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Cell-Permeable Near-Infrared Fluorogenic Substrates for Imaging β-Lactamase Activity

Bengang Xing, Ashot Khanamiryan, and Jianghong Rao*

Department of Radiology, Biophysics, Bio-X, Cancer Biology, and Molecular Imaging Programs at Stanford, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, California 94305-5344

Received November 29, 2004; E-mail: jrao@stanford.edu

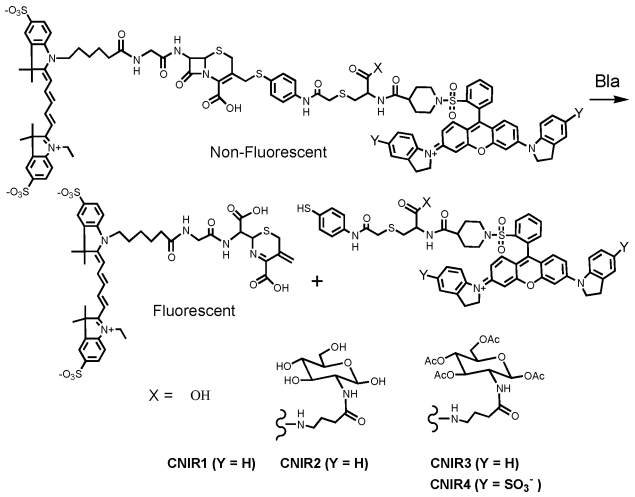
TEM-1 β-lactamase (Bla), the monomeric isoform product of the ampicillin resistance gene (*amp*^{r}), is a small enzyme (29 kDa) that efficiently hydrolyzes β-lactam substrates. It does not exist in eukaryotes but can be easily expressed in eukaryotic cells without any noticeable toxicity,[1−3] and it has become an attractive biosensor for detecting biological processes and interactions in vivo, such as monitoring the promoter/regulatory elements' activity,[4−6] constitutive and inducible protein interactions,[7−9] and ribozyme in vivo splicing reactions.[10,11]

Several fluorogenic substrates for Bla have been reported,[4,12] but none work for infrared or near-infrared fluorescence imaging. Infrared/near-infrared light is preferred in molecular imaging studies of living subjects because its long wavelength causes less photo damage to cells, produces less autofluorescence background, and offers better sensitivity. Furthermore, its longer wavelength has better tissue penetration and less light scattering than visible light, therefore it is more suitable for living animal imaging.[13] Common near-infrared dyes, including the carbocyanine series of compounds, are widely used for labeling proteins and nucleic acids, but their poor membrane permeability due to high molecular weights and multiple charges makes it difficult to apply them for imaging living subjects. Methods that include using synthetic grafted copolymers and nanoparticles have been developed to assist their cellular delivery.[14,15] Here, we report a design of a cell-permeable small near-infrared fluorogenic substrate for Bla and its application in imaging gene expression in living mammalian cells. Availability of such a new substrate should further facilitate the application of Bla as a biological reporter in living cells and even in whole living animals.

Our designed substrates are shown in Scheme 1. Cy5 emits maximally at 670 nm when excited at 650 nm. A quenching group, QSY21, is not fluorescent and has a wide absorption spectrum from 540 to 730 nm with a peak at 660 nm. Therefore, they were chosen as a fluorescence resonance energy transfer (FRET) pair so emission from Cy5 can be efficiently quenched by QSY21. Cy5 was tethered to the 7-amino of the cephalosporin through a glycyl linkage, and QSY21 was attached to the 3′-position via a linker, which contained an amino thiophenol and a cysteine residue. As an excellent leaving group, the amino thiophenol at the 3′-position would facilitate the fragmentation after Bla hydrolysis, and the cysteine introduces a coupling site for adding any potential new functionality. The substrate should have little or no fluorescence due to the FRET quenching effect. Bla hydrolysis will break the FRET quenching by releasing QSY21 and, thus, rendering a fluorescent product containing Cy5 (Scheme 1). Preparation and characterization of **CNIR1** are detailed in the Supporting Information.

Indeed, **CNIR1** itself was essentially nonfluorescent, but produced a highly fluorescent product with a 57-fold increase in the emission intensity at the wavelength of 660 nm upon treatment with purified Bla (Figure 1). This large increase confirmed our design that Bla hydrolysis of the substrate can lead to activation of the fluorescence of Cy5. Further analysis of the hydrolysis kinetics of **CNIR1** by Bla in phosphate-buffered saline (PBS) at pH 7.1 revealed the catalytic constant, $k_{cat} = 0.8 \pm 0.1\ s^{-1}$, and Michaelis constant, $K_m = 6.7 \pm 1.5\ \mu M$ (Figure 2). Its catalytic efficiency (k_{cat}/K_m) is $7.4 \times 10^5\ M^{-1}\ s^{-1}$. The value of k_{cat} is smaller than that of a previously reported fluorogenic substrate, CCF2 ($k_{cat} = 29\ s^{-1}$),[4] despite the chemical similarity in their 3′-leaving groups, which might be related to the much larger size of **CNIR1** as a substrate for Bla. **CNIR1** is highly stable with the spontaneous hydrolysis rate constant in PBS of $\sim 1.7 \times 10^{-7}\ s^{-1}$, and the enzymatic acceleration (k_{cat}/k_{uncat}) is $\sim 5 \times 10^6$. A concentration as low as 190 fM Bla is readily detectable with **CNIR1**.

Scheme 1. Structures of **CNIR1**, **CNIR2**, **CNIR3**, and **CNIR4** and their Hydrolysis by Bla



Because of its hydrophilic and charged nature, **CNIR1** is not cell-permeable. It has been reported that D-glucosamine can carry molecules into cells through the attachment to its 2-amino group.[16] To test this strategy, we decided to link D-glucosamine to the

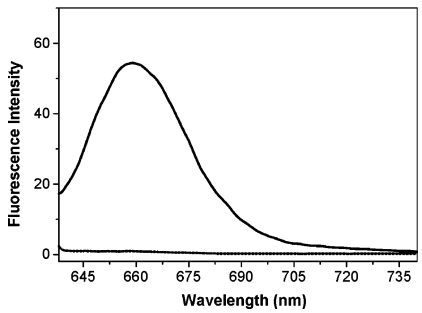


Figure 1. Fluorescence emission of **CNIR1** (100 nM in PBS) before (· · ·) and after (—) addition of Bla (excitation: 620 nm).

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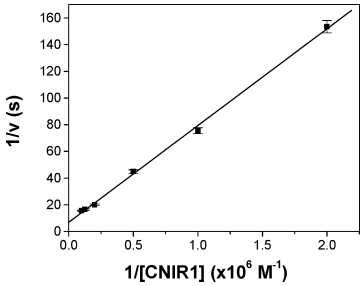

Figure 2. Double-reciprocal plot of **CNIR1** hydrolyzed per enzyme molecule per second (v) versus substrate concentration. Error bars indicate standard errors. Refer to Supporting Information for details.

carboxylate of cysteine on **CNIR1** to facilitate its membrane penetration. A γ-amino butyric acid was inserted as a spacer to minimize any potential steric interactions between the transporter and the enzyme. We have successfully prepared **CNIR2** from D-glucosamine. **CNIR2** can efficiently detect Bla in vitro with a 58-fold increase in the fluorescence emission at 660 nm after the treatment of Bla (Figure S2C in Supporting Information), which suggested that the introduction of D-glucosamine has little interference with its activity.

We then tested the applicability of **CNIR2** in imaging Bla activity in living cells, **CNIR2** was able to enter into cells but with just moderate efficiency (data not shown). However, to our surprise, a derivative of **CNIR2** containing fully acetylated D-glucosamine, **CNIR3** (Scheme 1), was found to be much more efficient. Incubation of both wild-type C6 glioma cells (no Bla expression; a negative control) and stably transfected C6 glioma cells with constitutive expression of Bla in a 4 μM solution of **CNIR3** for 1 h at room temperature revealed a clear difference; in wild-type cells, little Cy5 fluorescence was observed, and stably transfected C6 glioma cells emitted bright fluorescence signals with a contrast of up to 10-fold higher.[17]

The peracetylated D-glucosamine in **CNIR3** is presumably not a substrate for glucosamine/glucose transporters, so its entrance into cells may have a path different from that of native D-glucosamine.[18] When incubated at 4 °C, little fluorescence signal was observed in the Bla stably transfected C6 glioma cells, which may suggest the possible involvement of endocytosis in the uptake.

Acetylation of D-glucosamine improves the membrane permeability of the substrate, but decreases its solubility. We, therefore, introduced two sulfonate groups to QSY21 of **CNIR3** and prepared **CNIR4** (Scheme 1).[19] **CNIR4** has a much improved solubility in aqueous media with the cell permeability still maintained. The images of Bla stably transfected C6 glioma cells were similar to that of **CNIR3**, but the uptake was more uniform (Figure 3). The fluorescence signals appeared to preferentially localize in endosomes and some nuclei. When incubated at 37 °C, the uptake was even higher and there was less background signal from wild-type cells; thus the contrast was further improved (Figure 3). The retention of the fluorescence signal in Bla-expressed cells seemed not to be compromised even with 1 h incubation at 37 °C.

In summary, we report here a design of cell-permeable near-infrared fluorogenic substrates for β-lactamase and have demonstrated their applicability in imaging β-lactamase activity in living mammalian cells. With both good excitability with near-infrared light and cell permeability, this new type of fluorogenic probe holds promises for imaging gene expression in living animals, which is currently ongoing in our laboratory.

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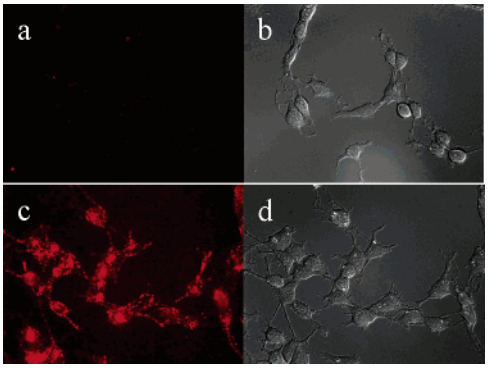

Figure 3. Fluorescence (a and c) and differential interference contrast (b and d) images of wild-type (a and b) and Bla stably transfected (c and d) C6 glioma cells loaded with **CNIR4** (4 μM) in Hank's balanced salts solution for 1 h at 37 °C. Cy5 emission is displayed in red. Fluorescence images are corrected with background subtraction. See Supporting Information for the detailed conditions.

Supporting Information Available: Synthetic procedures and characterizations of all four near-infrared substrates, and procedures for determining kinetic parameters and imaging Bla activity in living cells (16 pages, PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) Refer to Figure S5 in Supporting Information for details.

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