

Mechanistic Studies of the Inactivation of TEM-1 and P99 by NXL104, a Novel Non- β -Lactam β -Lactamase Inhibitor[∇]

Thérèse Stachyra,¹ Marie-Claude Péchereau,¹ Jean-Michel Bruneau,¹ Monique Claudon,¹ Jean-Marie Frère,² Christine Miossec,¹ Kenneth Coleman,¹ and Michael T. Black^{1*}

Novoxel SA, Parc Biocitech, 102 Avenue Gaston Roussel, 93230 Romainville, France,¹ and CIP/Enzymology, Université de Liège, Institute of Chemistry B6, Sart-Tilman B4000 Liège, Belgium²

Received 26 April 2010/Returned for modification 25 July 2010/Accepted 25 September 2010

NXL104 is a potent inhibitor of class A and C serine β -lactamases, including KPC carbapenemases. Native and NXL104-inhibited TEM-1 and P99 β -lactamases analyzed by liquid chromatography-electrospray ionization-time of flight mass spectrometry revealed that the inactivated enzymes formed a covalent adduct with NXL104. The principal inhibitory characteristics of NXL104 against TEM-1 and P99 β -lactamases were determined, including partition ratios, dissociation constants (K), rate constants for deactivation (k_2), and reactivation rates. NXL104 is a potent inhibitor of TEM-1 and P99, characterized by high carbamylation efficiencies (k_2/K of $3.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for TEM-1 and $1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for P99) and slow decarbamylation. Complete loss of β -lactamase activity was obtained at a 1/1 enzyme/NXL104 ratio, with a k_3 value (rate constant for formation of product and free enzyme) close to zero for TEM-1 and P99. Fifty percent inhibitory concentrations (IC_{50} s) were evaluated on selected β -lactamases, and NXL104 was shown to be a very potent inhibitor of class A and C β -lactamases. IC_{50} s obtained with NXL104 (from 3 nM to 170 nM) were globally comparable on the β -lactamases CTX-M-15 and SHV-4 with those obtained with the comparators (clavulanate, tazobactam, and sulbactam) but were far lower on TEM-1, KPC-2, P99, and AmpC than those of the comparators. In-depth studies on TEM-1 and P99 demonstrated that NXL104 had a comparable or better affinity and inactivation rate than clavulanate and tazobactam and in all cases an improved stability of the covalent enzyme/inhibitor complex.

The β -lactam antibiotics constitute the largest and most diverse structural class of antibiotic. Gram-negative bacteria have become resistant to β -lactam antibiotics mainly as a result of mechanisms for reducing the concentration of inhibitor at the target (penicillin-binding proteins). Although such mechanisms include drug efflux and reduction in permeation of the drug through the outer membrane, hydrolytic inactivation of the antibiotic by β -lactamases is the most widespread and is the most problematic clinically although all mechanisms are frequently combined in Gram-negative β -lactam-resistant organisms.

The Ambler classification scheme in its evolved form separates β -lactamases into four classes, of which A, C, and D are serine hydrolases, and B encompasses metallo- β -lactamases (16). During several decades, not only have the class A and C enzymes become widely disseminated so as to become the most widespread causes of β -lactam antibiotic-resistant Gram-negative infections in Europe and North America, but many mutant forms have also evolved which are capable of hydrolyzing the expanded-spectrum β -lactam antibiotics. The class A enzymes are mainly plasmid encoded, of which the first to be described at amino acid sequence level were the enzymes TEM-1 and TEM-2 (1, 39). These and closely related enzymes have given rise over the years to inhibitor-resistant TEM variants which possess various levels of resistance to inhibition by clinically available β -lactamase inhibitors such as clavulanic acid. The class C enzymes include P99, a chromosome-en-

coded cephalosporinase from *Enterobacter cloacae* P99 (26) that was identified as a clavulanate-resistant enzyme more than 30 years ago (31). The structural and enzymological properties of both enzymes and their naturally occurring and laboratory-generated mutant variants have been intensively studied for many years, and thus they present as model enzymes for investigation of inhibition by novel compounds (8, 17, 24, 37, 38).

The β -lactamase inhibitors currently available, coadministered with a β -lactam antibiotic, are clavulanic acid (CLA), tazobactam (TZB), and sulbactam (SUL), all of which structurally are β -lactam inhibitors. Although such inhibitors have been of considerable clinical utility, they all have relatively limited activity against the class C enzymes and against some class A enzymes such as the clinically important KPC carbapenemases (for excellent recent reviews of β -lactamase inhibitors, see references 2, 10, 30, and 32).

NXL104 [*trans*-7-oxo-6-(sulfoxy)-1,6-diazabicyclo[3.2.1]octan-2-carboxamide; also previously known as AVE 1330A] (4) is the first of a series of diaza-bicyclo-octane β -lactamase inhibitors (Fig. 1). NXL104 has been demonstrated to restore the activity of β -lactam antibiotics against bacterial strains expressing class C enzymes and KPC carbapenemases (12, 23, 36). It is the first and only non- β -lactam inhibitor of β -lactamases to advance to the clinical phase of drug development and is currently undergoing phase II clinical trials in combination with ceftazidime (<http://www.clinicaltrials.gov/>). As NXL104 is not a β -lactam, it was considered of interest to define the principal features of interaction of NXL104 with the TEM-1 and P99 β -lactamases. A subset of experiments was also performed with additional β -lactamases

* Corresponding author. Mailing address: Novoxel SA, Parc Biocitech, 102 Avenue Gaston Roussel, 93230 Romainville, France. Phone: 33 6 04478583. E-mail: mick.black128@gmail.com.

[∇] Published ahead of print on 4 October 2010.

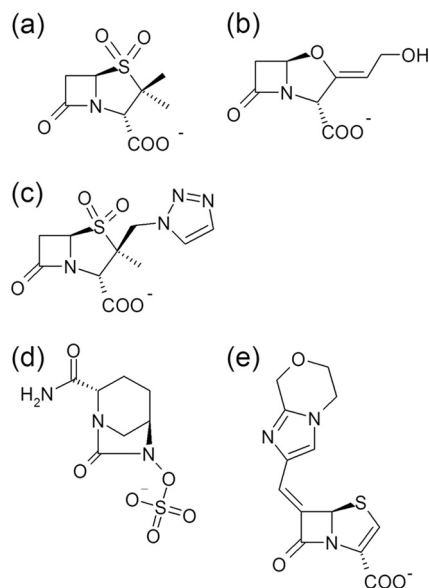


FIG. 1. Molecular structures of β -lactamase inhibitors: SUL (a), CLA (b), TZB (d), NXL104 (d), and BLI-489 (e).

and with the inhibitors TZB, SUL, and CLA for purposes of comparison.

MATERIALS AND METHODS

Protein purification. A pET-24 vector containing the sequence encoding TEM-1 fused to the leader sequence of OmpA was used to overexpress the enzyme (35). TEM-1 was purified from the culture supernatant (33). After ammonium sulfate precipitation between 35 and 70% saturation, the protein precipitate was solubilized in 50 mM Na-acetate, pH 7.5, and subjected to zinc chelate chromatography. TEM-1 was eluted with 50 mM Na-acetate (pH 4)–0.5 M NaCl buffer. The Zn chelate eluate was concentrated and reduced to a volume of 1.5 ml by dialysis and loaded onto a Superdex 75 16/60 (GE Healthcare) chromatography column previously equilibrated in 50 mM HEPES–150 mM NaCl, pH 7.5. After separation, the enzyme was finally concentrated to 7.4 mg \cdot ml⁻¹. Purity was >95% as measured by SDS-PAGE.

β -Lactamase from *E. cloacae* P99 was prepared after disruption by French press and purified by phenyl boronic acid affinity chromatography (7) and ion exchange chromatography to a purity of >95% as measured by SDS-PAGE, at 6.5 mg \cdot ml⁻¹.

KPC-2 β -lactamase was purified from a periplasm extract of an *Escherichia coli* BL21 strain overexpressing a pET-29-encoded protein by chromatography on Prosep-PB glass beads (Millipore). Elution was achieved using a 20 mM Tris (pH 8)–0.5 M sorbitol buffer. After concentration/dialysis with 20 mM morpholineethanesulfonic acid (MES), pH 5.5, buffer, the KPC-2 enzyme was purified using cation exchange chromatography on Resource-S (GE Healthcare), followed by gel filtration on a Superdex 75 column as for TEM-1. Purity was >98% as measured by SDS-PAGE.

The sequence encoding the β -lactamase CTX-M-15 was cloned in pET29 and overexpressed in *E. coli* BL21. After 24 h of isopropyl- β -D-thiogalactopyranoside (IPTG) induction, cells were collected, and CTX-M-15 was purified from the periplasmic fraction. The periplasm extract was loaded on a Q-Sepharose fast-flow (GE Healthcare) column previously equilibrated with 10 mM Tris-HCl (pH 9.0), 0.5 mM MgCl₂, and 1 mM EDTA (osmotic shock buffer). The unbound fraction was concentrated/dialyzed with a 20 mM MES (pH 6) buffer and loaded onto a Resource-S chromatography column. Proteins were eluted with a linear NaCl gradient. Fractions containing CTX-M-15 were pooled, concentrated to 1.5 ml, and subjected to gel filtration on a Superdex 75 column, as for TEM-1. Purity was estimated to be >98% by SDS-PAGE.

Pseudomonas aeruginosa AmpC β -lactamase was provided by J.-D. Docquier (University of Siena), and SHV-4 β -lactamase was provided by F. Michoux (Aventis).

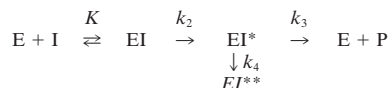
β -Lactamase activity and IC₅₀ determination. Enzyme activity was quantitated by spectrophotometric measurement of nitrocefin (NCF) hydrolysis at 485 nm and at 37°C ($\Delta\epsilon = 20,500 \text{ M}^{-1} \text{ cm}^{-1}$). NCF was present at 100 μM , TEM-1 was at 0.25 nM, and P99 was at 0.2 nM in 50 mM phosphate buffer, pH 7.0, with 0.1 g \cdot liter⁻¹ bovine serum albumin (buffer A) in a final volume of 200 μl . Initial rates were monitored for 2 min on a microplate reader (SpectraMaxPlus; Molecular Devices) which was used also for determination of the 50% inhibitory concentration (IC₅₀), partition ratio, and deacylation determination.

IC₅₀s were determined in buffer A after a 5-min preincubation of the enzyme with compound at 37°C. Final concentrations of β -lactamase in the assays were 0.25, 0.35, 3, 6, 0.2, and 0.4 nM for TEM-1, CTX-M-15, KPC-2, SHV-4, P99, and AmpC, respectively. NXL104, clavulanic acid from USP Rockville, and tazobactam and sulbactam from Sigma were tested at 11 concentrations from 1 nM to 100 μM . Initial rates of NCF hydrolysis were determined during the first minute of reaction during which the rates were constant. Data were processed using Grafit (Erithacus Software Ltd.). All kinetic parameters presented in this paper are mean values obtained from at least two independent experiments.

Partition ratio determination. Enzyme inactivation was performed at 37°C with 1 μM TEM-1 or P99, various molar inhibitor/enzyme ratios, and by a 5- or 30-min incubation with inhibitor. Enzyme activity was measured after a subsequent 4,000-fold (TEM-1) or 5,000-fold (P99) dilution. A plot of fractional activity against the ratio of $[I]/[E]$, where $[I]$ is the concentration of inhibitor and $[E]$ is the concentration of enzyme, was constructed in order to determine the partition ratio. The intercept with the abscissa gives the partition ratio.

Acylation/carbamylation reaction: determination of the dissociation constants (K , K_m , or K_i) and the inactivation rate constants [k_2 or $(k_i)_{\text{lim}}$]. The dissociation constant K (k_{-1}/k_{+1}) and inactivation rate constant k_2 (k_{inact}) were calculated and processed according to the reporter substrate method (9) by spectrophotometric measurement of NCF hydrolysis in the presence of various concentrations of inhibitor.

Scheme 1 represents a simple inactivation pathway (horizontal route):



When $[I]$ is much larger than the total enzyme concentration $[E]_0$, then in the presence of the reporter substrate, equation 1 applies:

$$k_{\text{obs}} = k_3 + k_2[I]/\{[I] + K(1 + [S]/K_{m,S})\} \quad (1)$$

where k_{obs} is the apparent pseudo-first-order rate constant, $[S]$ is the concentration of substrate, and k_3 is the rate constant for generation of product and free enzyme. If k_3 is $\ll k_{\text{obs}}$, K and k_2 can be determined from a plot of $[I]/k_{\text{obs}}$ against $[I]$.

When EI^* progresses by an alternative postacylation route to formation of a stable inhibited complex, EI^{**} (branched pathway, vertical route), at a rate characterized by k_4 (27), the partition ratio is $(k_3 + k_4)/k_4$. The k_{cat} and K_m values, characteristic of the horizontal route, have the usual values and can be determined with short incubation times at low inactivator/enzyme ratios while inactivation experiments performed at high inactivator/enzyme ratios and with longer incubation times yield $(k_i)_{\text{lim}}$ according to the following: $k_{\text{obs}} = (k_i)_{\text{lim}} [I]/([I] + K_m)$, where K_m is that of the horizontal route and $(k_i)_{\text{lim}} = k_2 k_4 / (k_2 + k_3 + k_4)$ or $k_2 k_4 / (k_2 + k_3)$ since k_4 is usually much less than k_3 (15). Moreover, $(k_i)_{\text{lim}}/K_m = k_2 k_4 / k_3 K$.

A direct competition assay was performed at 37°C to determine the dissociation constant, K and k_2 (with NXL104), or the K_m value and $(k_i)_{\text{lim}}$ of the horizontal branch (with TZB or CLA). For that, k_{obs} was evaluated: various concentrations of inhibitor were mixed with 100 μM NCF, and reactions were started by the addition of 0.25 nM TEM-1 or 0.2 nM P99 in a final volume of 500 μl . Kinetics were followed during 60 s. k_{obs} was deduced from time courses of NCF hydrolysis (9). Measurements were performed on a UV-2501PC Shimadzu spectrophotometer. From the same experiments, the dissociation constant for the preacylation complex, K_i , could also be determined. In this case, the initial velocity (v_0) was evaluated during the first 5 s of NCF hydrolysis, and K_i was determined with the help of equation 2. The $v_{0\text{control}}$ corresponds to the initial velocity in the absence of inhibitor:

$$v_{0\text{control}}/v_0 = \{K_m \text{NCF}(1 + [I]/K_i) + [\text{NCF}]\}/\{K_m \text{NCF} + [\text{NCF}]\} \quad (2)$$

Deacylation/decarbamylation reaction: determination of the reactivation rate constant (k_3). The time course of recovery of enzymatic activity was measured after complete inactivation by excess inhibitor. Enzyme (1 μM) was incubated for

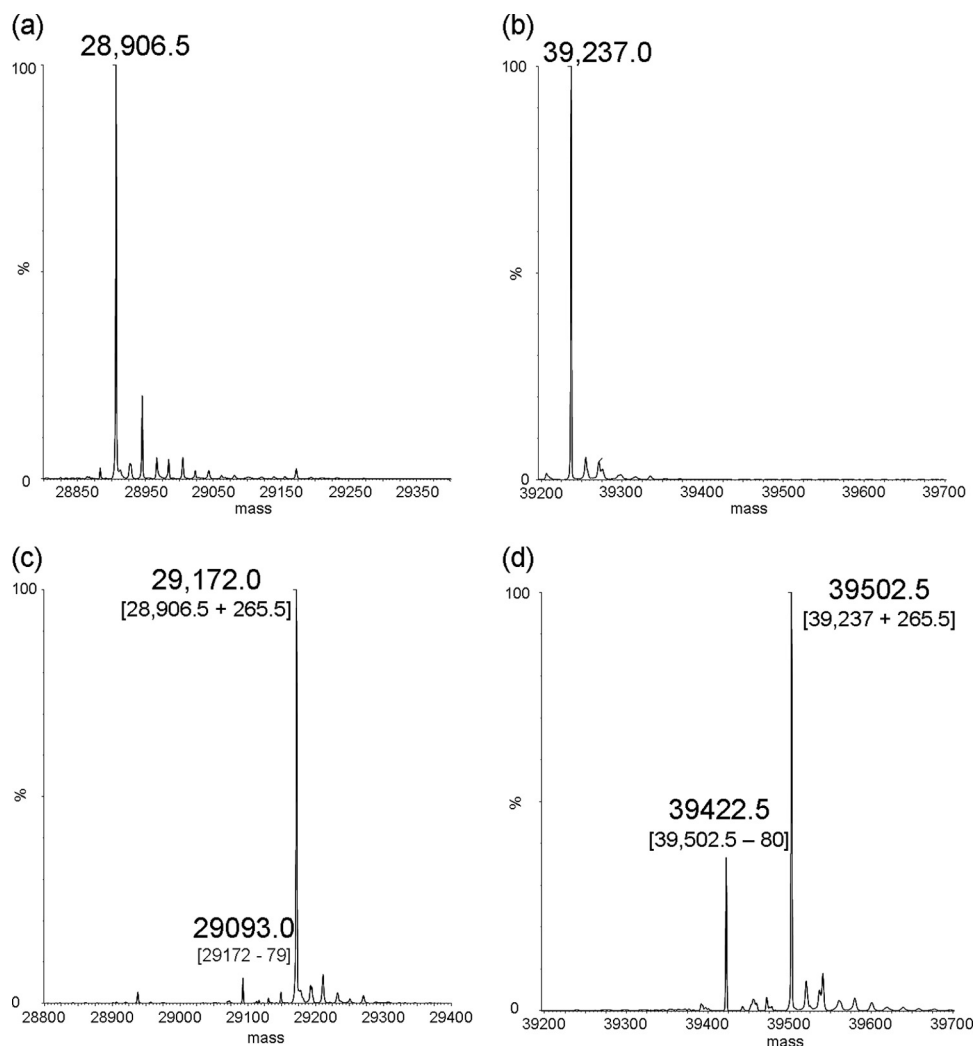


FIG. 2. Mass spectra of native (a) and inhibited (c) TEM-1 β -lactamase and of native (b) and inhibited (d) P99 β -lactamase.

10 min with NXL104 (5 μ M) or with CLA or TZB (0.5 mM). Excess inhibitor was removed by successive ultrafiltrations by centrifugation (cutoff of 5 kDa; Biomax; Millipore), such that less than 0.02 μ M free inhibitor remained. Samples were withdrawn at regular intervals and immediately diluted 4,000-fold to obtain 0.25 nM TEM-1 and 5,000-fold to obtain 0.2 nM P99, and recovery of β -lactamase activity was measured in 100 μ M solutions of NCF.

Characterization of the production of a covalent enzyme-inhibitor complex. TEM-1 or P99 was incubated at 40 μ M for 15 min at 37°C in the presence and absence of 4 mM NXL104, dialyzed at 4°C, and analyzed by electrospray ionization-quadrupole time of flight (ESI-Q-TOF) mass spectrometry (MS). Under the same conditions, a time dependence experiment was performed with P99/NXL104. Samples were withdrawn after 5-min, 30-min, 2-h, and 24-h periods of enzyme incubation with NXL104 and analyzed by ESI-Q-TOF MS.

Hydrolysis of TZB by TEM-1. A UV difference spectrum of native and hydrolyzed TZB was obtained by complete β -lactam hydrolysis of a 200 μ M solution of TZB with FEZ-1 metallo- β -lactamase or KPC-2 in order to ensure reaction completion. An extinction coefficient at 233 nm was determined ($\Delta\epsilon_{233}$ of 2,500 $M^{-1} cm^{-1}$). Hydrolysis of TZB by TEM-1 was followed by monitoring the variation in the absorbance of the β -lactam solution in buffer A on a UV-2501PC Shimadzu spectrophotometer and in a final volume of 500 μ l.

Mass spectrometry (ESI-Q-TOF). The molecular masses of the enzymes were determined in positive-ion mode by electrospray ionization mass spectrometry on a Q-TOF Ultima global (Quattro Ultima mass spectrometer; Waters). Proteins were analyzed in 50% acetonitrile, 25 mM ammonium acetate, and 0.5% formic acid (final concentration). Raw spectra were de-

convoluted by the method of maximum entropy, using the MaxEnt1 program of MassLynx, version 4.0.

RESULTS

Production of a β -lactamase-NXL104 covalent complex demonstrated by LC-ESI-Q-TOF. The steady-state kinetic parameters of purified P99 and TEM-1 were first evaluated using NCF as substrate. k_{cat} and K_m were $480 \pm 30 s^{-1}$ and $54 \pm 11 \mu$ M, respectively, for TEM-1, and $450 \pm 90 s^{-1}$ and $53 \pm 3 \mu$ M, respectively, for P99. Native TEM-1 or P99 and NXL104-inhibited TEM-1 or P99 were separated and characterized using liquid chromatography (LC)-ESI-Q-TOF. The deconvoluted mass spectra of the chromatographic peaks of the enzymes are shown in Fig. 2. The molecular masses of the native TEM-1 and NXL104-inhibited TEM-1 β -lactamases measured by ESI-Q-TOF were 28,906.5 and 29,172 Da, respectively. Those of the native P99 and NXL104-inhibited P99 β -lactamases were 39,237 and 39,502.5 Da, respectively. For each enzyme, this represents a mass increase of 265.5 Da. This additional mass corresponds to the molecular mass of

TABLE 1. Concentrations of inhibitors required to reduce active enzyme concentration by 50% after a 5-min enzyme-inhibitor preincubation

Enzyme class and name	IC ₅₀ (μ M) ^a			
	CLA	TZB	SUL	NXL104
Class A				
TEM-1	0.058	0.032	1.56	0.008
CTX-M-15	0.012	0.006	0.23	0.005
KPC-2	>100	50	57	0.170
SHV-4	0.004	0.055	0.26	0.003
Class C				
P99	>100	1.3	21.1	0.1
AmpC ^b	>100	4.6	27	0.128

^a Standard deviation of <20% for all IC₅₀s except CTX-M-15, for which the standard deviation was 0.082 μ M.

^b From *P. aeruginosa*.

NXL104, indicating that the inhibitor was covalently attached in the NXL104- β -lactamase complex. For TEM-1 in the presence of NXL104, a minor peak with a molecular mass of 29,093 Da corresponds to the molecular mass of a NXL104-TEM-1 complex with a loss of 79 ± 1 Da (\equiv SO₃). For P99, an equivalent peak is observed with a molecular mass of 39,422.5 Da corresponding to the molecular mass of a NXL104-P99 complex with a loss of 80 ± 1 Da (\equiv SO₃).

The proportion of 39,422.5 Da species did not change when samples were incubated for various times between 5 min and 24 h. However, when a sample corresponding to a 5-min incubation was analyzed by LC-ESI-Q-TOF at different collision energies, the peak area of the entity corresponding to 39,423 Da increased proportionally, demonstrating that the loss of 80 Da mass was an artifact due to MS and not a spontaneous degradation of NXL104 in the active site.

IC₅₀ determination on classes A and C β -lactamases. IC₅₀s are not entirely appropriate for assessing suicide substrates as the values are time dependent. However, they can be useful for initial ranking of compounds provided that experimental conditions are carefully controlled. The IC₅₀s derived from a 5-min exposure of NXL104, TZB, CLA, and SUL to four class A and two class C β -lactamases are shown in Table 1.

Against all four class A enzymes, NXL104 displays the lowest IC₅₀, and SUL has the highest although in some cases the apparent difference in potency is insignificant. With respect to SHV-4 and CTX-M-15, NXL104 has a value very similar to that of CLA or to TZB, respectively, and IC₅₀s that are improved by less than 10-fold against TEM-1. The activity against KPC-2, however, is very substantially greater than that of any of the β -lactam inhibitors, all of which have inadequate activity against KPC-2 (29), corresponding to an improvement in the IC₅₀ of >300-fold. The activity against KPC is particularly interesting as the KPC enzymes of the four recognized class A carbapenemase groups currently pose the greatest clinical threat and have been disseminated widely within the *Enterobacteriaceae* and have recently been detected in *P. aeruginosa* (43).

In addition, NXL104 is considerably more active than the β -lactam inhibitors against both class C enzymes and is the only compound with a submicromolar IC₅₀. The NXL104 IC₅₀s are improved more than 10-fold compared with the TZB IC₅₀

and by 2 and 3 orders of magnitude compared with SUL and CLA IC₅₀s, respectively. These data are consistent with the demonstrated capacity of NXL104 to restore the antibiotic susceptibility of *Enterobacteriaceae* bearing KPC-2, AmpC, and extended-spectrum β -lactamases (ESBLs) (12, 23, 36).

Partition ratio. Figure 3 shows that both TEM-1 and P99 are titrated by NXL104 with a complete loss of activity at a 1/1 ratio, indicating the validity of scheme 1 with a k_3 value close to zero. A potential rearrangement of EI* to EI** cannot be visualized by this method. In fair agreement with the published data (6, 13) the partition ratios were >150 for TZB/TEM, >100 for CLA/TEM, and about 11 for TZB/P99. In the latter case, there was a clear time dependence of the inactivation, indicating a rather slow reaction. CLA did not significantly inactivate P99. With respect to the partition ratio, NXL104 is thus a more efficient inactivator since very little, if any, turnover takes place.

Determination of the kinetic constants of β -lactamase inactivation. From the k_{obs} values, the parameters characteristic of the inactivation reactions could be derived [i.e., k_2 and K for NXL104 and both enzymes and $(k_i)_{\text{lim}}$ and K_m in the other cases] (Table 2). With TZB, the K_i value (which corresponds in fact to the K_m value of the horizontal branch) was evaluated at 0.030 ± 0.004 μ M, in excellent agreement with that measured in the inactivation experiments (0.014 μ M) (Table 2). When TZB was treated as a substrate, its hydrolysis was monitored at 233 nm over very short incubation periods (less than 60 s). The maximal rate of hydrolysis of TZB by TEM-1 was significant (V_{max} of 3.6 μ M \cdot min⁻¹ at 0.8 μ M TEM-1) with a calculated k_{cat} value [for the horizontal branch again, i.e., $k_2k_3/(k_2 + k_3)$] of 0.07 ± 0.006 s⁻¹.

Determination of the kinetic constants of β -lactamase reactivation. The extent of reactivation of P99 and TEM-1 subsequent to inhibition by NXL104 was very low (Fig. 4). A recovery of less than 10% activity was effected after ~10 to 15 min, after which the covalent complex was stable, with no further significant recovery of activity during 24 h. In contrast, the reactivation of P99 following TZB inhibition was a monophasic first-order process with a half-life ($t_{1/2}$) of ~5 h ($k_3 = 0.14 \pm 0.03$ h⁻¹). CLA was also studied as an alternative comparator against TEM-1. In this case there was a rapid recovery of ~40% activity that was complete after 15 min, followed by a much slower phase during 24 h wherein no, or a very small proportion of, additional activity was recovered.

DISCUSSION

Although NXL104 is not a β -lactam, a structural similarity to the β -lactam inhibitors around the carbonyl group is evident (Fig. 1). This immediately suggests inhibition of β -lactamases by a similar mechanism involving the formation of a stable covalent adduct. The MS data presented in Fig. 2 are consistent with this proposal. Hydrolysis of β -lactam antibiotics by, or interaction of β -lactam inhibitors with, the serine β -lactamases involves the nucleophilic attack of an active-site serine O γ (Ser70 according to the TEM numbering system) on the carbonyl carbon of the β -lactam ring, resulting in formation of the acyl enzyme. In the case of interaction with antibiotics that are considered substrates, the deacylation rate is very substantial, and the active enzyme is rapidly regenerated, whereas

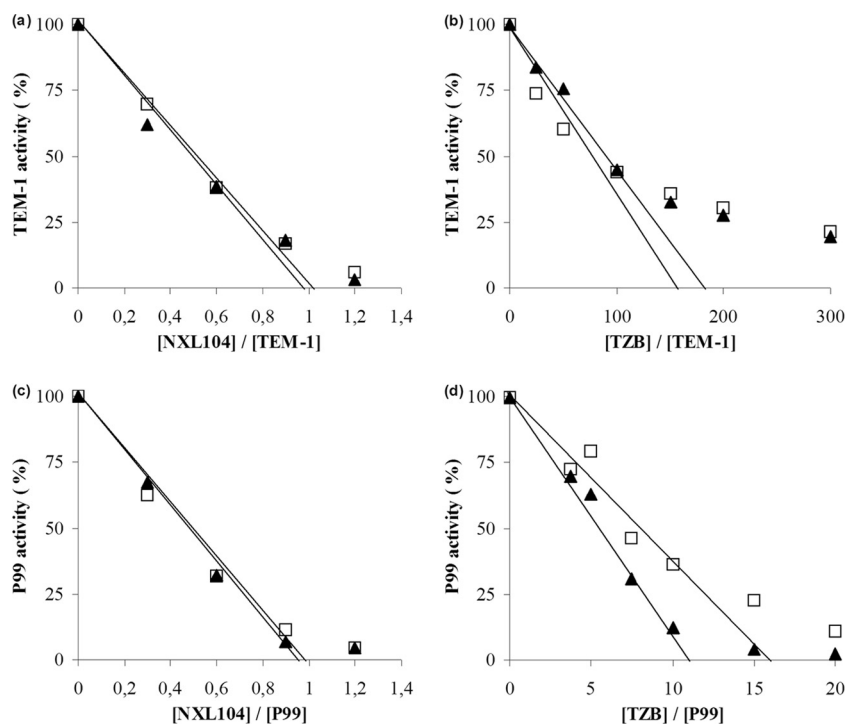


FIG. 3. Inactivation of TEM-1 and P99 by NXL104 (a and c) and TZB (b and d) at various inhibitor/enzyme ratios after 5-min (□) or 30-min (▲) incubation. Each point of these graphs is the mean value obtained from at least two independent experiments.

interaction with β -lactam inhibitors results in an acyl enzyme complex of substantially greater stability.

However, in this case NXL104 is not bound via an acyl group but by a carbamyl link (Fig. 5). A comparison of the kinetic parameters of NXL104 inhibition of β -lactamases with those of the β -lactam inhibitors indicates that the unusually high level of potency of NXL104 as characterized by IC_{50} s and the full inactivation of TEM and P99 at a 1/1 ratio was to a great extent due to the temporal stability of the carbamyl enzyme. The very small extent of reactivation of TEM-1 and P99 following inhibition with NXL104, which may result from experimental error, presents the possibility of a heterogeneity in the decarbamylation pathway. Heterogeneity in decylation pathways of each of the β -lactam inhibitors with various β -lactamases has been well documented. There are multiple branch points after

acylation with CLA, SUL, or TZB, and the acyl enzyme intermediate can undergo reversible changes (e.g., tautomerization) or hydrolysis to yield active enzyme or can form a permanent acyl enzyme species by various mechanisms, e.g., by further covalent modification or by displacement of hydrolytic water molecules (2, 10, 14, 30, 32). Of particular relevance to the temporal stability of the acyl enzymes seems to be the formation of a relatively high proportion of *trans*-enamine species as opposed to the more labile *cis*-enamines and imines (18, 19, 40, 41), species which are of course not possible with NXL104. A β -lactam inhibitor has reached phase I clinical trials (BLI-489), a methyldene bicyclic penem. This inhibitor rearranges to form a seven-membered dihydrothiazepine product which leads to low occupancy, disorder, or poor activation of the hydrolytic water molecule after formation of this post-

TABLE 2. Principal kinetic parameters associated with inhibition of TEM-1 and P99 β -lactamases by NXL104, TZB, and CLA as derived from the k_{obs} values^a

Enzyme	Inactivator	PR ^b	k_2 (s ⁻¹)	$(k_i)_{lim}$ (s ⁻¹)	K (μ M) ^c	K_m (μ M) ^c	k_2/K (M ⁻¹ s ⁻¹)	$(k_i)_{lim}/K_m$ (M ⁻¹ s ⁻¹)
TEM-1	NXL104	—	0.11		0.3 (0.26)		370,000	
TEM-1	TZB	>150		0.02		0.014 ^d (0.030)		1,400,000
TEM-1	CLA	>100		0.03		0.5 ^e (0.8)		60,000
P99	NXL104	—	0.05		5.1 (7.7)		10,000	
P99	TZB	11		0.15		300 (170)		500

^a Standard deviation of <20% for all the values except as noted.

^b PR, partition ratio. This ratio does not apply (—) to a simple linear scheme where stability of the EI complex results from a k_3 value that is close to zero. If a rearrangement does in fact take place with NXL104 (rate constant k_4), then the k_3/k_4 ratio values for both P99 and TEM-1 would be close to zero according to the data shown in Fig. 3a and c. NXL104 obeys scheme 1 with $k_3 = 0$ (or close to) for both enzymes; TZB and CLA obey the branched-pathway model.

^c The value in parentheses is the K_i value obtained by competition with nitrocefirin during a very short incubation (5 s).

^d Standard deviation of 0.006 μ M.

^e Standard deviation of 0.2 μ M.

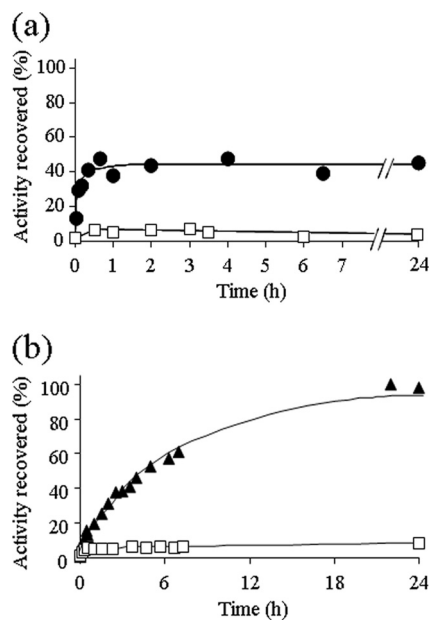


FIG. 4. Time course of TEM-1 (a) and P99 (b) reactivation following quasi-total enzyme inhibition by NXL104 (□), CLA (●), or TZB (▲). Typical curves are presented in the graphs. Each experiment was performed three times, and the shapes of the curves were similar in all cases.

acylation adduct (28). Thus, it seems that in most cases where a β -lactam inhibitor has achieved an advanced status, postacylation rearrangements play a major role in stabilization of the covalent adduct.

Although the formation of covalent adducts with TEM-1 and P99 indicates no subsequent loss of mass, it is not possible to exclude the possibility of transitions after ring opening that may impact the capacity of the enzyme to hydrolyze the carbamyl linkage, e.g., disruption of the interactions required for activation of the hydrolytic water or simply exclusion of the water.

Mechanisms of hydrolysis of amide and ester bonds that require the generation of a nucleophile at the active site, often a serine hydroxyl or a cysteine thiol, are a recurring theme in biology. Not only is the canonical serine protease catalytic triad of Ser-His-Asp found operative in lipases, cholinesterases, fatty acid hydrolases, and cholesterol esterases (for example), but some serine proteases also possess alternatives to the canonical triad and include variants with Lys as the active-site base (3, 11), as might be the case with some β -lactamases. Similarly, and perhaps predictably, it is known that the conservation of mechanism is reflected in commonalities of a mechanism-based inhibitor class. For example fluoroketones, boronic acids, β -lactones, and β -lactams are known to inhibit a variety of "serine/cysteine enzymes," not just proteases (20). Among such inhibitors are various alkyl- and aryl-carbamates and ureas responsible for the mechanism-based inhibition of enzymes by formation of carbamyl enzymes. Among these enzymes are fatty acid amide hydrolase (34, 42), monoacylglycerol lipase (25), acetyl cholinesterase (21), and *Pseudomonas* species lipase (22). In all of these cases the enzyme forms a covalent carbamyl enzyme via the active-site serine, including

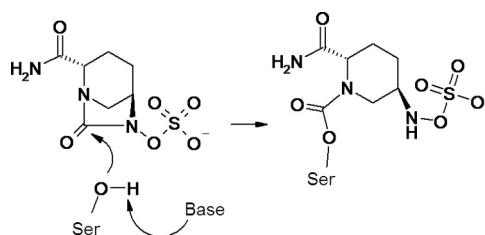


FIG. 5. Adduct formed between NXL104 and the active-site serine of β -lactamases.

in some cases via a substituted piperidine carbamyl link (25) as is the case with NXL104. Although some quantitative data are available concerning the carbamylation rate, little seems to be available concerning stability of the carbamyl enzyme. One exception to this is a study of the decarbamylation of cathepsin K, a member of the papain cysteine protease family, following inhibition by a 1,5-diacylcarbohydrazide (5). As for NXL104, the inhibitor is not itself a carbamate but forms a carbamyl linkage after collapse of the tetrahedral intermediate although in the case of cathepsin K the link is via a sulfur, and it is via oxygen in the case of the β -lactamases (and therefore in the latter case, *stricto sensu*, a carbamoyl linkage). The decarbamylation of cathepsin K proceeded at a low rate ($t_{1/2}$ of 11.5 min) that was approximately the same rate as for cathepsin S and for papain, suggesting that the decarbamylation is governed by the innate stability of the carbamyl bond rather than specific interactions at the various active sites of the carbamyl enzymes. Such appears not to be the case with NXL104 and β -lactamases as the decarbamylation rates are several orders of magnitude lower. An explanation for the low rate of decarbamylation is not known with certainty. Contributory factors may include the stability of the carbamyl link relative to an acyl link, exclusion of the hydrolytic water during carbamylation, or disruption of the network of interactions required for activation of the hydrolytic water. Combinations of all of these and additional unknown factors may play a role, and the relative importance of such factors may vary widely according to the enzyme in question, both within and between serine β -lactamase classes. The details of the decarbamylation and reactivation process will require an intense structural biology effort in order to increase understanding.

ACKNOWLEDGMENTS

We thank S. Petrella (University of Pierre and Marie Curie, Paris, France) and W. Sougakoff (Pitié-Salpêtrière Hospital, Paris, France) for the KPC-2 expression plasmid, J.-D. Docquier (University of Siena) for *P. aeruginosa* AmpC, and François Michoux (Sanofi-Aventis) for SHV-4.

We gratefully acknowledge the skilled contribution of M.-A. Meuwis and G. Mazzuchelli of the GIGA proteomic facility and laboratory of mass spectrometry, CART, at the University of Liège, Belgium, for collection of the mass spectrometry data.

REFERENCES

1. Ambler, R. P., and G. K. Scott. 1978. Partial amino acid sequence of penicillinase coded by *Escherichia coli* plasmid R6K. Proc. Natl. Acad. Sci. U. S. A. **75**:3732–3736.
2. Bassetti, M., E. Righi, and C. Viscoli. 2008. Novel beta-lactam antibiotics and inhibitor combinations. Expert Opin. Invest. Drugs **17**:285–296.
3. Black, M. T. 1993. Evidence that the catalytic activity of prokaryote leader peptidase depends upon the operation of a serine-lysine catalytic dyad. J. Bacteriol. **175**:4957–4961.

4. Bonnefoy, A., C. Dupuis-Hamelin, V. Steier, C. Delachaux, C. Seys, T. Stachyra, M. Fairley, M. Guittin, and M. Lampilas. 2004. *In vitro* activity of AVE1330A, an innovative broad-spectrum non-beta-lactam beta-lactamase inhibitor. *J. Antimicrob. Chemother.* **54**:410–417.
5. Bossard, M. J., T. A. Tomaszek, M. A. Levy, C. F. James, M. J. Huddleston, J. Briand, S. Thompson, S. Halpert, D. F. Veber, S. A. Carr, T. D. Meek, and D. G. Tew. 1999. Mechanism of inhibition of cathepsin K by potent, selective 1,5-diacylcarbohydrazides: a new class of mechanism-based inhibitors of thiol proteases. *Biochemistry* **38**:15893–15902.
6. Bush, K., C. Macalintal, B. A. Rasmussen, V. J. Lee, and Y. Yang. 1993. Kinetic interactions of tazobactam with beta-lactamases from all major structural classes. *Antimicrob. Agents Chemother.* **37**:851–858.
7. Cartwright, S. J., and S. G. Waley. 1984. Purification of beta-lactamases by affinity chromatography on phenylboronic acid-agarose. *Biochem. J.* **221**:505–512.
8. Charlier, P., O. Dideberg, J. M. Frère, P. C. Moews, and J. R. Knox. 1983. Crystallographic data for the beta-lactamase from *Enterobacter cloacae* P99. *J. Mol. Biol.* **171**:237–238.
9. De Meester, F., B. Joris, G. Reckinger, C. Bellefroid-Bourguignon, J. M. Frère, and S. G. Waley. 1987. Automated analysis of enzyme inactivation phenomena. Application to beta-lactamases and DD-peptidases. *Biochem. Pharmacol.* **36**:2393–2403.
10. Drawz, S. M., and R. A. Bonomo. 2010. Three decades of beta-lactamase inhibitors. *Clin. Microbiol. Rev.* **23**:160–201.
11. Ekici, O. D., M. Paetzel, and R. E. Dalbey. 2008. Unconventional serine proteases: variations on the catalytic Ser/His/Asp triad configuration. *Protein Sci.* **17**:2023–2037.
12. Endimiani, A., Y. Choudhary, and R. A. Bonomo. 2009. *In vitro* activity of NXL104 in combination with beta-lactams against *Klebsiella pneumoniae* isolates producing KPC carbapenemases. *Antimicrob. Agents Chemother.* **53**:5599–5601.
13. Fisher, J., R. L. Charnas, and J. R. Knowles. 1978. Kinetic studies on the inactivation of *Escherichia coli* RTEM beta-lactamase by clavulanic acid. *Biochemistry* **30**:2180–2184.
14. Fisher, J. F., S. O. Meroueh, and S. Mobashery. 2005. Bacterial resistance to beta-lactam antibiotics: compelling opportunism, compelling opportunity. *Chem. Rev.* **105**:395–424.
15. Frère, J.-M., C. Dormans, V. M. Lenzini, and C. Duyckaerts. 1982. Interaction of clavulanate with the beta-lactamases of *Streptomyces albus* G and *Actinomadura R39*. *Biochem. J.* **207**:429–436.
16. Hall, B. G., and M. Barlow. 2005. Revised Ambler classification of beta-lactamases. *J. Antimicrob. Chemother.* **55**:1050–1051.
17. Jelsch, C., F. Lenfant, J. M. Masson, and J. P. Samama. 1992. Beta-lactamase TEM1 of *E. coli*. Crystal structure determination at 2.5 Å resolution. *FEBS Lett.* **299**:135–142.
18. Kalp, M., C. R. Bethel, R. A. Bonomo, and P. R. Carey. 2009. Why the extended-spectrum beta-lactamases SHV-2 and SHV-5 are “hypersusceptible” to mechanism-based inhibitors. *Biochemistry* **48**:9912–9920.
19. Kalp, M., M. A. Totir, J. D. Buynak, and P. R. Carey. 2009. Different intermediate populations formed by tazobactam, sulbactam, and clavulanate reacting with SHV-1 beta-lactamases: Raman crystallographic evidence. *J. Am. Chem. Soc.* **131**:2338–2347.
20. Lin, G., W. C. Liao, and S. Y. Chiou. 2000. Quantitative structure-activity relationships for the pre-steady-state inhibition of cholesterol esterase by 4-nitrophenyl-N-substituted carbamates. *Bioorg. Med. Chem.* **8**:2601–2607.
21. Lin, G., W. C. Liao, C. H. Chan, Y. H. Wu, H. J. Tsai, and C. W. Hsieh. 2004. Quantitative structure-activity relationships for the pre-steady state acetylcholinesterase inhibition by carbamates. *J. Biochem. Mol. Toxicol.* **18**:353–360.
22. Lin, M. C., C. P. Lu, Y. R. Cheng, Y. F. Lin, C. S. Lin, and G. Lin. 2007. Inhibition or activation of *Pseudomonas* species lipase by 1,2-ethylene-di-N-alkylcarbamates in detergents. *Chem. Phys. Lipids* **146**:85–93.
23. Livermore, D. M., S. Mushtaq, M. Warner, C. Miossec, and N. Woodford. 2008. NXL104 combinations versus *Enterobacteriaceae* with CTX-M extended-spectrum beta-lactamases and carbapenemases. *J. Antimicrob. Chemother.* **62**:1053–1056.
24. Lobkovsky, E., E. M. Billings, P. C. Moews, J. Rahil, R. F. Pratt, and J. R. Knox. 1994. Crystallographic structure of a phosphonate derivative of the *Enterobacter cloacae* P99 cephalosporinase: mechanistic interpretation of a beta-lactamase transition-state analog. *Biochemistry* **33**:6762–6772.
25. Long, J. Z., X. Jin, A. Adibekian, W. Li, and B. F. Cravatt. 2010. Characterization of tunable piperidine and piperazine carbamates as inhibitors of endocannabinoid hydrolases. *J. Med. Chem.* **53**:1830–1842.
26. Marshall, M. J., G. W. Ross, K. V. Chanter, and A. M. Harris. 1972. Comparison of the substrate specificities of the beta-lactamases from *Klebsiella aerogenes* 1082E and *Enterobacter cloacae* P99. *Appl. Microbiol.* **23**:765–769.
27. Monnaie, D., and J.-M. Frere. 1993. Interaction of clavulanate with class C beta-lactamases. *FEBS Lett.* **334**:269–271.
28. Nukaga, M., T. Abe, A. M. Venkatesan, T. S., Mansour, R. A. Bonomo, and J. R. Knox. 2003. Inhibition of class A and class C beta-lactamases by penems: crystallographic structures of a novel 1,4-thiazepine intermediate. *Biochemistry* **42**:13152–13159.
29. Papp-Wallace, K. M., C. R. Bethel, A. M. Distler, C. Kasuboski, M. Taracila, and R. A. Bonomo. 2010. Inhibitor resistance in the KPC-2 beta-lactamase, a preeminent property of this class A beta-lactamase. *Antimicrob. Agents Chemother.* **54**:890–897.
30. Pérez-Llarena, F. J., and G. Bou. 2009. beta-Lactamase inhibitors: the story so far. *Curr. Med. Chem.* **16**:3740–3765.
31. Reading, C., and M. Cole. 1977. Clavulanic acid: a beta-lactamase-inhibiting beta-lactam from *Streptomyces clavuligerus*. *Antimicrob. Agents Chemother.* **11**:852–857.
32. Shahid, M., F. Sobia, A. Singh, A. Malik, H. M. Khan, D. Jonas, and P. M. Hawkey. 2009. Beta-lactams and beta-lactamase-inhibitors in current- or potential-clinical practice: a comprehensive update. *Crit. Rev. Microbiol.* **35**:81–108.
33. Sideraki, V., W. Huang, T. Palzkill, and H. F. Gilbert. 2001. A secondary drug resistance mutation of TEM-1 beta-lactamase that suppresses misfolding and aggregation. *Proc. Natl. Acad. Sci. U. S. A.* **98**:283–288.
34. Sit, S. Y., C. M. Conway, K. Xie, R. Bertekap, C. Bourin, and K. D. Burris. 2010. Oxime carbamate—discovery of a series of novel FAAH inhibitors. *Bioorg. Med. Chem. Lett.* **20**:1272–1277.
35. Sosa-Peinado, A., D. Mustafi, and M. W. Makinen. 2000. Overexpression and biosynthetic deuterium enrichment of TEM-1 beta-lactamase for structural characterization by magnetic resonance methods. *Protein Expr. Purif.* **19**:235–245.
36. Stachyra, T., P. Levasseur, M. C. Pêchereau, A. M. Girard, M. Claudon, C. Miossec, and M. T. Black. 2009. *In vitro* activity of the beta-lactamase inhibitor NXL104 against KPC-2 carbapenemase and *Enterobacteriaceae* expressing KPC carbapenemases. *J. Antimicrob. Chemother.* **64**:326–329.
37. Stec, B., K. M. Holtz, C. L. Wojciechowski, and E. R. Kantrowitz. 2005. Structure of the wild-type TEM-1 beta-lactamase at 1.55 Å and the mutant enzyme Ser70Ala at 2.1 Å suggest the mode of noncovalent catalysis for the mutant enzyme. *Acta Crystallogr. D Biol. Crystallogr.* **61**:1072–1079.
38. Strynadka, N. C., H. Adachi, S. E. Jensen, K. Johns, A. Sielecki, C. Betzel, K. Sutogh, and M. N. James. 1992. Molecular structure of the acyl-enzyme intermediate in beta-lactam hydrolysis at 1.7 Å resolution. *Nature* **359**:700–705.
39. Sutcliffe, J. G. 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. *Proc. Natl. Acad. Sci. U. S. A.* **75**:3737–3741.
40. Totir, M. A., J. Cha, A. Ishiwata, B. Wang, A. Sheri, V. E. Anderson, J. Buynak, S. Mobashery, and P. R. Carey. 2008. Why clinically used tazobactam and sulbactam are poor inhibitors of OXA-10 beta-lactamase: Raman crystallographic evidence. *Biochemistry* **47**:4094–4101.
41. Totir, M. A., M. S. Helfand, M. P. Carey, A. Sheri, J. D. Buynak, R. A. Bonomo, and P. R. Carey. 2007. Sulbactam forms only minimal amounts of irreversible acrylate-enzyme with SHV-1 beta-lactamase. *Biochemistry* **46**:8980–8987.
42. Vacondio, F., C. Silva, A. Lodola, A. Fioni, S. Rivara, A. Duranti, A. Tontini, S. Sanchini, J. R. Clapper, D. Piomelli, M. Mor, and G. Tarzia. 2009. Structure-property relationships of a class of carbamate-based fatty acid amide hydrolase (FAAH) inhibitors: chemical and biological stability. *Chem. Med. Chem.* **4**:1495–1504.
43. Villegas, M. V., K. Lolans, A. Correa, J. N. Kattan, J. A. Lopez, and J. P. Quinn. 2007. First identification of *Pseudomonas aeruginosa* isolates producing a KPC-type carbapenem-hydrolyzing beta-lactamase. *Antimicrob. Agents Chemother.* **51**:1553–1555.