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Research Article

A fluorescein-labeled AmpC β-lactamase allows rapid characterization of β-lactamase inhibitors by real-time fluorescence monitoring of the β-lactamase-inhibitor interactions

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Rapid emergence of class C β-lactamases has urged an immediate need for developing class C β-lactamase specific inhibitors for effective clinical treatment. To facilitate the development of effective class C β-lactamase inhibitors, we propose a new approach for a rapid analysis of the interaction of AmpC β-lactamase and its inhibitors using our recently developed V211Cf fluorescent β-lactamase biosensor during drug screening. Since the fluorescein of V211Cf can report the local environment change in the active site of AmpC β-lactamase, fluorescence responses of V211Cf toward its substrates/inhibitors can provide real-time traces of the dynamic change of the interaction of the β-lactamase with its substrates/inhibitors. In this study, we found that V211Cf displayed distinct fluorescence signal patterns toward different kinds of inhibitors (including clavulanic acid, sulbactam, tazobactam and 2-thiopheneboronic acid) due to the differences in their interactions with β-lactamase. V211Cf not only enables a high throughput screening for inhibitors but can also provide a rapid preliminary indication on the inhibitor's potency and stability to β-lactamase's hydrolytic action as well as how the inhibitors interact with the target enzyme, thereby speeding up the drug discovery and development cycle of class C β-lactamase inhibitors.

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1 Introduction

β-Lactam antibiotics are the most popular therapeutics for treatment for bacterial infectious diseases. However, bacteria have evolved to produce β-lactamases to hydrolyze β-lactam antibiotics, lowering the effectiveness of these drugs [1]. Nowadays, the problem of antibiotic resistance has become severe owing to the rapid emergence of various kinds of β-lactamases [2, 3]. Notably, rapid emergence of class C β-lactamases, which are often produced in pathogenic gram-negative bacteria, have led to a big clinical challenge because of their high efficiency in hydrolyzing a broad spectrum of β-lactam antibiotics and the lack of their specific potent inhibitors [4]. This highlights an immediate need for introducing effective therapeutic strategies to combat the class C β-lactamase-producing causative pathogens. In clinical practice, to overcome the

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Abbreviations: 7-ACA, 7-aminocephalosporanic acid; **BHY**, medium composed of 37 g/L brain heart infusion and 5 g/L yeast extract; **CD**, circular dichroism; **f**, fluorescein; **IC_{50}**, inhibitory concentration at 50% inhibition; **k_{cat}**, catalytic constant; **K_i**, inhibition constant; **K_m**, Michaelis constant

deleterious effect of β-lactamase on β-lactam antibiotic, a β-lactamase inhibitor is often co-administrated with a partner β-lactam antibiotic so as to protect the antibiotic from the hydrolysis by β-lactamase and/or potentiate the bacteria-killing activity of the antibiotic [5–8]. Because of the lack of effective class C β-lactamase inhibitors, recently, enormous efforts have been made in discovering new β-lactam-based inhibitors as well as seeking novel non-β-lactam-based inhibitors including boronic acids, phosphonates, other organic compounds and inorganic complexes for the inactivation of the class C β-lactamases [9–17].

Concomitant with immense interests in the novel inhibitor discovery are the demands for efficient approaches for screening and characterizing a vast array of potential candidates that comes from nature or is generated from structure-guided in-parallel synthesis. Conventional approaches for identifying the potential drug candidates are microbial susceptibility testing and β-lactamase inhibition assay [18–21]. These methods determine the minimal inhibitory concentration (MIC) and inhibition constants (IC_{50} or K_i) of the candidates respectively, thus providing indices for the drug potency. In addition, characterization techniques such as radioactive labeling of inhibitors, UV difference spectral studies, mass spectrometry and crystallographic studies, which provide kinetic and structural analyses on the enzyme-inhibitor complexes, are well-established to advance a better understanding of the inactivation chemistry of the candidates with desirable efficacy for further drug design and lead compound optimization [22–28].

Recently, our laboratory has fabricated a class C β-lactamase-based fluorescent V211Cf biosensor for high throughput screening of class C β-lactamase inhibitors [29]. In V211Cf, a fluorescein molecule is covalently tethered to position 211 in an AmpC β-lactamase from *Enterobacter cloacae* P99 at which this site is resided on a long flexible loop of the β-lactamase and it is in close proximity to the entrance of the active site pocket. According to the molecular modeling of the structure of V211Cf, the fluorescein molecule is buried inside the hydrophobic pocket of the AmpC β-lactamase mutant [29]. In the presence of substances that can specifically bind to the enyzme's active site, the fluorescein molecule has to move outward from the active site to provide space for the entry of the β-lactamase-binding substance and the flexibility of the loop permits such movement. The fluorescein is then in a more solvent exposable environment, resulting in an enhancement of the fluorescence intensity of V211Cf. In this sense, the enhanced fluorescence intensity of V211Cf indicates the presence of active-site binding substance. In our previous study [29], we suggested to pre-incubate V211Cf with the inhibitor candidate followed by a subsequent addition of penicillin G (a β-lactam antibiotic). The pre-incubated inhibitor occupies the active site pocket of V211Cf, preventing the penicillin from binding to the active site. We then compared the fluorescence intensity of penicillin-pre-incubated V211Cf with that of the without pre-incubation control to determine the presence and potency of the inhibitor. This drug screening assay not just helps identify the class C β-lactamase inhibitor but also determines the combination of antibiotic and inhibitor for effective treatment.

In this study, we further explored the possibility of the use of V211Cf in the drug discovery and development for class C β-lactamase inhibitors. Here we illustrated that V211Cf not only enables efficient inhibitor screening but can also allow rapid characterization of the inhibitor candidate based on the ability of V211Cf to report the interaction between the AmpC β-lactamase and its inhibitor. In this study, we examined the fluorescence properties of V211Cf toward three currently available β-lactamase inhibitors including clavulanic acid, sulbactam, tazobactam as well as a transition state analog, 2-thiopheneboronic acid. From our results, V211Cf showed distinct fluorescence signals in response to these compounds, which adopt different mechanisms in inactivating β-lactamases [6]. It is thus envisioned that these differential fluorescence signals can provide valuable information about the interaction between the inhibitors and the β-lactamase drug target, facilitating the characterization of the identified inhibitor candidates and structure-based drug design for class C β-lactamase inhibitors.

2 Materials and methods

2.1 Chemicals

7-Aminocephalosporanic acid (7-ACA), ampicillin, cephalothin, chloramphenicol, penicillin G and 2-thiopheneboronic acid were purchased from Sigma (St. Louis, MO). Nitrocefin was obtained from Becton Dickson and Company (Cockeyville, Md.). Clavulanic acid was generously provided by Prof. Yoshikazu Ishii (Toho University, Japan). Sulbactam and tazobactam were gifts from Qilu Pharmaceutical Co. Ltd. (Jinan, China). Fluorescein-5-maleimide was obtained from Molecular Probes Inc. (Eugene, O. R.). Brain heart infusion (BHI) and yeast extract were purchased from Oxoid Ltd. (Nepean, Ontario, Canada). Chemical structures of the chemicals used in the study are shown in Supporting information, Fig. S1.

2.2 Preparation of V211Cf

V211Cf was prepared as previously described [29]. Briefly, *Bacillus subtilis* strain IA304 (ϕ105MU331) containing the expression construct for N-terminally $(His)_6$-tagged *E. cloacae* P99 AmpC β-lactamase V211C mutant was cultivated in BHY medium (37 g/L brain heart infusion and 5 g/L yeast extract) at 37 °C with shaking at 300 rpm for 11–12 h. Afterwards, 5 mL of the inoculum

was transferred to a 1 L-flask containing 100 mL of fresh BHY medium and then incubated at 37°C with shaking at 300 rpm. When the OD_{600} reached 3.0–3.5, expression of the V211C mutant protein was thermo-induced by incubating the bacterial culture at 50°C for 5 min. After thermo-induction, the bacterial culture was grown at 37°C with shaking at 300 rpm for a further 5 h. After that, cell pellet was obtained by centrifugation at 9000 rpm and 4°C for 20 min.

For purification of the V211C mutant protein, cell pellet was re-suspended in solubilization buffer (50 mM Tris-HCl, 0.1 M NaCl, pH 8.0) and lysed by incubation with lysozyme (75 μg/mL) at 30°C for 1 h and a subsequent sonication. Afterwards, the cell lysate was clarified by centrifugation at 10 000 rpm and 4°C for 1 h. Supernatant was loaded onto a nickel-charged HiTrap chelating column (GE Healthcare), which was pre-equilibrated with 20 mM sodium phosphate buffer, 0.5 M NaCl (pH 7.4). Then the non-specific binding proteins on the column were removed by washing the column with six column volumes of 20 mM sodium phosphate buffer, 0.5 M NaCl (pH 7.4) and then the $(His)_6$-tagged V211C mutant was eluted by a linear gradient of 0–0.2 M imidazole. The purified V211C protein was buffer-exchanged with 50 mM potassium phosphate buffer (pH 7.0) at 4°C.

To label the V211C mutant protein with fluorescein, 1 mg of V211C protein was incubated with a 10-fold molar excess of fluorescein-5-maleimide in the dark with shaking at 300 rpm for 30 min. After the labeling reaction, excess dye was removed by buffer-exchange with 50 mM potassium phosphate (pH 7.0) by ultracentrifugation using an Amicon Ultra-15 (NMWL = 10 000) centrifugal filter device. The labeling efficiency and the identity of the labeled sample were checked by mass spectrometry. The labeled mutant (V211Cf) was then aliquoted and stored at -80°C.

2.3 Far-UV CD spectrometry

Secondary structures of the wild-type AmpC β-lactamase, V211C and V211Cf were assessed by far-UV CD spectrometry using JASCO J-810 spectropolarimeter. Prior to the measurement, the CD spectropolarimeter was purged with nitrogen for 30 min. The protein samples were dissolved in 50 mM potassium phosphate (pH 7.0) at the concentration of 50 μg/mL and added to a quartz cuvette of 0.1-cm light absorption path length. The CD spectrum of the protein was obtained by scanning the far UV region from 190 to 250 nm and the CD measurement for each protein was performed in triplicate.

2.4 IC_{50} determination

1 nM enzyme was first incubated with different concentrations of inhibitors in 50 mM potassium phosphate (pH 7.0) at 25°C for 5 min. After the incubation, nitrocefin (100 μM) was added to each enzyme/inhibitor mixture. The product formation arising from nitrocefin hydrolysis was then monitored at 500 nm over the first 2 min by UV-visible spectrophotometry to determine the initial velocity. The residual activity at each inhibitor concentration was determined by dividing the initial velocity with the inhibitor (v_i) by the initial velocity without the inhibitor (v_0). The inhibitor concentration, at which the enzymatic activity was reduced by 50% (IC_{50}), was determined from the plot of residual activity against inhibitor concentration using Origin 6.0 Professional.

2.5 Fluorescence measurements

Fluorescence measurements were performed as previously described [29]. 0.1 μM V211Cf was added with various concentrations of antibiotics or inhibitors in 50 mM potassium phosphate buffer (pH 7.0). The reaction mixture was mixed immediately and the fluorescence signal was recorded by Perkin-Elmer LS50B spectrofluorimeter (Beaconsfield, Buckinghamshire, England). Excitation and emission wavelengths were set at 498 nm and 517 nm, respectively. Both excitation and emission slit widths were 5 nm. For stopped-flow experiments, fluorescence measurements of V211Cf were carried out by using Applied Photophysics SX.18MV-R stopped-flow spectrophotometer (Leatherhead, UK). Excitation wavelength was set at 498 nm and the emission was observed by using a 515 nm cutoff filter. Both excitation and emission slit widths were 5 nm. All fluorescence measurements were carried out at 20°C.

3 Results

3.1 Fluorescein tethered to V211Cf can report the dynamic changes in the enzyme's active site

An introduction of a fluorescein to position 211 of a flexible loop on an AmpC β-lactamase did not impose any severe effects on the secondary structure (as revealed by the CD spectra shown in Supporting information, Fig. S2) and the catalytic properties of the enzyme [29]. From our previous studies, it was also demonstrated that this fluorescein-modified β-lactamase with preserved catalytic properties enabled the detection of the local environment change in the active site pocket. Thus, the fluorescence trace of V211Cf in response to β-lactamase-binding compound represents a real-time dynamic reaction happening in the active site pocket [29].

In general, in response to a class C β-lactamase hydrolyzable β-lactam analyte, V211Cf shows a typical fluorescence signal which is composed of three phases: (i) an initial rising phase; (ii) a plateau phase; and (iii) a declining phase to baseline (Fig. 1A). As illustrated by molecular modeling of V211Cf with and without cefoxitin

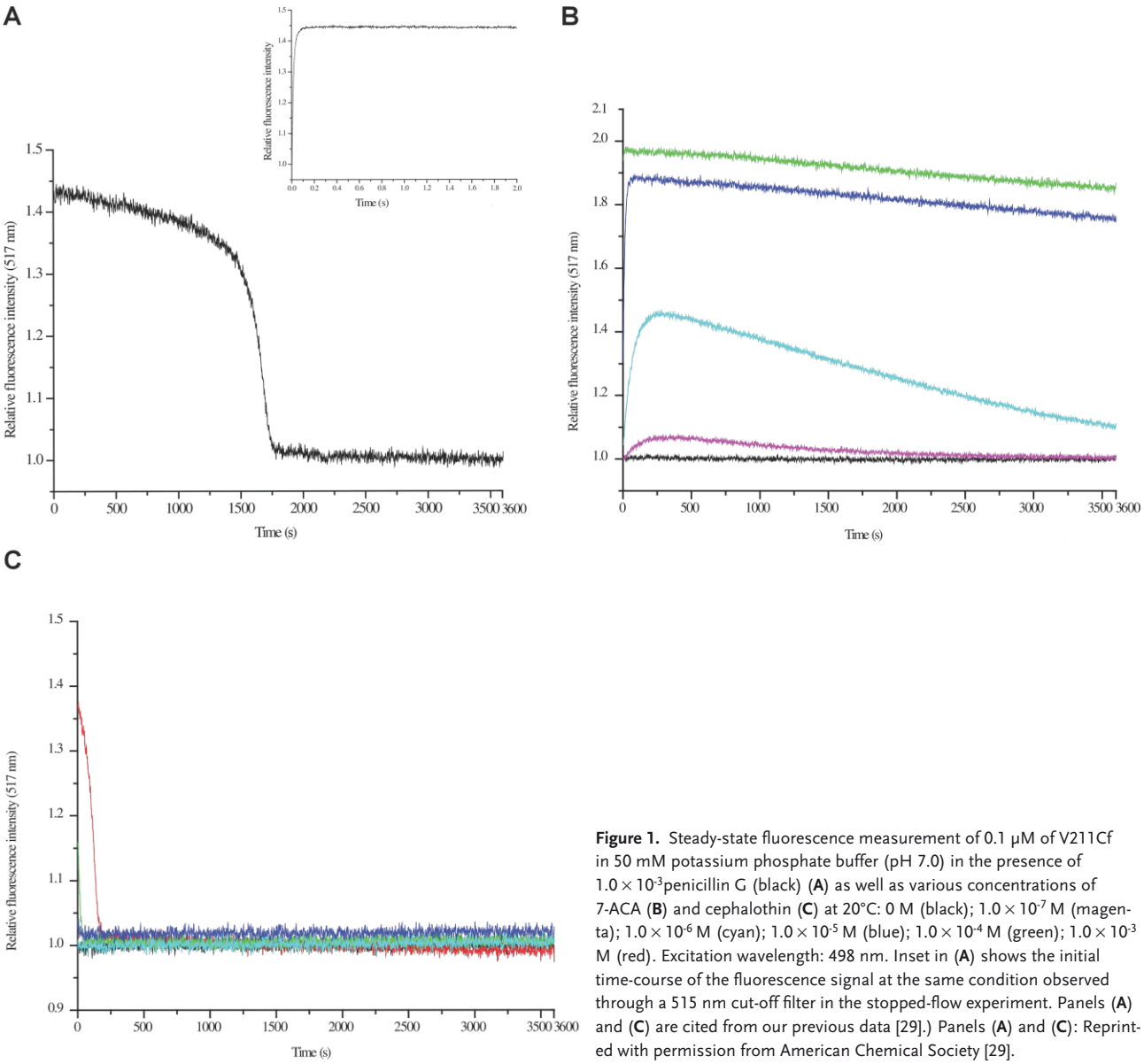

Figure 1. Steady-state fluorescence measurement of 0.1 μM of V211Cf in 50 mM potassium phosphate buffer (pH 7.0) in the presence of 1.0×10^{-3}penicillin G (black) (**A**) as well as various concentrations of 7-ACA (**B**) and cephalothin (**C**) at 20°C: 0 M (black); 1.0×10^{-7} M (magenta); 1.0×10^{-6} M (cyan); 1.0×10^{-5} M (blue); 1.0×10^{-4} M (green); 1.0×10^{-3} M (red). Excitation wavelength: 498 nm. Inset in (**A**) shows the initial time-course of the fluorescence signal at the same condition observed through a 515 nm cut-off filter in the stopped-flow experiment. Panels (**A**) and (**C**) are cited from our previous data [29].) Panels (**A**) and (**C**): Reprinted with permission from American Chemical Society [29].

in our previous work, upon the binding of an analyte to the active site of V211Cf, the entry of the analyte will dislocate the fluorescein molecule from its original position to a more solvent accessible environment [29], leading to an increase in the fluorescence intensity of V211Cf. This increase in fluorescence intensity due to the analyte binding contributes to a rising phase in the fluorescence signal. Once all the active site pockets are completely occupied by the analyte molecules, the increase of fluorescence intensity ceases. The fluorescence intensity remains at the same level as long as the active site pockets are occupied by the analyte molecules and/or enzyme-analyte intermediate molecules, resulting in a plateau phase of the fluorescence signal. With the hydrolytic ability of β-lactamase comparable to the parental wild-type AmpC β-lactamase, V211Cf can hydrolyze the β-lactam analyte. When the hydrolyzed product molecules are leaving the active site clefts, the exposed fluorescein returns back to its original position, giving rise to a decrease in the fluorescence intensity of V211Cf. After the release of the product molecules, the active sites are regenerated and the fluorescence intensity of V211Cf returns back to its basal level. The intensity and duration of the fluorescence signal are analyte-concentration dependent.

The duration of the various phases in a fluorescence trace varies according to the type of β-lactam substrates in a manner that the ease of the substrate to be hydrolyzed determines the longevity of the fluorescence signal. 7-ACA, a scaffold for the semi-synthesis of cephalosporin, is a poor substrate of class C β-lactamase with high K_m

and low k_{cat} values [30]. Poor affinity of the AmpC enzyme with 7-ACA (as reflected by high value of K_m) resulted in a slow increase in the fluorescence intensity at the rising phase whereas low deacylation rate contributed to a slow decline of the fluorescence signal (Fig. 1B). This signal pattern is remarkably different from that for the cephalothin which is a first generation cephalosporin. Cephalothin, as a preferred substrate for the class C β-lactamase [31], exhibited a comparably short-lived signal with rapid rising phase (which is too fast to be monitored by the steady-state fluorescence measurement) and a sharp decreasing phase (Fig. 1C). Therefore, from the fluorescence signals of V211Cf, information about the binding rate of the analyte to the active site of the AmpC β-lactamase, the stability of the enzyme-analyte intermediates and the stability of the analyte toward the hydrolysis by the AmpC β-lactamase can be easily deciphered.

3.2 V211Cf displayed distinct fluorescence signal patterns toward clavam type- and sulfone type-inhibitors

Clavulanic acid, sulbactam and tazobactam are the only three β-lactamase inhibitors that are clinically approved and they are formulated into combinations with various β-lactam partners for different clinical settings [5]. They are β-lactam compounds that can serve as suicide inhibitors by inactivating the β-lactamases through the covalent modification at the enzyme's active site [32, 33]. Their inhibition mechanism follows complicated branched pathways (as depicted in Supporting information, Fig. S3). The inhibitor first gets bound with the enzyme-active site and then undergoes acylation to give the covalent acyl-enzyme adduct (EI*). Before inactivation, some of these complexes would dissociate through deacylation, thus a small portion of inhibitor molecules would sacrifice in this normal enzyme turnover. The remaining acyl-enzyme complexes would either convert into various transiently inhibited forms of the enzyme (E-T) or yield irreversibly inactivated enzymes (EI**) through the rearrangement events, eventually leading to either transient or irreversible inhibition of the enzyme. Despite the general inactivation mechanism by the clavam (clavulanic acid) and the penicillanic acid sulfones (sulbactam and tazobactam), slight differences in the partition of the branched pathways leading to inhibition are found between these two groups of inhibitors (Supporting information, Figs. S4 and S5).

Among the three inhibitors, while tazobactam was the best inhibitor inactivating the class C β-lactamase in micromolar ranges, clavulanic acid showed the poorest inhibitory activity toward the enzyme by exhibiting IC_{50} values higher in one- to two-orders of magnitude than the other two penicillanic acid sulfones did (Table 1). Furthermore, it was noted that those values for V211C and V211Cf were comparable to those for the wild-type enzyme, indicating that the genetic and chemical modifications at the position of 211 for the AmpC β-lactamase posed no effects on the enzyme's properties toward the inhibitors. And this high similarity in the characteristics between V211Cf and its wild-type enzyme toward the inhibitors' activities implied the likeliness of V211Cf to reflect the real situation of the enzyme inactivation by the inhibitors. This finding underlined the potential of V211Cf as a useful system for determining the enzyme-inhibitor interaction which is the fundamental basis of inhibitor screening as well as providing important information for the inhibition mechanistic study.

Table 1. IC_{50} values of various inhibitors for the wild-type AmpC P99 β-lactamase, V211C and V211Cf.

	IC_{50} (μM)		
	Wild-type	**V211C**	**V211Cf**
Clavulanic acid[a)]	1322.0	722.0	651.0
Sulbactam	22.4	23.9	17.4
Tazobactam	3.5	3.5	4.0
2-thiopheneoboronic acid[a)]	28.2	32.0	5.2

a) IC_{50} results were cited from our previously published data [29].

As shown in Fig. 2, V211Cf displayed distinct fluorescence signals for the clavulanic acid and the two penam sulfone compounds over a 1-h time-course. For clavulanic acid, there was a gradual increase in the fluorescence intensity by the addition of the inhibitor (Fig. 2A). As shown in the trace for the concentration of 1.0×10^{-3} M of clavulanic acid, the fluorescence signal tended to level-off to a plateau level after its rise to its fluorescence maximum. In contrast, for sulbactam and tazobactam, fluorescence intensities instantaneously increased after mixing V211Cf with the inhibitors and the signals subsided rapidly after the initial fluorescence spikes (Figs. 2B and 2C).

The fluorescence results indicated that V211Cf could give rise to characteristic signals toward two groups of β-lactam inhibitors, the clavam and the penam sulfone which operate their own specific inactivation mechanisms according to different partitions in the routes to inhibition. However, because of the various possible forms of the inhibitor-enzyme adducts along the specific inactivation pathways for each class of inhibitors, the exact components ascribed to the distinct fluorescence signals for these inhibitors could not be resolved by interpreting these fluorescence signals. Indeed, as the fluorescence signals were due to the synergistic effects of the reaction products formed from each branching reaction in the inactivation process, the traces reflected the integrated dynamics of various branched pathways along the inhibition event.

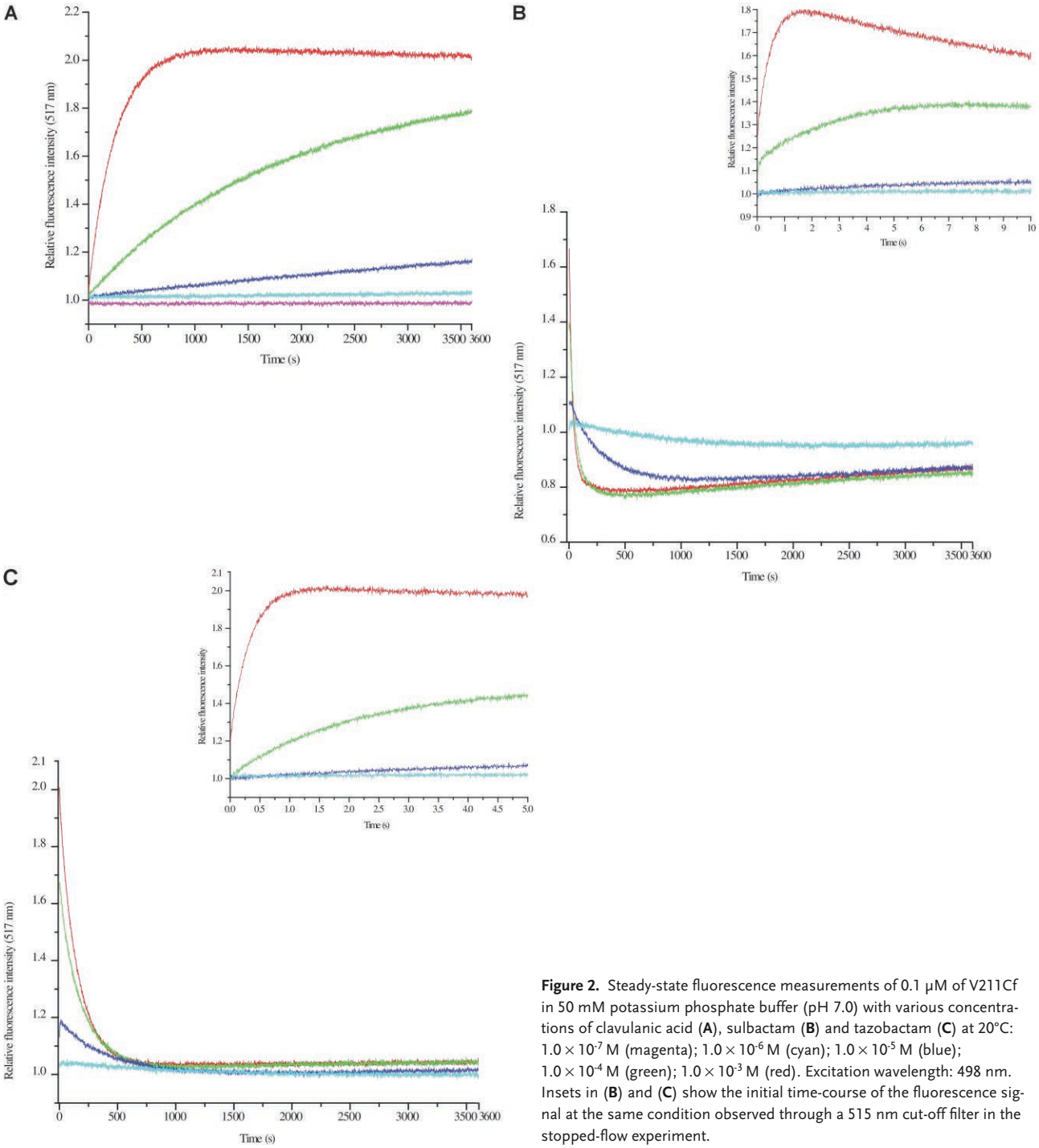

Figure 2. Steady-state fluorescence measurements of 0.1 μM of V211Cf in 50 mM potassium phosphate buffer (pH 7.0) with various concentrations of clavulanic acid (**A**), sulbactam (**B**) and tazobactam (**C**) at 20°C: 1.0×10^{-7} M (magenta); 1.0×10^{-6} M (cyan); 1.0×10^{-5} M (blue); 1.0×10^{-4} M (green); 1.0×10^{-3} M (red). Excitation wavelength: 498 nm. Insets in (**B**) and (**C**) show the initial time-course of the fluorescence signal at the same condition observed through a 515 nm cut-off filter in the stopped-flow experiment.

3.3 Fluorescence signal of V211Cf toward 2-thiopheneboronic acid reflected the two-step inactivation mechanism of boronic acid on AmpC β-lactamase

Boronic acid is an intriguing class of non-β-lactam compounds that can inactivate serine β-lactamases by acting as a transition-state analog. Inactivation by boronic acid process proceeds with an initial binding of a boronic acid to the enzyme active site and a subsequent step of dative covalent bond formation between the active site serine hydroxyl group and the boronic acid to give a reversible adduct [34]. This resultant adduct, which adopts a geometry resembling to that of the high energy tetrahedral intermediates along the hydrolytic reaction pathway, occupies the active site of the β-lactamase, thus deacti-

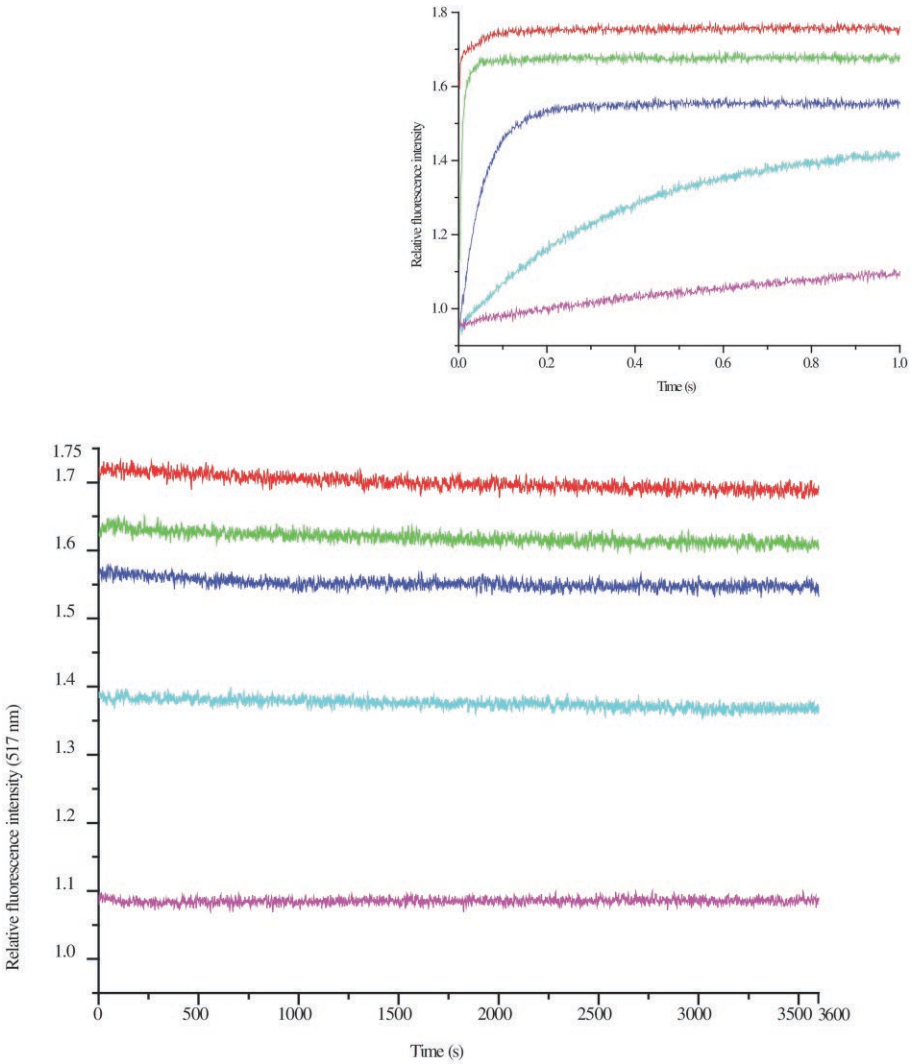

Figure 3. Steady-state fluorescence measurements of 0.1M of V211Cf in 50mM potassium phosphate buffer (pH7.0) with various concentrations of 2-thiopheneboronic acid at 20°C: 1.0×10^{-7} M (magenta); 1.0×10^{-6} M (cyan); 1.0×10^{-5}M (blue); 1×10^{-4} M (green); 1.0×10^{-3}M (red).

vating the enzyme (as shown in Supporting information, Fig. S6) [35].

The fluorescence signal of V211Cf in response to 2-thiopheneboronic acid was comprised of two phases: an initial rising phase and a succeeding plateau phase (Fig. 3). It was observed that the plateau phase was stable over the 1 h-fluorescence detection. This result was in good agreement with the two-step inhibition mechanism of boronic acid on serine-type β-lactamase in which the initial phase corresponded to the binding of the boronic acid to the active site whereas the following plateau phase referred to the stage at which all the enzyme molecules with their active sites saturated with the transition-state analog.

4 Discussion

Since the introduction of penicillin, the first β-lactam antibiotic, the use of β-lactam antibiotics for infectious disease treatment has been greatly challenged by the rapid emergence of bacterial β-lactamases [1]. To ensure effective treatment to control infections caused by β-lactamase-producing bacteria, it is necessary to develop new β-lactam antibiotics that are not susceptible to β-lactamase's hydrolysis and/or new β-lactam inhibitors that are used in combination with the antibiotics to protect them from the hydrolytic action of β-lactamases. In particular, there is a high clinical priority to develop new therapeutics that can inactivate the rapidly emerged class C β-lactamases that currently have no effective inhibitors for clinical use. Therefore, a lot of efforts have been made in developing novel potent class C β-lactamase inhibitors.

To keep pace with the increasing loads of potential inhibitor candidates and to shorten the time-investment for the drug development, it is noteworthy to have efficient systems that can allow rapid exploration for the novel therapeutics. This prompted our interests in exploring the potential of using our V211Cf as a versatile tool for novel drug screening and characterization for class

C β-lactamase inhibitors. Our previous study has indicated that V211Cf can offer high throughput screening for potential inhibitors. Because of its ability to detect the active-site binding substances, inhibitor screening using V211Cf can eliminate the false-positive results caused by the substances that bind to class C β-lactamase non-specifically [29]. Apart from this, since V211Cf has the ability to report the dynamic reactions inside the active site, in this study, we want to show that time-resolved measurement of V211Cf can also allow a simple method of direct, real-time monitoring of the enzyme-inhibitor interaction.

As revealed by our results, V211Cf displayed characteristic signals toward different classes of inhibitors in which these signals may serve as fingerprints for particular groups of inhibitors. This undoubtedly helps provide valuable information important for optimizing the lead compounds in the structure-based drug design. Although for some inhibitors with complicated inactivation pathways (for examples, clavulanic acid, sulbactam and tazobactam), their inhibition mechanisms may not be directly deciphered from the fluorescence spectra, these fluorescence traces did provide complementary information in the comprehensive mechanistic analyses which make use of a series of techniques such as spectrometric analyses and crystallographic studies. Furthermore, as demonstrated in our previous study, V211Cf can be amenable to a micro-titer plate format, shedding light on its potential for high-throughput inhibitor screening. Taken together, V211Cf illustrated attractive features for being an efficient tool for inhibitor screening and mechanistic studies, meriting the discovery for class C β-lactamase inhibitors.

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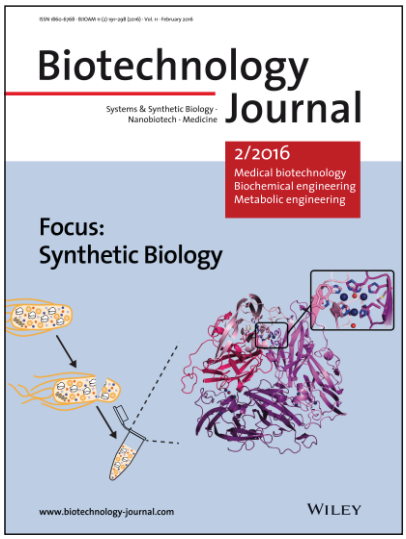

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