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NXL104 Irreversibly Inhibits the β-Lactamase from *Mycobacterium tuberculosis*

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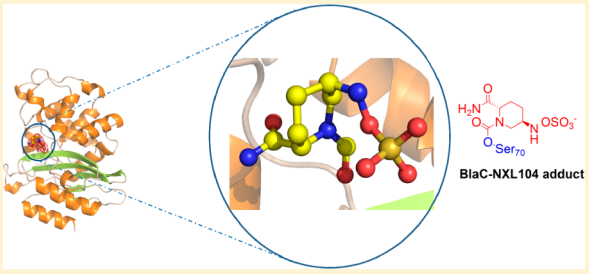
Supporting Information

ABSTRACT: NXL104 is a novel β-lactamase inhibitor with a non-lactam structural scaffold. Our kinetic and mass spectrometric analysis demonstrates that NXL104 quantitatively inhibits BlaC, the only chromosomally encoded β-lactamase from *Mycobacterium tuberculosis*, by forming a carbamyl adduct with the enzyme. The inhibition efficiency (k_2/K) of NXL104 was shown to be more than 100-fold lower than that of clavulanate, a classical β-lactamase inhibitor, which is probably caused by the bulky rings of NXL104. However, the decarbamylation rate constant (k_3) was determined to be close to zero. The BlaC−NXL104 adduct remained stable for at least 48 h, while the hydrolysis of the BlaC−clavulanate adduct was observed after 2 days. The three-dimensional crystal structure of the BlaC−-NXL104 carbamyl adduct was determined at a resolution of 2.3 Å. Interestingly, the sulfate group of NXL104 occupies the position of a phosphate ion in the structure of the BlaC−clavulanate adduct and is hydrogen bonded to residues Ser128, Thr237, and Thr239. Favorable interactions are also seen in the electrostatic potential map. We propose that these additional interactions, as well as the intrinsic stability of the carbamyl linkage, contribute to the extraordinary stability of the BlaC−NXL104 adduct.



Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), which causes nearly 2 million deaths each year.[1] It is estimated that one-third of the world's population is currently infected with *M. tuberculosis*, and new infections occur at an alarming rate of approximately 1 s^{-1}.[2] Current TB treatments are time-consuming and not effective against the nonreplicating forms of *M. tuberculosis* that are responsible for the persistence of TB infections. In addition, the increasing emergence of multidrug resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* strains[3] makes it important to develop novel therapies against this bacterium.

β-Lactam antibiotics are among the most clinically prescribed antibacterial drugs and act by inhibiting the D,D-transpeptidases involved in the biosynthesis of the bacterial cell wall.[4] A major strategy of bacterial resistance to β-lactams is the production of β-lactamases that catalyze the hydrolysis of β-lactams, leading to the inactivation of the antibiotics. β-Lactams have not been used in clinical practice to treat TB infections, because an active penicillinase was reported in *M. tuberculosis*.[5] Recently, it has been shown that this penicillinase is a chromosomally encoded β-lactamase (BlaC), rendering *M. tuberculosis* resistant to β-lactam antibiotics.[6]

The Ambler classification divides β-lactamases into four classes based on their amino acid sequences.[7] Class A, C, and D enzymes employ a nucleophilic serine residue to attack the β-lactam ring, while class B enzymes are metallo-β-lactamases binding one or two zinc ions in the active site. BlaC is a class A β-lactamase that contains a nucleophilic serine residue (Ser70) and shares sequence homology with the penicillin-binding protein (PBP) domain of the ancestral D,D-transpeptidases. Previously, we have shown that BlaC hydrolyzes a broad spectrum of β-lactam antibiotics, including penicillins, cephalosporins, and even carbapenems.[8] Our recent structural and kinetic studies of two BlaC mutants (K73A and E166A) suggest that K73 and E166 are essential for acylation and deacylation of β-lactams, respectively.[9] Specifically, Lys73 activates Ser70 by deprotonating its hydroxyl group, which then attacks the carbonyl carbon in the β-lactam ring, resulting in the acylated enzyme. During deacylation, Glu166 activates a highly conserved active site water molecule that attacks acylated BlaC and consequently releases the inactive ring-opened compound from the enzyme.

Additionally, we have shown that BlaC is irreversibly inhibited by clavulanate (Figure 1), a β-lactamase inhibitor approved by

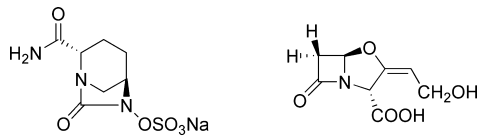


Figure 1. Chemical structures of NXL104 and clavulanic acid.

the Food and Drug Administration,[8,10] and the combination of clavulanate and meropenem, a poor β-lactam substrate of BlaC, exhibited potent inhibitory activity against laboratory strains of *M. tuberculosis* as well as 13 clinical XDR strains,[11] suggesting that

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the combination of a β-lactam antibiotic and a β-lactamase inhibitor might represent an effective TB chemotherapy.

NXL104 [*trans*-7-oxo-6-(sulfooxy)-1,6-diazabicyclo-[3.2.1]octan-2-carboxamide sodium salt (Avibactam)] is a novel non-lactam inhibitor of β-lactamases (Figure 1). Previous studies show that NXL104 inhibits both Class A and Class C β-lactamases,[12−14] making the bacterial strains expressing these β-lactamases susceptible to β-lactam antibiotics.[15−17] The combination of NXL104 and ceftazidime, a cephalosporin antibiotic, is currently in phase II clinical trials for the treatment of complicated urinary tract infections (cUTIs), which are caused by various pathogenic bacteria.[18]

In this work, we conducted kinetic and mass spectrometric analysis of NXL104 and showed that it quantitatively inactivates BlaC by forming a carbamyl linkage with the enzyme. In addition, we determined the three-dimensional structure of the BlaC−NXL104 adduct, providing molecular insight into the slow decarbamylation mechanism.

EXPERIMENTAL PROCEDURES

Materials. NXL104 was a generous gift from from Anacor Pharmaceuticals (Palo Alto, CA). BlaC was expressed and purified as described previously.[8] Buffer reagents for crystallography were purchased from Hampton Research (Aliso Viejo, CA). Unless noted, other chemicals were from Sigma-Aldrich (St. Louis, MO).

Inhibition Assays. BlaC (1.5 μM) was incubated with various inhibitor to enzyme ratios (0−2.5) at room temperature for 30 min (clavulanate) and for 2 or 10 h (NXL104). The activity of BlaC was measured by following the hydrolysis of 100 μM nitrocefin at 486 nm (ε = 20500 M^{-1} cm^{-1}) after a 500-fold dilution. The fractional enzyme activity was then plotted versus the inhibitor:enzyme ratio.

To measure the rate constants of carbamylation and decarbamylation, we continuously monitored the hydrolysis of 100 μM nitrocefin with 0.6 nM BlaC in the presence of 72−540 μM NXL104. The progress curve was fit to eq 1

$$[P] = v_s t + \frac{v_i - v_s}{k_{iso}}[1 - \exp(-k_{iso}t)] \quad (1)$$

where [P] is the concentration of the product, v_i and v_s represent the initial and final reaction velocities, respectively, and k_{iso} is the apparent first-order rate constant.

The general inhibition mechanism is modeled as

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E^*I \overset{k_2}{\rightarrow} E{-}I \overset{k_3}{\rightarrow} E + P \quad (2)$$

where k_1 and k_{-1} represent the rate constants of the binding of NXL104 to and dissociation of NXL104 from BlaC, respectively, while k_2 and k_3 correspond to the rate constants of carbamylation and decarbamylation, respectively.

For this model, k_{iso} determined from eq 1 was fit to the eq 3 to obtain the values of k_2 and k_3, where K equals k_{-1}/k_1.

$$k_{iso} = k_3 + \frac{k_2[I]}{K + [I]} \quad (3)$$

When k_3 is close to zero and K is much larger than [I], eq 3 could be simplified as eq 4

$$k_{iso} = k_3 + \frac{k_2}{K}[I] \quad (4)$$

Table 1. Data Collection and Refinement Statistics

Data Collection[a]	
X-ray source	NSLS beamline X29
wavelength (Å)	1.0
temperature (K)	100
resolution range (Å)	2.29−75.0
no. of reflections	11647 (598)
completeness (%)	95.44 (93.62)
R_{merge}	0.14 (0.81)
$I/\sigma(I)$	11.9 (3.5)
redundancy	7.0 (7.2)
space group	$P2_12_12_1$
unit cell	
a (Å)	49.960
b (Å)	68.004
c (Å)	75.705
$\alpha = \beta = \gamma$ (deg)	90.0
no. of molecules per asymmetric unit	1
Refinement	
R_{work}	0.170
R_{free}	0.261
no. of atoms	
protein	1989
NXL104	17
PO_4	10
water	236
rmsd	
bond lengths (Å)	0.020
bond angles (deg)	1.667
average *B* factor (Å^2)	
overall	22.31
protein main chain	21.53
protein side chain	23.20
NXL104	41.38
PO_4	42.60
water	36.80
Ramachandran	
favored	91.4% + 7.6%
outliers	0.0%
PDB entry	4DF6

[a]Values for the highest-resolution shell are given in parentheses.

Reactivation Assay. BlaC (2 μM) was completely inactivated by incubation with 5 μM NXL104 for 24 h or 5 μM clavulanate for 2 h at room temperature. The excess inhibitors were removed by ultracentrifugation using an Amicon 3K cutoff filter device (Millipore). Then the reactivation of the acylated or carbamylated enzyme was monitored by measuring the BlaC activity with the nitrocefin activity assay described above.

Mass Spectrometric Analysis. The mass spectra were recorded as described previously with minor modifications.[8,19] BlaC was dialyzed into 20 mM ammonium bicarbonate (pH 6.5) to minimize salt inference. Then 50 μM BlaC was incubated with 50 μM NXL104 at room temperature. One microliter of the reaction mixture was withdrawn at various incubation times (0, 10, 120, and 600 min), mixed with 9 μL of a 50% acetonitrile/0.1% formic acid solution, and then injected into a 9.6 T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Ionspec, Lake Forest, CA). The molecular weight of the protein sample was determined for the +25 charge state using the equation $m = (m/z \times 25) - 25$ on the isotopic centroid.

Crystallization. Hanging drop vapor diffusion was used for crystallization of BlaC. The composition of the well consisted of

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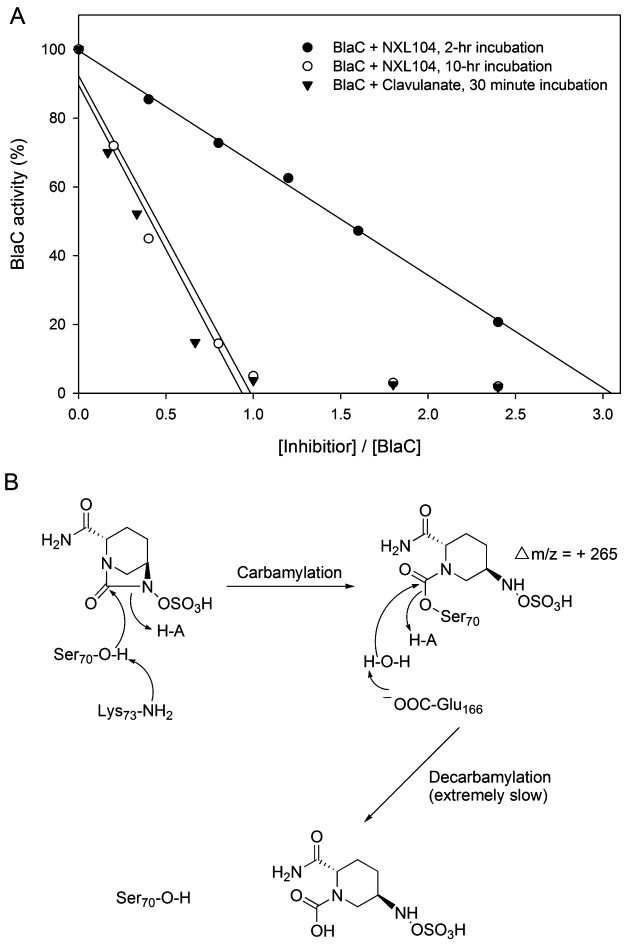

Figure 2. Inhibition of NXL104 vs BlaC. (A) Inhibition of BlaC with NXL104 and clavulanate. BlaC was incubated with various concentrations of NXL104 or clavulanate. Then the fractional BlaC activity was plotted vs the inhibitor:BlaC ratio. (B) Proposed mechanism of inhibition of BlaC by NXL104. Upon deprotonation of Ser70 by Lys73, the hydroxyl group of Ser70 attacks the carbonyl carbon of NXL104, resulting in an inactive carbamyl BlaC intermediate. The decarbamylation of this intermediate mediated by Glu166 and a catalytic water molecule is much slower than carbamylation, which leads to the inactivation of BlaC.

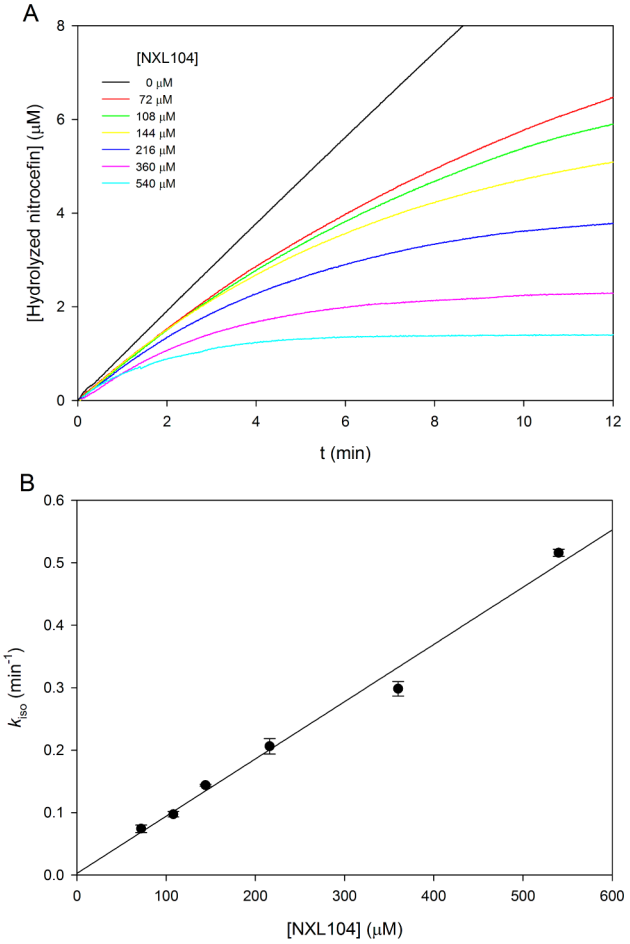

Figure 3. Determination of the inhibition rate constants. (A) Time-dependent hydrolysis of nitrocefin in the presence of various concentrations of NXL104. The apparent first-order rate constants (k_{iso}) were calculated by fitting the curves to eq 1. (B) Plot of k_{iso} vs NXL104 concentration. The data were fit to eq 4 to obtain k_2/K and k_3 values.

0.1 M HEPES (pH 7.5) and 2 M $NH_4H_2PO_4$, which makes the final pH of the well solution 4.1. The protein at a concentration of 12 mg/mL was mixed 1:1 with the well solution and incubated at 10 °C. Initial crystals grew within 4−5 days but were small, were needle-shaped, and numbered in the thousands. Repeated microseeding was performed, which resulted in efficient crystal growth as well as improved morphology, to produce diffraction quality crystals of the active enzyme.

Data Collection and Refinement. BlaC crystals were soaked with 40 mM NXL104 in mother liquor. Before the crystals were frozen, 20% glycerol was added to the solution as a cryoprotectant. Data were collected at Brookhaven National Laboratory on crystals frozen on 120 min soaks with NXL104. Beamlines X12C and X29 were used; various resolutions of diffraction were obtained depending on the soaking times and beamline. The data were processed using HKL2000.[20] Our previous structure of clavulanate-bound BlaC[10] [Protein Data Bank (PDB) entry 3CG5] was used to phase the data, using the CCP4 software suite.[21] Multiple rounds of structural refinement and model building were performed in Refmac5[22,23] and Coot.[24]

Table 1 lists the data collection statistics for the structures as well as the final refinement statistics.

RESULTS AND DISCUSSION

Inhibition Studies. Inhibition studies were performed by measuring the residual BlaC activity after incubation with various concentrations of the inhibitor, revealing that only 1 equiv of NXL104 is needed to inactivate BlaC (Figure 2A) after a 10 h incubation. Complete inactivation of BlaC by 1 equiv of NXL104 was not realized after a 2 h incubation, while clavulanate achieved 100% inhibition of BlaC within 30 min (Figure 2A), indicative of slow onset inhibition by NXL104 compared to that of clavulanate. NXL104-inhibited BlaC remained inactive after extensive dialysis, suggesting the formation of a covalent adduct via carbamylation, followed by a slow decarbamylation step (Figure 2B).

To measure the rate constants of carbamylation (k_2) and decarbamylation (k_3), we conducted the reporter substrate assay by monitoring the hydrolysis of nitrocefin by BlaC,[8] in the presence of various concentrations of NXL104. As shown in Figure 3A, NXL104 exhibited time-dependent inhibition against BlaC. The apparent first-order rate constants (k_{iso}) were calculated from the progress curves in Figure 3A and then plotted versus NXL104 concentration (Figure 3B), revealing an intercept indistinguishable from zero, which suggests that the value of rate of

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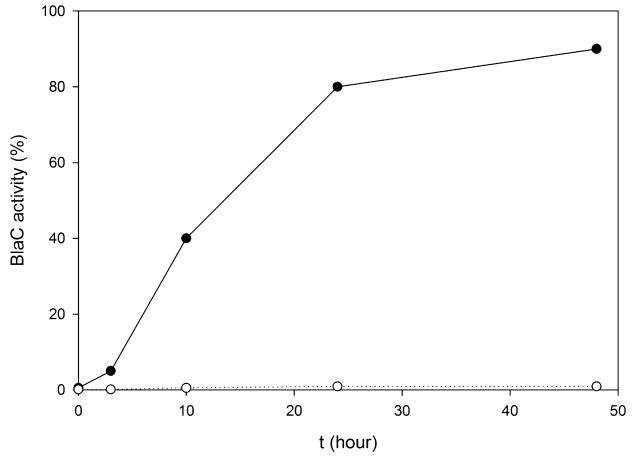

Figure 4. Recovery of NXL104- or clavulanate-inhibited BlaC. BlaC activity was monitored for 48 h using the nitrocefin hydrolysis assay, after complete inactivation of BlaC by NXL104 (○) or clavulanate (●).

decarbamylation, k_3, is close to zero. The plot is quite linear at the concentrations of NXL-104 used, indicating that the dissociation constant K (defined as k_{-1}/k_1) is much higher than 500 μM and k_2 is greater than 0.5 min^{-1}. The poor reversible binding of NXL104 to BlaC is probably caused by the bulky fused rings and the lack of a β-lactam feature in NXL104. Although the precise values of k_2 and K could not be determined, we were able to measure the value of k_2/K (0.92 ± 0.03 min^{-1} mM^{-1}) based on the slope of the plot. The fact that this value is more than 100-fold lower than that of clavulanate (k_2/K = 132 ± 4 min^{-1} mM^{-1})[8] suggests that NXL104 is less efficient than clavulanate in converting BlaC into the acylated or carbamylated species.

To further investigate the stability of the adduct, we followed the recovery of the β-lactamase activity of the carbamylated BlaC using the nitrocefin hydrolysis assay, which revealed that more than 98% of BlaC remained inactive after 48 h (Figure 4). In contrast, BlaC activity was almost completely restored 2 days after inactivation by clavulanate. Therefore, the BlaC−NXL104 adduct is much more stable than the BlaC−clavulanate adduct and the k_3 value of NXL104 much smaller than that of clavulanate.

Mass Spectrometry. To identify the NXL104 adduct formed during the incubation, we performed Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry by incubating

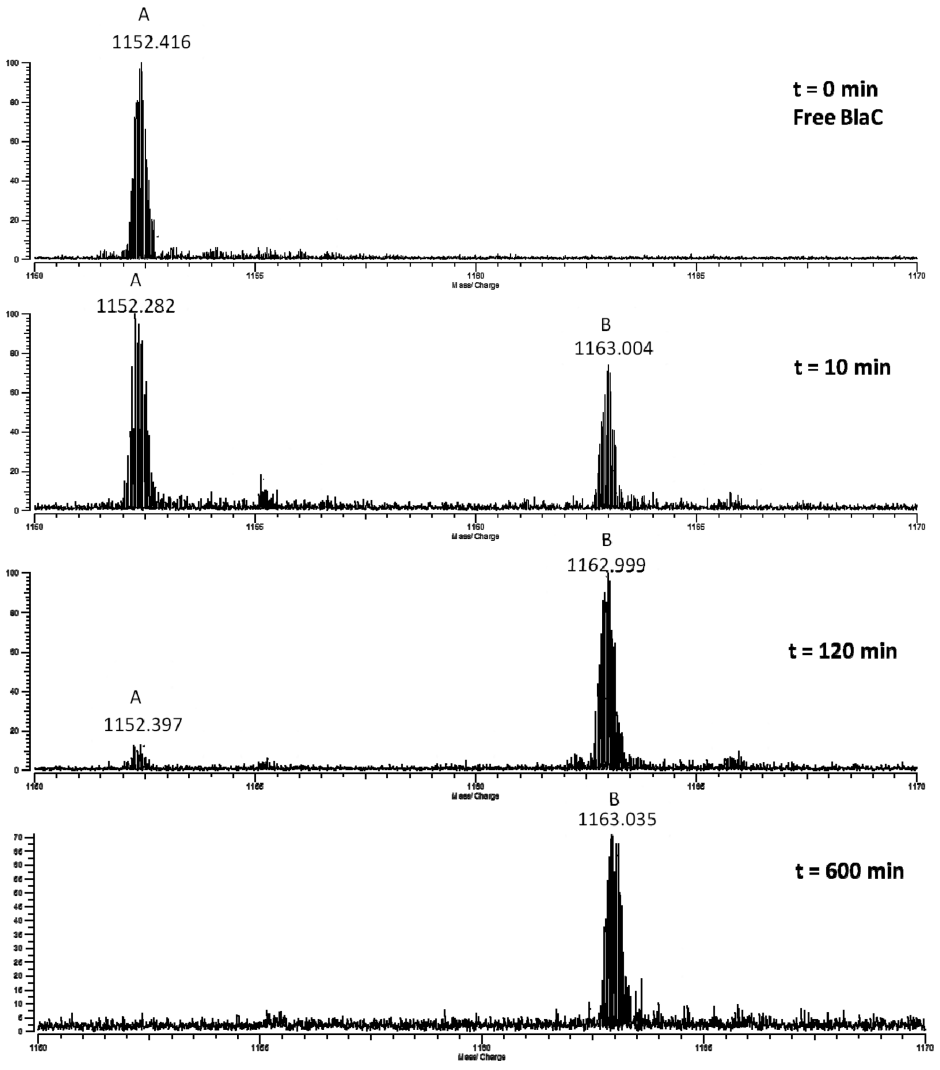

Figure 5. FT-ICR mass spectra of the BlaC−NXL104 covalent adduct. Only the +25 charge states are shown. Peak A is the free BlaC enzyme with an experimental molecular weight of 28784.95, while peak B corresponds to the BlaC−NXL104 adduct with an experimental molecular weight of 29050.12.

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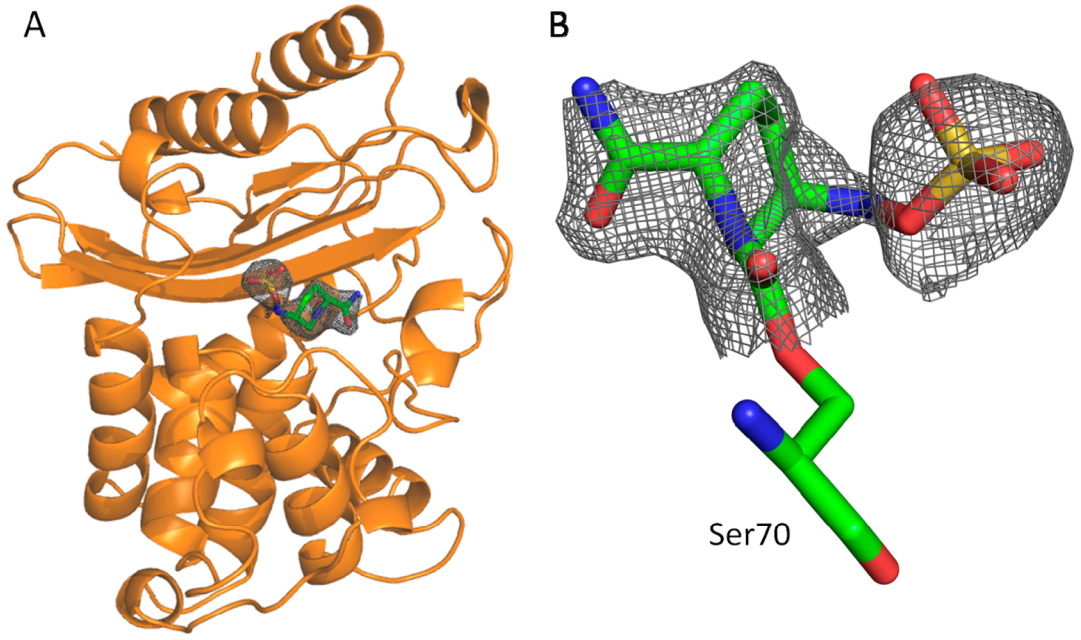

Figure 6. Crystal structure of the BlaC−NXL104 adduct. (A) Overall structure of BlaC with the NXL104 adduct displayed as gray mesh. (B) $F_o - F_c$ omit density (2.3 Å) of the BlaC−NXL104 adduct formed at the Ambler active site residue Ser70. These structures were produced with Pymol and contoured at 2.2σ.

BlaC with an equal amount of NXL104. Free BlaC showed an experimental molecular weight of 28784.95 (peak A in Figure 5). Ten minutes after incubation, peak B with a molecular weight of 29050.12 was observed (Figure 5). The molecular weight increase of 265.18 compared to that of the free enzyme corresponds to the addition of a ring-opened NXL104 molecule (predicted increase of 265.04), which further supports the fact that NXL104 inhibits BlaC by forming a covalent carbamyl linkage with the enzyme. The free enzyme was still observed after 2 h (Figure 5), confirming the unusually slow reaction of apo-BlaC and NXL104.

X-ray Crystallography. The extremely slow decarbamylation of the adduct allowed us to trap the carbamylated BlaC intermediate by soaking the apo BlaC crystal with NXL104. The three-dimensional structure was resolved at a resolution of 2.3 Å with an R_{work} of 0.170 and an R_{free} of 0.261 (Figure 6A and Table 1). In the structure, the nucleophilic active residue Ser70 is covalently linked to the ring-opened form of NXL104 (Figure 6B), substantiating the inhibition mechanism depicted in Figure 2B. The quality of the electron density of the ligand is shown under the calculated $F_o - F_c$ omit maps contoured at 2.2σ (Figure 6B).

To provide molecular insight into the unusually slow decarbamylation of the BlaC−NXL104 covalent intermediate, we superimposed the structures of the BlaC−NXL104 and BlaC−clavulanate adducts (PDB entry 3CG5). The two BlaC structures are quite similar with an rmsd of 0.2 Å. The conserved active site hydrolytic water molecule is seen in the BlaC−NXL104 adduct (water541 in Figure 7A). In addition, the distance between the carboxylate oxygen of E166 and the catalytic water in the BlaC−NXL104 adduct (2.7 Å) is quite close to that in the BlaC−clavulanate adduct [2.8 Å (Figure 7A)], as well as the distance between the water molecule and the carbonyl carbon of the adduct [2.8 Å in the BlaC−NXL104 adduct vs 3.1 Å in the BlaC−clavulanate adduct (Figure 7A)], ruling out the possibility that the slow decarbamylation is due to the exclusion of the hydrolytic water molecule from the active site or the misalignment of E166 and the water molecule. The sulfate group

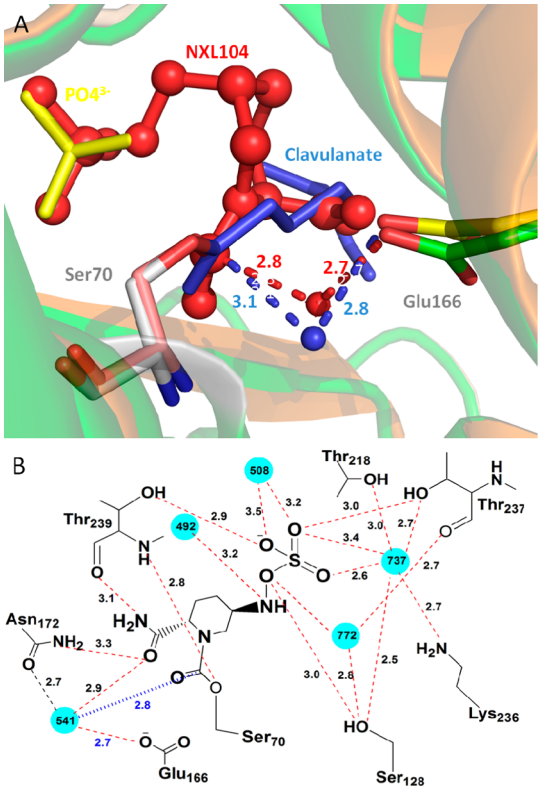

Figure 7. Structures of the BlaC active site. (A) Overlay of structures of the BlaC−NXL104 and BlaC−clavulanate adducts (PDB entry 3CG5). NXL104 is colored red, clavulanate blue, and phosphate ion from the clavulanate structure yellow. The distance between the carboxylate oxygen and catalytic water as well as the distance between the catalytic water and carbonyl carbon of the adduct is represented by a blue dashed line for the BlaC−clavulanate adduct and a red dashed line for the BlaC−NXL104 adduct. (B) Interactions between BlaC and NXL104. Water molecules are colored cyan. The distance between the catalytic water and carbonyl carbon of the adduct is represented by a blue dashed line.

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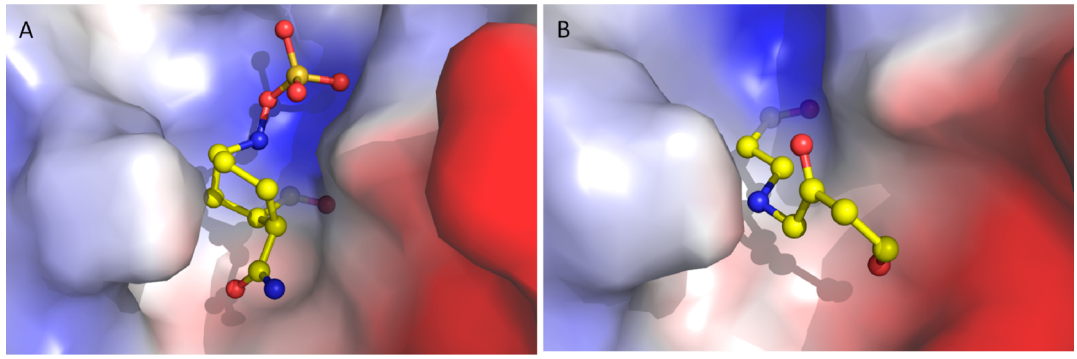

Figure 8. Calculated electrostatic interaction maps of the BlaC−NXL104 (A) and BlaC−clavulanate (B) adducts. Positive electrostatic potentials are colored blue and negative potentials red. Hydrophobic surfaces are colored white.

of NXL104 occupies the position of a nearby phosphate ion in the structure of the BlaC−clavulanate adduct and is hydrogen bonded to Ser128, Thr237, and Thr239 (Figure 7B and Table S1 of the Supporting Information). In addition, a number of favorable interactions are seen in the calculated electrostatic interaction map for the BlaC−NXL104 complex (Figure 8A). The piperidine ring of NXL104 is oriented toward the hydrophobic residues in the active site of BlaC. Moreover, the negatively charged sulfate moiety is oriented toward a positively charged region on the enzyme, and these interactions are absent in the BlaC−clavunalate complex (Figure 8B). We believe that these interactions that are absent in the BlaC−clavulanate adduct, together with the intrinsic stability of the carbamyl linkage, may contribute to the exceptional stability of the BlaC−NXL104 adduct. This information may be useful in designing NXL104 derivatives as novel BlaC inhibitors.

This work demonstrates that NXL104 quantitatively inactivates BlaC by forming a carbamyl adduct with the enzyme. The carbamyl adduct exhibits extraordinary stability beyond that observed with clavulanate. Unfortunately, the very low binding affinity of NXL104 for BlaC will likely preclude its use as a β-lactamase inhibitor that can be partnered with a β-lactam antibiotic for the treatment of tuberculosis.

■ ASSOCIATED CONTENT

Supporting Information

Interactions between NXL104 and clavulanate with BlaC (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BlaC, *M. tuberculosis* β-lactamase; FT-ICR, Fourier transform ion cyclotron resonance; PBP, penicillin-binding protein; rmsd, root-mean-square deviation; TB, tuberculosis.

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