×10^4	1.1 $\pm$ 0.1
3 CDC-3	69 $\pm$ 7	6 $\pm$ 0.2	8.7×10^4	59 $\pm$ 10	77 $\pm$ 6.6	1.3×10^6	1.8 $\pm$ 0.2
4 CDC-OMe	47 $\pm$ 9.4	1 $\pm$ 0.1	2.1×10^4	50 $\pm$ 2	$8 \pm 0.3 \times 10^{-4}$	16	2.8 $\pm$ 0.1
5 CDC-OEt	131 $\pm$ 40	0.026 $\pm$ 0.005	1.9×10^2	ND	ND	ND	2.3 $\pm$ 0.2
6 CDC-4	148 $\pm$ 50	10 $\pm$ 1.9	6.8×10^4	133 $\pm$ 18	10 $\pm$ 1	7.5×10^4	1.9 $\pm$ 0.1
7 CDC-5	139 $\pm$ 22	0.52 $\pm$ 0.05	3.6×10^3	ND	ND	ND	2.6 $\pm$ 0.3
8 CDG-1*	2.1 $\pm$ 0.3 (2 $\pm$ 0.4)	4 $\pm$ 0.2 (1.0 $\pm$ 0.1)	1.9×10^6 (5×10^5)	1.0 $\pm$ 0.1 (2 $\pm$ 0.2)	9 $\pm$ 0.4 (5 $\pm$ 0.1)	9×10^6 (2.5×10^6)	3.3 $\pm$ 0.1 (6.0 $\pm$ 0.1)
9 CDG-OMe*	3 $\pm$ 0.3 (5 $\pm$ 0.3)	0.7 $\pm$ 0.04 (0.8 $\pm$ 0.01)	2.3×10^5 (1.6×10^5)	30 $\pm$ 8 (40 $\pm$ 4)	1 $\pm$ 0.2 $\times 10^{23}$ ($7 \pm 0.6 \times 10^{24}$)	33 (18)	2.5 $\pm$ 0.1 (1.9 $\pm$ 0.1)

Kinetic data were measured in PBS buffer (1 $\times$, pH = 7.4) at room temperature (22 °C) unless otherwise noted. All data indicate averages of three replicate experiments.

* Data in parentheses were measured in MES buffer (0.1 M, pH 6.6) at 22 °C. ND = not determined due to extremely slow kinetics.