Strategic Design of an Effective β-Lactamase Inhibitor: LN-1-255, a 6-Alkylidene-2’-Substituted Penicillin Sulfone

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ABSTRACT

In an effort to devise strategies for overcoming bacterial β-lactamases, we studied LN-1-255, a 6-alkylidene-2’-substituted penicillin sulfone inhibitor. By possessing a catecholic functionality that resembles a natural bacterial siderophore, LN-1-255 is unique among β-lactamase inhibitors. LN-1-255 combined with piperacillin was more potent against *Escherichia coli* DH10B strains bearing bla<sub>SHV</sub> extended-spectrum and inhibitor resistant β-lactamases than an equivalent amount of tazobactam and piperacillin. In addition, LN-1-255 significantly enhanced the activity of ceftazidime and cefpirome against extended-spectrum cephalosporin and Sme-1 containing carbapenem resistant clinical strains. LN-1-255 inhibited SHV-1 and SHV-2 β-lactamases with nM affinity ($K_i = 110 \pm 10$ and $100 \pm 10$ nM, respectively). When LN-1-255 inactivated SHV β-lactamases a single intermediate was detected by mass spectroscopy. To gain insight into the mechanism of inactivation of this potent inhibitor, the crystal structure of LN-1-255 in complex with SHV-1 was determined at 1.55 Å resolution. Interestingly, this novel inhibitor forms a bicyclic aromatic intermediate with its carbonyl oxygen pointing out of the oxyanion hole and forming hydrogen bonds with K234 and S130 in the active site. Electron density for the “tail” of LN-1-255 is less ordered and modeled in two conformations. Both conformations have LN-1-255’s carboxyl group interacting with R244, yet the remaining tails of the two conformations diverge. The observed presence of the bicyclic aromatic intermediate with its carbonyl oxygen positioned outside the oxyanion hole provides a rationale for the stability of this inhibitory intermediate. The 2’ substituted penicillin sulfone, LN-1-255, is proving to be an important lead compound for novel β-lactamase inhibitor design.

INTRODUCTION

The rapidly increasing number of antibiotic-resistant Gram-negative microorganisms, including the Enterobacteriaceae family and the *Pseudomonas, Acinetobacter*, and *Klebsiella* genera, represents a grave threat to human health. The first-line treatments for such infections are β-lactam antibiotics and the most common mechanism of resistance to such agents is bacterial production of β-lactamases. Regrettably, Enterobacteriaceae resistant to penicillins and extended-spectrum cephalosporins (e.g.,
ceftazidime, ceftriaxone and cefepime) are continuing to threaten the efficacy of our available \(\beta\)-lactam antibiotics (1-4). Currently, up to 30% of *Enterobacter* spp., *Escherichia coli*, and *Klebsiella* spp. recovered from patients with infections in hospitals, long-term care facilities, and intensive care units in the United States are resistant to ceftazidime (1,2,5-9). Most concerning, is the emerging number of community-acquired *E. coli* and *Klebsiella* spp. that are resistant to these cephalosporins (3,4,8-11). This latter group presents a very significant future danger to the current use of \(\beta\)-lactam antibiotics to treat common infections in the ambulatory setting (11).

Resistance to extended-spectrum cephalosporins in *E. coli* and *K. pneumoniae* is typically caused by a plasmid or chromosomal class A *bla* gene that encodes for an extended-spectrum \(\beta\)-lactamasmes (ESBLs) (1,5,12,13). Among the \(\beta\)-lactam class of antibiotics, only carbapenems are recommended for the therapy of infections caused by ESBL-producing bacteria (1,2,7). Many ESBL-producing bacteria possess \(\beta\)-lactamasmes that are susceptible to \(\beta\)-lactamase inhibitors, but the clinical efficacy of \(\beta\)-lactam/\(\beta\)-lactamase inhibitor combinations still remains to be conclusively established (5,12). \(\beta\)-Lactam/\(\beta\)-lactamase inhibitor combinations offer an extremely attractive approach to combating infections caused by ESBL-producing bacteria. Unfortunately, current commercial \(\beta\)-lactamase inhibitors (clavulanate, sulbactam and tazobactam) narrowly target class A enzymes. Thus, there is an urgent need for the design and development of more clinically useful \(\beta\)-lactam antibiotic/\(\beta\)-lactamase inhibitor combinations.

Early studies demonstrated that selected 6-alkylidene-2'-substituted penicillin sulfones and 7-alkylidene-3-substituted cephalosporin sulfones (Fig. 1A-B) are effective \(\beta\)-lactamase inhibitors (14-17). In an effort to assess the activity and understand the efficacy of these compounds, we investigated one of the most potent of these derivatives, a 6-alkylidene-2'-substituted penicillin sulfone, which has become known as LN-1-255 (18). This inhibitor was designed with a catecholic functionality which could potentially resemble a natural bacterial siderophore, thus enabling LN-1-255 to utilize the iron uptake system to traverse the outer membrane (18).

In this report, LN-1-255, was evaluated as a class A \(\beta\)-lactamase inhibitor and compared to tazobactam against SHV-1, an ESBL variant of SHV-1 (SHV-2), and selected "inhibitor resistant" SHV \(\beta\)-lactamasmes. In addition, we tested LN-1-255 as a broad-spectrum inhibitor against representative clinical isolates possessing a variety of \(\beta\)-lactamasmes. In order to elucidate the mechanism of inactivation, the crystal structure of SHV-1 in complex with LN-1-255 was determined at 1.55 Å resolution. We show that LN-1-255 inactivates SHV-1 by forming a bicyclic aromatic intermediate that resists deacylation.

**MATERIALS AND METHODS**

**Chemical synthesis**

The chemical structure of LN-1-255 and tazobactam are shown in Fig. 1. The synthesis, and initial evaluation, of LN-1-255 was reported (18) and reviewed (15) by Buynak et al..

**Bacterial strains, plasmids and mutagenesis to create ESBL variants of SHV-1**

As described previously, *bla*<sub>SHV-1</sub> was first cloned into phagemid vector pBC SK(-) (Stratagene, La Jolla, CA) from a clinical strain of *K. pneumoniae* 15571 and maintained in *E. coli* DH10B<sup>TM</sup> cells (Invitrogen, Carlsbad, CA) (19). This *E. coli* strain with *bla*<sub>SHV-1</sub> was used for minimum inhibitory concentration determinations (MICs), mutagenesis, and protein purification. The construction of *bla*<sub>SHV-2</sub>, *bla*<sub>SHV-5</sub> and other inhibitor resistant *bla*<sub>SHV</sub> enzymes was previously described (20-22).

**Antibiotic susceptibility**

The minimum inhibitory concentrations, MICs, of *Escherichia coli* DH10B expressing *bla*<sub>SHV</sub> \(\beta\)-lactamasmes were performed in Luria Bertani (LB) agar. MICs were determined using a “Steers Replicator” that delivered 10 µl containing 10<sup>4</sup> CFU/spot. The inhibitors were screened using piperacillin as the partner \(\beta\)-lactam. In the first set of MICs, both \(\beta\)-lactam/ \(\beta\)-lactamase inhibitors were tested using an 8/1 ratio of piperacillin/\(\beta\)-lactamase inhibitor. Piperacillin was obtained...
from (Sigma, St. Louis, MO) and sodium tazobactam was a kind gift from Wyeth Pharmaceuticals (Pearl River, New York).

Additional susceptibility testing was performed to assess the potency of LN-1-255 with candidate broad-spectrum β-lactam antibiotics using a collection of well-defined clinical isolates. These isolates represent a world-wide collection of important patient-derived strains that possess contemporary β-lactamases used in screening studies. Against these strains, MICs were performed according to Clinical Laboratory Standards Institute, CLSI, guidelines. Here, we used microdilution panels with cation adjusted Mueller Hinton broth. The β-lactam antibiotics and tazobactam employed here were acquired from Sigma Chemical Co. LN-1-255 was used in a fixed (4 µg/ml) ratio with ceftazidime, cefpirome, and meropenem. Tazobactam was tested at a fixed ratio of 4 µg/ml combined with increasing concentrations of piperacillin.

β-Lactamase purification
The SHV-1 and SHV-2 β-lactamases were harvested from E. coli DH10B according to a previously published method employing preparative isoelectric focusing, pIEF (20-22). Cell lysis was achieved with the addition of lysozyme and EDTA (Sigma). Crude enzyme preparations were further purified using pIEF. We assessed the homogeneity of these preparations of SHV-1 and SHV-2 using 5% stacking, 12% resolving sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE. Gels were stained with Coomassie brilliant blue R250 (Fisher, Pittsburgh, Pa.) to visualize proteins. In certain instances we were required to further purify SHV-1 and SHV-2 β-lactamases using size exclusion chromatography with a Pharmacia AKTA Purifier System (GE Healthcare, Piscataway, NJ). We employed a Hi Load 16/60 Superdex 75 column (GE Healthcare) and eluted with 20 mM phosphate buffered saline, PBS, (pH 7.4). We determined protein concentrations using Bio-Rad’s Protein Assay with bovine serum albumin as a standard.

Kinetic parameters
Kinetic parameters (V\text{max}, K\text{m}, k\text{cat}) for SHV-1 and SHV-2 β-lactamases were determined from initial steady state velocities using an Agilent™ 8453 Diode Array spectrophotometer. The kinetic determinations were performed at room temperature (23 ºC) in 20 mM PBS, at pH 7.4. V\text{max} and K\text{m} were obtained with non-linear least squares fit of the data (Henri-Michaelis equation) using Enzfitter™ (Biosoft Corporation, Ferguson, MO) (20).

We measured the ability of LN-1-255 to inhibit and inactivate SHV-1 and SHV-2. We first determined the dissociation constants for the preacylation complex (K_D) according to two published methods (23-26). Firstly, we performed a direct competition assay using 100 µM nitrocefin (ncf) (Becton Dickinson, Cockeysville, MD) as the indicator substrate (Δε 482 = 17,400 M⁻¹ cm⁻¹), 21 nM SHV-1 or SHV-2, and varying concentration of inhibitor (tazobactam 0.3→1.2 µM or LN-1-255 10→500 nM). After the data were obtained, the K_D was "corrected" according to the following equation to account for the affinity of ncf for SHV-1 and SHV-2 β-lactamases:

\[
K_D\text{(corrected)} = K_D\text{(observed)} / [1 + ([S] / K_m \text{ncf})] \quad \text{(Eq. 1)}
\]

Next, we also determined the K_D by measuring the initial velocity, v_i, in the presence of increasing concentrations of LN-1-255 (0→50 nM) or tazobactam (0→500 nM) against the colorimetric substrate ncf (0→100 µM). Care was taken to initiate the reaction with the addition of both ncf and the inhibitor and to limit the initial velocity to the first 5-10 seconds so that the only inhibition observed would be derived from formation of the noncovalent Henri complex. Three experimental determinations were made for each unique ncf/inhibitor concentration and progress curves in the presence of varying concentrations of inhibitor were analyzed by non-linear regression using a competitive model of inhibition and corrected as above. Each value was presented.

The first-order rate constant for enzyme and inhibitor complex inactivation, k_{inact}, was measured directly by monitoring the reaction time courses in the presence of inhibitor and ncf. A fixed concentration of enzyme E (21 nM), 100 µM ncf, and increasing concentrations of tazobactam (0.5→6 µM) or LN-1-255 (50→600
(inactivator, $I$) were used in each assay. The $k_{\text{obs}}$ values were determined using a non-linear least squares fit of the data to Equation 2 employing Origin 7.5®:

$$A = A_0 + v_0 t + (v_0 - v_f) \left[1 - \exp(-k_{\text{obs}} t)\right] / k_{\text{obs}} \quad \text{(Eq. 2)}$$

Here, $A$ is absorbance, $v_0$ (expressed in variation of absorbance per unit time) is initial velocity, $v_f$ is final velocity, and $t$ is time. Each $k_{\text{obs}}$ was plotted versus $I$ and fit to determine $k_{\text{inact}}$ and $K_I$ (27). The 24 hour turnover number, $t_n$, (i.e., partitioning of the initial enzyme inhibitor complex between hydrolysis and enzyme inactivation, $k_{\text{cat}} / k_{\text{inact}}$ or partition ratio) was determined as previously reported (23, 26, 28).

**Ultraviolet Difference (UVD) Spectroscopy**

According to a previously published method, UV difference spectra (absorption spectra of LN-1-255 reacted with SHV-1 and SHV-2 β-lactamases in a 1000:1 Inhibitor to Enzyme ($I : E$) ratio) were measured from $\lambda = 190$ to 400 nm (29, 30).

**Chelation protocol**

Five mls of 1 N HCl (Fisher Health Care, Pittsburgh, PA) was used to activate one gram of chelating resin (iminodiacetic acid) (Sigma Co.) in order to remove ambient Fe. HCl was then removed using 10 ml 20 mM PBS (pH 7.4) after which 5 ml of 1 M LN-1-255 in 20 mM PBS was passed thru the column. Flow through was collected and LN-1-255 recovery was measured based on inhibitory activity of SHV-1 vs. LN-1-255 which was not passed through the column. Recovery was approximately 60%. LN-1-255 passed thru the chelating resin was then used for mass spectrometry analysis.

**Electrospray ionization (ESI) Mass spectrometry (MS)**

ESI-MS of the intact SHV-1 and SHV-2 β-lactamases inactivated by LN-1-255 was performed on an Applied Biosystems (Framingham, MA) Q-STAR XL quadrupole-time-of-flight mass spectrometer equipped with a nanospray source as described previously (26). Experiments were performed by first desalting the reaction mixtures using C18 ZipTips (Millipore, Bedford MA) according to the manufacturer’s protocol. The protein sample was then diluted with acetonitrile/1% formic acid to a concentration of 10 µM. This protein solution was infused at a rate of 0.5 µL/min and data were collected for 2 min. Spectra were deconvoluted using the Applied Biosystems (Framingham, MA) Analyst program.

**Crystallization and data collection**

Crystals for X-ray diffraction analysis of SHV-1 β-lactamase were obtained as described previously (31, 32). One crystal was soaked for 24 h with 50mM LN-1-255 in mother liquor containing CYMAL-6 detergent also present in the crystallization condition. The crystal was cryo-protected in 30%MPD before immersing into liquid nitrogen for data collection. A 1.55 Å data set were collected at the NSLS synchrotron X-29 and processed using HKL2000 (33, 34). Refinement was carried out in CNS (35) and model building was done using COOT (36). After initial refinement and model fitting, strong density was observed for a bound bicyclic aromatic LN-1-255 intermediate covalently attached to S70. Topology and parameter files for this LN-1-255 ligand intermediate were obtained using PRODRG2 for further refinement in CNS (37). Two conformations were modeled for the LN-1-255 atoms beyond the bicyclic aromatic system with occupancies of 0.6 and 0.4, respectively as determined using B-factor refinement while varying their relative occupancies and monitoring difference density maps. The final model refined to an R/Rfree of 16.8/19.7% and contains residues 26-292, 248 water molecules, 1 complete and 1 partial CYMAL-6 molecule, and one LN-1-255 intermediate of which a subset of the atoms are in two alternative conformations.

**RESULTS**

**Antibiotic susceptibility**

We compared the in vitro efficacy of piperacillin/LN-1-255 to piperacillin/tazobactam (Table 1). Against the $\text{bla}_{\text{SHV-1}}$ and $\text{bla}_{\text{SHV-2}}$ in E. coli DH10B, susceptibility tests revealed that LN-1-255 was much more potent than an equal amount of tazobactam combined with piperacillin. In addition, we also demonstrated that LN-1-255 and piperacillin were very effective at lowering MICs against E. coli containing $\text{bla}_{\text{SHV-5}}$ and a
series of 5 different inhibitor resistant \textit{bla}_{SHV} enzymes (\textit{bla}_{SHV} S130G, R244S, M69I, M69L, and M69V). This broad effect is extremely notable because piperacillin/tazobactam does not lower MIC significantly when tested against \textit{E. coli} strains containing inhibitor resistant \textit{SHV} \(\beta\)-lactamases.

Further susceptibility testing was performed using LN-1-255 with ceftazidime, cefpirome, meropenem, and piperacillin. Against this selection of clinical isolates (\textit{E. coli} and \textit{K. pneumoniae}) that represent a Gram-negative bacteria harboring contemporary class A and D \(\beta\)-lactamase enzymes (e.g., TEM-10, SHV-5 and -7, OXA-2, and CTX-M-3 \(\beta\)-lactamases), LN-1-255 combined with ceftazidime and cefpirome markedly lowered MICs (Table 2). Moreover LN-1-255 improved the activity of ceftazidime against \textit{E. coli} and \textit{K. pneumoniae} containing the class C \(\beta\)-lactamases, CMY-6 and CMY-7. The overall effect seen here was substantial when compared to piperacillin alone and piperacillin/tazobactam. In contrast, improvement was not evident against AmpC cephalosporinase producing \textit{Enterobacter cloacae}, \textit{Citrobacter freundii}, and \textit{Serratia marcescens}.

The median MIC value for meropenem alone and in combination remained unchanged. Noteworthy, LN-1-255 combined with meropenem readily inhibited bacteria possessing the class A carbapenemase, Sme-1, produced by a strain of \textit{S. marcescens} (MIC decrease from 32 to 1 \(\mu\)g/ml). This represents an important extension of the spectrum of LN-1-255.

\textit{Kinetic parameters}

\textit{SHV-1} and \textit{SHV-2} \(\beta\)-lactamases were purified to homogeneity by pIEF and size exclusion chromatography. Using increasing concentration of \textit{ncf} as an indicator substrate in a competition reaction with increasing amounts of inhibitor, \(I\), we show that LN-1-255 inhibited \textit{SHV-1} and \textit{SHV-2} \(\beta\)-lactamases at lower concentrations than tazobactam: LN-1-255, \(K_D = 0.019 \pm 0.003\) \(\mu\)M and LN-1-255, \(K_D = 0.009 \pm 0.002\) \(\mu\)M, respectively (Table 3 and Supplemental Figs. 1a-1d). The alternative determination of the dissociation constants for the preacylation complex, \(K_D\), followed a similar trend (Table 3).

Comparing \(k_{\text{inact}}\) determinations, there was a highly significant difference between these 2 inhibitors. LN-1-255 inactivated \textit{SHV-1} and \textit{SHV-2} \(\beta\)-lactamases more rapidly than tazobactam (Table 3, Supplemental Figs 1e-1h). Although the \(K_I\) values reveal that both tazobactam and LN-1-255 are nM inhibitors, the \(k_{\text{cat}}/k_{\text{inact}}\) ratio explains why LN-1-255 inactivates \textit{SHV-1} and \textit{SHV-2} more “efficiently” than tazobactam in this test system. In determining the partition ratio, \(t_o\), of LN-1-255 for \textit{SHV-1} and for \textit{SHV-2}, we see that there is nearly stoichiometric inactivation: LN-1-255 and \textit{SHV-1}, \(k_{\text{cat}}/k_{\text{inact}} = 4\); LN-1-255 and \textit{SHV-2}, \(k_{\text{cat}}/k_{\text{inact}} = 0\).

There is a dramatic “real time” inhibition effect when adding the inhibitor LN-1-255 on the rate of hydrolysis of \textit{ncf} by \textit{SHV-2} \(\beta\)-lactamase: 7.5 fold less LN-1-255 inactivated \textit{SHV-2} more rapidly (note arrows) when compared to tazobactam. In addition, there is no apparent reactivation (see Supplemental Fig. 2).

\textit{UVD spectroscopy}

In previous work, UVD spectroscopy has been used to provide insight into the nature of reactive intermediates formed during the inactivation process (28,30,38). This approach was useful in detecting a major peak at 280 nm consistent with the formation of the \(\beta\)-aminoacrylate or enamine intermediate in our studies with clavulanate (38).

UVD spectroscopy measured every 12 s using LN-1-255 (Inhibitor, \(I\)) reacted with \textit{SHV-1} or \textit{SHV-2} \(\beta\)-lactamase (Enzyme, \(E\)) at an \(I/E\) ratio of 1000/1 identified intense chromophores at 258 nm and 307 nm that formed within 900 s and increased in intensity up to 1 h (Fig. 2a and 2b). We tentatively assign the chromophore at 307 nm to a conjugated aromatic ring (\textit{vide infra}).

\textit{ESI-MS}

To gain insight into the nature of the inactivation products, ESI-MS was performed with a Q-STAR quadrupole-time-of-flight mass spectrometer equipped with a nanospray source. The \(m/z\) analyses of the \textit{SHV-1} and \textit{SHV-2} \(\beta\)-lactamases inactivation experiments are summarized in Table 4. The MW of \textit{SHV-1} \(\beta\)-lactamase was determined as 28,871 \pm 3 Da (Fig. 3a). The mass of \textit{SHV-2} was 28,901 \pm 3 Da (Fig. 3b). ESI-MS measurements were in agreement with theoretical masses of \textit{SHV-1} and \textit{SHV-2} \(\beta\)-lactamases.
Using the partition ratios ($k_{cat}/k_{inact}$) as a guide, we inactivated SHV-1 and SHV-2 β-lactamases with a 10-fold excess of LN-1-255 (900 sec) in 20 mM PBS, pH 7.4, and removed any ambient Fe by passage through a chelation column. The deconvoluted spectra for LN-1-255 inactivated SHV-1 and SHV-2 β-lactamases are presented in Figs. 3a and 3b.

This analysis shows that there is covalent attachment of LN-1-255 to the SHV-1 and SHV-2 β-lactamases. In the time period studied, we did not find evidence of fragmentation of LN-1-255 as was seen in the inactivation of TEM-1, CMY-2, SHV-1, Arg244Ser and the Ser130Gly variant of SHV-1 with tazobactam and clavulanate (20,29,30,39). Taken together these mass spectrometry data indicate that there is formation of a stable inactivation product that corresponds to the addition of intact (unfragmented) LN-1-255 to SHV-1 and -2 β-lactamases.

**X-ray crystallographic refinement and structure analysis.**

The initial unbiased omit electron density map in the active site of SHV-1 revealed strong density of a covalently bound LN-1-255 molecule (Fig. 4). The formation of a LN-1-255 intermediate containing a bicyclic aromatic ring system was evident from the density holes for both the 6 and 5-membered ring depending on the density contour levels. This ring system is pointing towards the solvent yet makes van der Waals interactions with A237 and partially occludes the oxyanion hole. The carbonyl oxygen of this intermediate is therefore not located in the oxyanion hole and is involved in hydrogen bonds with the sidechains of K234 and S130 (Fig. 5). The tail of the LN-1-255 intermediate displays weaker electron density and is refined in two alternative conformations. The major conformation of LN-1-255’s tail has its catechol moiety in close proximity to the hydrophobic ring of one of the CYMAL-6 molecules (Fig. 5). This conformation agrees with, and is the only conformation, when SHV-1 is soaked with an analog of LN-1-255 that lacks the two hydroxyls of the catechol moiety (data not shown). The second (0.4 occupancy) conformation (Fig. 6) has its catechol moiety sandwiched in between the LN-1-255’s bicyclic aromatic ring system and Y105 but its density is also relatively weak.

**DISCUSSION**

Major developments in the 6-alkylidenedepenam sulfone structural series of β-lactamase inhibitors are shown in Fig. 7. The first example was the 6-benzylidenedepenicillin sulfone (Fig. 7A) reported by Foulds *et al.* (40). This was rapidly followed by the work of Chen *et al.* (41) demonstrating superiority of the 6-pyridylmethylidene analog (Fig. 7B). These authors proposed a general inhibitory mechanism shown in Fig. 8, based on a methanalysis of the inhibitor and isolation of the corresponding methyl ester. Buynak *et al.* (18) independently varied both the C6 and C2 substituents, again demonstrating that the optimal inhibitory substituent at C6 was the pyridylmethylidene. In addition, they also showed that substantially improved inhibitory activity could be obtained with selected C2 substituents (Fig. 7C), such as R = O2CCH2Ph. In addition, *in vitro* activity could be substantially improved by the catecholic functionality of LN-1-255 (18).

Current commercial β-lactamase inhibitors narrowly target the class A enzymes. A clinically useful new β-lactamase inhibitor must have a significantly broader inhibitory spectrum, presumably targeting all serine β-lactamases (classes A, C, and D). To achieve clinical efficacy, the inhibitor should meet specific molecular, microbiological, and physiological requirements. This new inhibitor will first need to be selectively recognized by the bacterial enzyme (i.e. have a high affinity for the active site) and, secondly, form a highly stabilized acyl-enzyme. Moreover, the inhibitor will need to demonstrate synergy with an appropriately selected β-lactam antibiotic, thereby significantly lowering MIC values. For Gram-negative microorganisms this will involve i) rapid penetration (of both inhibitor and antibiotic) through the outer membrane and ii) resistance to efflux. Lastly, the inhibitor/antibiotic combination will need to display appropriate pharmacokinetic and pharmacodynamic characteristics.
LN-1-255 clearly meets several of the criteria for an effective β-lactamase inhibitor. This compound satisfies the standards of: (i) efficacy by MIC testing against SHV-1, inhibitor resistant SHV variants, and ESBLs produced in laboratory and clinical strains; (ii) affinity for the active site of SHV-1 and ESBL variant enzymes, and (iii) stability of the resultant acyl-enzyme. It is also clear from kinetics, mass spectrometry, and X-ray crystallography that the dynamics and details of the inactivation process are unique when compared to commercially available inhibitors like tazobactam.

This work represents the first crystallographic confirmation of Chen’s proposed inhibitory mechanism in the penicillin series, the individual steps of which are shown in Fig. 8. Regarding the effect of the 2’-substituent, Buynak (18) initially observed a 10-100 fold improvement in the IC<sub>50</sub> values of specific 2’-substituted-6-alkylidenepencillins sulfones against representative class A and C enzymes, particularly when utilizing a phenylacetoxo substituent at C2’. Catecholic analogs were next designed (eg LN-1-255) to simultaneously take advantage of this improvement in activity and to facilitate passage through the outer membrane via the iron-uptake pathway. Recent data indicate that the improved enzymatic inhibitory activity may be due to tight binding in the initial Michaelis complex (27). As in the case of sulbactam, once the acyl-enzyme is formed, the now more highly basic amine nitrogen electrons facilitate the departure of the sulfinate sulfur, leading to fragmentation of the dioxothizolidine system, and formation of an intermediate imine. While in the case of sulbactam this imine would either tautomerize to the β-aminoacrylate (enamine) or serve as an electrophile to capture a second nucleophile (e.g., Ser130), in the case of LN-1-255, the favorable disposition of the basic pyridyl nitrogen atom allows rapid intramolecular capture. Loss of a proton from C5 results in the formation of the bicyclic 10 π electron aromatic indolizine sytem. The acyl-enzyme ester carbonyl is now stabilized not only by the adjacent aromatic system, but also by favorable resonance interactions with both nitrogens. Presumably the bulk and planar geometry demanded by the favorable π interactions in this bicyclic system result in movement to a different location in the active site, thereby removing the carbonyl oxygen from the oxyanion hole. Additionally, we observe favorable interactions with Tyr105 in SHV (see below).

Why does the combination piperacillin/LN-1-255 lower MICs more effectively when compared to piperacillin/tazobactam? The addition of the di-hydroxyl-phenyl catechol moiety may provide LN-1-255 with enhanced bacterial uptake via siderophore channels. This pathway is used by bacteria to incorporate iron that is complexed with bacterially synthesized siderophores (42). This feature can be exploited to improve drug uptake (14,15). Secondly, the addition of the di-hydroxyl-phenyl catechol moiety may improve affinity since this moiety has a large hydrophobic side and can stack onto aromatic protein residues that enhance binding by making additional hydrophobic and/or van der Waals interactions.

The impact of the catechol moiety is further highlighted when LN-1-255 is tested against clinical isolates. LN-1-255 enhances the efficacy of ceftazidime against isolates of <i>E. coli</i> containing TEM-1, TEM-10, TEM-12, and TEM-26 β-lactamases (the latter three are clinically important ESBLs) (Table 2) and isolates of <i>K. pneumoniae</i> containing SHV ESBLs, OXA-2, and CTX-M ESBLs. This dramatic increase effectively expands the spectrum of the partner cephem to equal that of a “fourth generation” cephalosporin (cefpirome). When combined with a “fourth generation” cephalosporin (cefpirome), LN-1-255 effectively expands the activity to approximately equal that of meropenem.

The observed bicyclic aromatic LN-1-255 intermediate is remarkably stable as demonstrated by mass spectrometry, UV difference absorption spectroscopy, and X-ray crystallography (Figs. 2-6). Two unique molecular features of this intermediate that are revealed by crystallography and explain the stability to deacylation. Of primary importance, the aromatic bicyclic moiety and the carbonyl oxygen are part of a large conjugated π system that stabilizes the acyl-enzyme complex against nucleophilic attack by the activated water molecule in the active site. Additionally, the carbonyl oxygen of the
intermediate is pushed out of the oxyanion hole; this is likely a result of this C=O moiety needing to be planar with the bicyclic aromatic ring system. A reoriented C=O group away from the oxyanion hole decreases the rate of deacylation for two reasons: a) this moiety is no longer primed, via the backbone NH hydrogen bonds in the oxyanion hole, to be receptive for nucleophilic attack by a water; and b) the carbonyl carbon atom position is now positioned ~1.7 Å further away from the deacylation water, a distance likely too large for efficient deacylation by a water molecule. Note that the carbonyl oxygen does not necessarily need to be positioned outside the oxyanion hole to slow down deacylation as observed for a cephem sulphone compound similar to LN-1-255 yielding a bicyclic aromatic intermediate with class C GC-1 β-lactamase (43). An additional difference between the LN-1-255 SHV-1 structure and the cephem sulphone class C structure is that the former intermediate is refined with having a planar bicyclic aromatic ring, in accord with the unbiased omit density (Fig. 4), whereas this aromatic group in the latter compound was not refined in a planar conformation.

It is important to communicate that the crystal structure of LN-1-255 covalently bound to SHV-1 provides inhibitory information, yet does not contribute insights into the initial (Henri-Michaelis) binding complex that likely determines the $K_i$. The obtained complex is that of a late-stage reaction intermediate after substantial molecular rearrangements have occurred (Figure 4-6).

Lastly, we emphasize that the binding of LN-1-255 also results in changes in the active site of SHV-1. To illustrate this, we superimposed SHV-1 bound LN-1-255 with our previously determined structure of SHV-1 bound SA-2-13 (32) (Fig. 6). This SA-2-13 bound structure is a high resolution 1.28 Å structure and SA-2-13 causes little active site changes except for forcing residue N170 into two conformations (one inward wt conformation priming the deacylation water, and an outward conformation). The superposition reveals that LN-1-255 bound to SHV-1 produces two significant active site shifts. The first is a shift in the S130 side chain position to accommodate the carbonyl oxygen of LN-1-255 which would otherwise yield a ~2.2 Å steric clash. Second, the (larger) LN-1-255 inhibitor pushes the loop region V216 outwards to accommodate the bicyclic aromatic double ring system (Fig. 6). These LN-1-255 induced active site shifts are intriguing and could possibly affect the rate at which the intermediate is formed ($k_{\text{max}}$) and also the rate at which it is deacylated ($k_{\text{cat}}$). In Scheme 1 (Fig. 9) we illustrate the major differences between tazobactam and LN-1-255.

In summary, LN-1-255 possesses key features that permit increased cell entry, enhances affinity and acylation efficiency for the active site, and slow deacylation. More widespread MIC testing of LN-1-255 against clinical strains is required; the affinity of LN-1-255 against other β-lactamases must be further studied. Nevertheless, the discovery of LN-1-255 is an important “first-step” towards defining characteristics for rapid cell penetration and optimal structure-activity relationships (SARs) in the quest for novel and versatile β-lactamase inhibitors.

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**TABLE 1.**

Minimum Inhibitory Concentration (µg/ml)

|                     | Piperacillin | Piperacillin/ | Piperacillin/ |
|---------------------|--------------|---------------|---------------|
|                     |              | Tazobactam    | LN-1-255      |
|                     |              | (8:1 ratio)   | (8:1 ratio)   |
| *E. coli* DH10B     | 2            | 2/0.25        | 2/0.25        |
| *bla*~SHV-1~        | 1024         | 512/64        | 32/4          |
| *bla*~SHV-2~        | 512          | 32/4          | 16/2          |
| *bla*~SHV-5~        | 1024         | 32/4          | 16/2          |
| *bla*~SHV-S130G~    | 128          | 128/16        | 64/8          |
| *bla*~SHV-R244S~    | 64           | 32/4          | 32/4          |
| *bla*~SHV-M69I~     | 256          | 64/8          | 16/2          |
| *bla*~SHV-M69L~     | 256          | 256/32        | 16/2          |
| *bla*~SHV-M69V~     | 256          | 256/32        | 16/2          |

According to the Clinical Laboratory Standards Institute (www.clsi.org) the breakpoints for piperacillin and piperacillin/tazobactam are: MICs ≤ 8 µg/ml is susceptible; 8-16 µg/ml is intermediate and ≥ 32 µg/ml is resistant.
### Table 2

Minimum Inhibitory Concentration (µg/ml)

| Genotype | Ceftazidime | Ceftazidime + LN-1-255 at 4 µg/ml | Cefpirome | Cefpirome + LN-1-255 at 4 µg/ml | Meropenem | Meropenem + LN-1-255 at 4 µg/ml | Piperacillin | Piperacillin + Tazobactam at 4 µg/ml |
|----------|--------------|----------------------------------|-----------|---------------------------------|-----------|---------------------------------|-------------|-----------------------------------|
| E. coli  | TEM-1        | 0.12                             | 0.12      | ≤0.06                           | ≤0.06     | ≤0.06                           | 128         | 2                                 |
|          | TEM-10       | >128                             | 1         | 2                               | 0.12      | ≤0.06                           | >128        | 16                                |
|          | TEM-12       | 16                               | 1         | 2                               | 0.25      | ≤0.06                           | >128        | 4                                 |
|          | TEM-26       | >128                             | 1         | 4                               | 0.12      | ≤0.06                           | >128        | 4                                 |
| K. pneumonia | SHV-3      | 64                               | 2         | 32                              | 0.12      | ≤0.06                           | >128        | >128                              |
|          | SHV-5        | >128                             | 0.5       | 8                               | 0.12      | ≤0.06                           | >128        | 64                                |
|          | SHV-5-1      | >128                             | 64        | 16                              | 8         | ≤0.06                           | >128        | >128                              |
|          | SHV-7, OXA-2 | 128                              | ≤0.06     | 2                               | ≤0.06     | ≤0.06                           | >128        | 4                                 |
|          | SHV-18       | 64                               | 1         | 2                               | 0.5       | ≤0.06                           | >128        | 16                                |
| K. pneumonia | CTX-M-3     | 32                               | 8         | 64                              | 16        | ≤0.06                           | >128        | 64                                |
|          | CTX-M-14     | 16                               | 2         | 64                              | 16        | ≤0.06                           | >128        | 32                                |
|          | CTX-M-24     | 2                                | 0.12      | 4                               | ≤0.06     | ≤0.06                           | 128         | 2                                 |
| E. cloacae | AmpC        | 64                               | 64        | 2                               | 1         | ≤0.06                           | >128        | 128                               |
| C. freundii | AmpC       | 64                               | 64        | 2                               | 0.12      | 0.12                            | >128        | >128                              |
| S. marcescens | AmpC    | 128                              | 64        | 1                               | 0.12      | ≤0.06                           | 16          | 8                                 |
| E. coli  | CMY-2        | 128                              | 64        | 4                               | 2         | 0.12                            | >128        | 64                                |
|          | CMY-6        | 128                              | 2         | >128                            | 0.5       | 0.12                            | >128        | 64                                |
| K. pneumonia | CMY-7     | >128                             | 4         | >128                            | 32        | ≤0.06                           | >128        | 128                               |
| S. marcescens | Sme-1    | 0.25                             | 0.25      | ≤0.06                           | 0.12      | 32                              | 1           | 32                                |

According to the Clinical Laboratory Standards Institute (www.clsi.org) breakpoints for ceftazidime are: MICs ≤ 8 µg/ml is susceptible; 8-16 µg/ml is intermediate and ≥ 32 µg/ml is resistant. For meropenem: MICs ≤ 4 µg/ml is susceptible; 8 µg/ml is intermediate and ≥ 16 µg/ml is resistant. Standards for cefpirome are not available in the US. We will regard MICs ≤ 8 µg/ml for cefpirome as susceptible; 8-16 µg/ml as intermediate and ≥ 32 µg/ml as resistant.
|            | Tazobactam | LN-1-255 |
|------------|------------|----------|
| $K_D^a$ $\mu$M |            |          |
| SHV-1      | 0.333 ± 0.033 | 0.027 ± 0.002 |
| SHV-2      | 0.182 ± 0.018 | 0.015 ± 0.002 |
| $K_D^b$ $\mu$M |            |          |
| SHV-1      | 0.206 ± 0.019 | 0.019 ± 0.003 |
| SHV-2      | 0.041 ± 0.009 | 0.009 ± 0.002 |
| $K_I$ $\mu$M |            |          |
| SHV-1      | 0.200 ± 0.035 | 0.11 ± 0.01 |
| SHV-2      | 0.030 ± 0.003 | 0.10 ± 0.01 |
| $k_{inact}$ s⁻¹ |      |        |
| SHV-1      | 0.14 ± 0.01  | 0.55 ± 0.03 |
| SHV-2      | 0.030 ± 0.003 | 0.71 ± 0.07 |
| $k_{inact}/K_I$ s⁻¹ $\mu$M⁻¹ |      |        |
| SHV-1      | 0.70 ± 0.12  | 5.0 ± 0.7  |
| SHV-2      | 1.00 ± 0.14  | 7.1 ± 1.1  |

|        | $t_n$ |
|--------|-------|
| SHV-1  | 9     |
| SHV-2  | 9     |

$^a$ $K_D$ measured using a direct completion reaction

$^b$ $K_D$ measured using progress curves
|                  | Mass spectrometry analysis (amu) |
|------------------|----------------------------------|
| LN-1-255         | 488                              |
| SHV-1 β-lactamase| 28,871 ± 4                       |
| SHV-1 β-lactamase + LN-1-255 | 29,359 ± 4                   |
| Δ Difference    | 488 ± 4                          |
| LN-1-255         | 488                              |
| SHV-2 β-lactamase| 28,901 ± 4                       |
| SHV-2 β-lactamase + LN-1-255 | 29,389 ± 4                   |
| Δ Difference    | 488 ± 4                          |
### Table 5
Data collection and refinement statistics for SHV-1:LN-1-255 structure

| Category                                      | Details                                           |
|-----------------------------------------------|---------------------------------------------------|
| Space group                                   | P2₁2₁2₁                                          |
| Unit cell dimensions (Å)                      | 49.61, 55.50, 83.50, 90, 90, 90                  |
| Wavelength (Å)                                | 1.1                                              |
| Resolution (Å)                                | 50-1.55 (1.61-1.55)                              |
| Redundancy                                    | 5.3                                              |
| Data cut-off (σ)                              | -3.0 (default)                                   |
| Unique reflections                            | 33,525                                           |
| <I>/<σ(I)>                                    | 20.8 (2.1)                                       |
| R<sub>merge</sub> (%)                         | 10.1 (41.0)                                      |
| Completeness (%)                              | 97.8 (96.3)                                      |

**Refinement**

| Category                                      | Details                                           |
|-----------------------------------------------|---------------------------------------------------|
| Resolution range (Å)                          | 50-1.55 (1.57-1.55)                              |
| Atoms in asymmetric unit                      | 6,174                                             |
| R-factor (%)                                  | 16.8 (28.1)                                      |
| R-free (%)                                    | 19.7 (30.7)                                      |
| RMSD deviations from ideality                 |                                                  |
| Bond lengths (Å)                              | 0.0094                                            |
| Angles (°)                                    | 1.57                                              |
| Average temperature factors (Å²)              |                                                  |
| Protein                                       | 14.9                                              |
| Cymal-6 molecules                             | 26.5                                              |
| LN-1-255                                      | 27.7                                              |
| Waters                                        | 30.7                                              |

**Ramanchandran plot statistics**

| Category                                      | Details                                           |
|-----------------------------------------------|---------------------------------------------------|
| Residues in -most favored regions            | 92.6%                                             |
| -additional allowed regions                   | 7.4%                                              |
| Region Type          | Percentage |
|---------------------|------------|
| Generously Allowed  | 0%         |
| Disallowed          | 0%         |
Figure legends

Fig. 1. Chemical structures of the β-lactamase inhibitors. A, general structure of 7-alkylidene-3-substituted cephalosporin sulfones; B, general structure of 6-alkylidene-2'-substituted penicillin sulfones; C, LN-1-255; and D, tazobactam sodium.

Fig. 2. UVD spectroscopy of SHV-1 (Fig. 2a) and SHV-2 (Fig. 2b) reacted with LN-1-255 and the formation of chromophores at 258 nm and 307 nm.

Fig. 3. Deconvoluted mass spectrometry of SHV-1, LN-1-255 inactivating SHV-1 (3a), and SHV-2, LN-1-255 inactivating SHV-2 (3b).

Fig. 4. Electron density of LN-1-255 in the active site of SHV-1 β-lactamase. Unbiased omit |Fo|-|Fc| difference density of LN-1-255 covalently bound to S70 contoured at 2.5σ (red) and 1.6σ (green) levels. The LN-1-255 ligand density shows clear density for the bicyclic aromatic double-ring moiety, yet the atoms beyond this moiety are modeled in two conformations (grey and black/thin) as the density is more consistent with two conformations (occupancies of 0.6 and 0.4, respectively). The catechol moiety of both conformations display poor electron density and their position cannot be accurately modeled. The CYMAL-6 molecules (yellow) are also depicted.

Fig. 5. Interactions of LN-1-255 in active site of SHV-1 β-lactamase. The bicyclic aromatic double-ring system forms a planar ring, and its conjugated acyl group attached to S70 is also found to be in the same plane. The carbonyl oxygen atom of this acyl moiety makes hydrogen bonds with K234 and S130. The remaining atoms of the LN-1-255 intermediate are modeled in two conformations (green and purple). Part of one of the CYMAL-6 molecules (yellow) is also depicted.

Fig. 6. Superposition of SHV-1 complexes with LN-1-255 and SA-2-13 bound. LN-1-255 is shown in green and purple color for the two conformations, SA-2-13 is depicted in black, and the SHV-1 protein atoms are in gray and black (thin lines), respectively. The superposition reveals that binding of LN-1-255 to SHV-1 affects the active site in several positions compared to SA-2-13. These shifts (indicated by red arrows) include the side chain of S130 and the loop region near V216 which shifts away to accommodate
LN-1-255 binding. In addition, minor difference is that LN-1-255 keeps residue N170 in the wt conformation, whereas SA-2-13 forces N170 to be in two conformations with the second being an outward conformation. Finally, the side chain of Y105 has also somewhat shifted.

Fig. 7. Evolution of the 6-alkyldenopenam sulfone structural series of β-lactamase inhibitors

Fig. 8. Proposed mechanism of enzymatic inhibition by the 6-(pyridylmethylidene)penicillin sulfone series of inhibitors.

Fig. 9. (Scheme 1). Model of the kinetic differences between LN-1-255 and tazobactam.
Fig. 1
Fig. 2

**SHV-1:LN-1-255 (1:1000)**

**SHV-2:LN-1-255 (1:1000)**
Fig. 3a

- SHV-1: Mass = 28,871 ± 3
- SHV-1 + LN-1-255: Mass = 29,359 ± 3
Fig. 4
Fig. 5
Fig. 7
Fig. 8
Scheme 1

Fig. 9
Strategic design of an effective β-lactamase Inhibitor: LN-1-255, a 6-alkylidene-2'-substituted penicillin sulfone

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