

# The Nature of Inhibition of TEM-1 $\beta$ -Lactamase by the Non- $\beta$ -Lactam Inhibitor NXL104

T. STACHYRA, M.-C. PÉCHEREAU, J.-M. BRUNEAU, C. MIOSSEC, J.M. FRÈRE\*, M.T. BLACK

Novexel S.A., France and \*University of Liège, Belgium

**novexel**  
novel therapies for infectious disease  
Contact Information:  
therese.stachyra@novexel.com

C1-1374

## ABSTRACT

**Background:** NXL104 is a new non- $\beta$ -lactam inhibitor of Classes A and C  $\beta$ -lactamases. For many years the Class A  $\beta$ -lactamase TEM-1 has been among the most intensively studied enzymes at both structural and mechanistic levels, and continues to serve as a model  $\beta$ -lactamase for such investigations. The principal parameters which define the efficiency of activity of NXL104 against TEM-1 have been determined.

**Methods:** TEM-1 was overexpressed in *E. coli* and purified to near homogeneity according to published procedures (1). All kinetic and inhibition parameters were determined spectrophotometrically using nitrocefin (NCF) as reporter substrate. TEM-1 was incubated for 15 min in the presence and absence of excess NXL104, dialysed, and mass spectra determined by ElectroSpray Ionization – quadrupole-Time of Flight spectrometry.

**Results:** The principal inhibitory characteristics of NXL104 against TEM-1  $\beta$ -lactamase were determined. These include partition ratio, dissociation constant (K), rate constant for deactivation ( $k_2$ ) and deacylation rate. NXL104 was a potent TEM-1 inhibitor, characterized by a high acylation efficiency ( $k_2/K = 3.4 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ), and slow deacylation. Mass spectrometry revealed that the enzyme was covalently linked to NXL104.

**Conclusion:** Inhibition of TEM-1 by NXL104 is characterized by efficient acylation and formation of a very stable covalent complex.

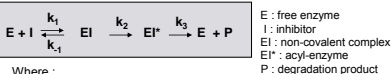
## INTRODUCTION

TEM-1 is a serine  $\beta$ -lactamase whose activity can be conceptually divided into 3 steps: formation of the non covalent Henri-Michaelis complex, acylation of the active site serine, and the subsequent deacylation. When the deacylation rate constant ( $k_3$ ) is very low or equal to zero, the substrate can be considered an irreversible inactivator (Model 1).

NXL104 (figure 1) is a new non- $\beta$ -lactam inhibitor of  $\beta$ -lactamases in Phase 2 clinical development. It displays a broad spectrum inhibition profile against both class A and class C enzymes (2, 3). NXL104 has virtually no intrinsic antibacterial activity, but efficiently protects  $\beta$ -lactams from hydrolysis in a variety of class A and class C producing strains, including ESBL producers (4).

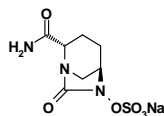
The objective of this study was the determination of the kinetic parameters of NXL104 interaction with TEM-1 in order to aid understanding of the mechanism of inhibition.

### Model 1: Reaction catalyzed by serine $\beta$ -lactamases



Where:  
 $k_2$  : first-order rate constant for acylation  
 $k_3$  : first-order rate constant for deacylation  
 $K = k_1/k_{-1}$  : dissociation constant (when  $k_2$  remains negligible)

Figure 1: Chemical structure of NXL104



## METHODS

### TEM-1 purification

A pET-24 vector containing the sequence encoding TEM-1 fused to the leader sequence of OmpA was used to overexpress the enzyme (5). TEM-1 was purified according to published procedures with modifications (1). The enzyme was concentrated to 2.5 mg/mL at a purity of > 95% as measured by SDS-PAGE, mass spectrometry and N-terminal sequence determination (data not shown).

### $\beta$ -lactamase activity

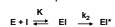
Enzyme activity was quantitated by spectrophotometric measurement of NCF hydrolysis at 485 nm and at 37°C. NCF was present at 100  $\mu\text{M}$  and TEM-1 at 0.25 nM in 50 mM phosphate pH 7.0 buffer, and 0.1 mg/mL bovine serum albumin. Initial rates were monitored for 2 min. The steady-state kinetic parameters for NCF,  $k_{\text{cat}}$  and  $K_m$ , were 455  $\text{s}^{-1}$  and 54  $\mu\text{M}$  respectively (data not shown). All kinetic parameters presented are mean values obtained from at least two independent experiments.

### Titration of TEM-1 (partition ratio, r)

Inactivation of TEM-1 was performed at 37°C, with 1  $\mu\text{M}$  TEM-1, different molar inhibitor/enzyme ratios, and by 5 or 30 min inhibitor-TEM-1 incubation.  $\beta$ -lactamase activity was measured after a subsequent 4,000-fold enzyme dilution (figure 2). A plot of fractional activity against  $[I]/[E]$  ratio was constructed in order to determine the number of inhibitor molecules required to inactivate one enzyme molecule (table 1).

### Acylation: K and $k_2$ kinetic parameters

The inactivation rates for NXL104 and clavulanate were measured at 37°C using NCF as the reporter substrate. The dissociation constant K and rate constant  $k_2$  (table 1), were calculated as previously described (6), and processed using software developed at the University of Liège. The following scheme represents a simple inactivation pathway.



When  $[I]_0$  is much larger than the total enzyme concentration  $[E]_0$ , then in the presence of the reporter substrate, equation 1 applies and permits K and  $k_2$  determination from a plot of  $[I]/k_{\text{obs}}$  against  $[I]$  (figure 3(b)). An example of progress curves of TEM-1 inactivation by 0.5  $\mu\text{M}$  NXL104 or clavulanate is presented in figure 3(a).

$$k_{\text{obs}} = \frac{k_2[I]}{[I] + K \left(1 + \frac{[NCF]}{K_{\text{NCF}}}\right)} \quad \text{Equation 1}$$

### Deacylation reaction

Time course of recovery of the enzymatic activity was measured after titration of concentrated enzyme (1  $\mu\text{M}$ ) to maximum inhibition. The excess of free inhibitor was removed by filtration (Biomax, Millipore, cut-off 5 kDa). Samples were withdrawn at regular intervals and immediately diluted 4,000-fold in a 100  $\mu\text{M}$  solution of NCF to measure the recovered  $\beta$ -lactamase activity (table 1).

### Acylenzyme characterization

40  $\mu\text{M}$  TEM-1 was incubated for 15 min in the presence and absence of 4 mM NXL104, dialyzed at 4°C for 24 h, and analyzed by ESI-Q-ToF mass spectrometry (figure 4).

### Mass spectrometry (ESI-Q-ToF)

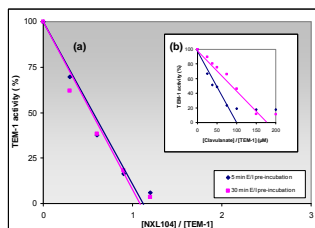
Mass spectrometry data were generated by Marie-Alice Meuwis and Gabriel Mazzucchelli at the CART/GIGA mass spectrometry facility at the University of Liège, using an ESI-Q-ToF instrument (Water, Micromass).

## REFERENCES

- Sideraki V, et al. Proc. Natl. Acad. Sci. USA (2001) 98, 283-288
- Bonnefoy A, et al. J. Antimicrob. Chemother. (2004) 54, 410-417.
- Stachyra T, et al. J. Antimicrob. Chemother. (2009) 54, 328-329.
- Shackcloth J, et al. 15<sup>th</sup> European Congress of Clinical Microbiology and Infectious Diseases (2005), Poster # 1348.
- Sosa-Peláez, A, et al. Protein expression (2000) 19, 235-245.
- De Meester, F, et al. Biochem. Pharmacol. (1987) 36, 2393-2403.

## RESULTS

Figure 2: Titration of TEM-1 with NXL104 and Clavulanate



→ 1  $\mu\text{mol}$  NXL104 was needed to completely inactivate 1  $\mu\text{mol}$  TEM-1 (100- to 175-fold more clavulanate was needed to obtain 80% TEM-1 inhibition).

→ TEM-1 inhibition by NXL104 was completed after 5 min and no deacylation was observed during the first 30 min of reaction (the same results were obtained with either 5 or 30 min incubation)

Figure 3: Acylation step - K and  $k_2$  determination

(a) Time course of NCF hydrolysis in the absence and in the presence of 0.5  $\mu\text{M}$  NXL104 or 0.5  $\mu\text{M}$  clavulanate.  
(b) Plot of  $[I]/k_{\text{obs}}$  against  $[I]$  obtained from inactivation experiments at different inhibitor concentrations. In this experiment,  $[I]$  corresponds to  $[NXL104]$ .

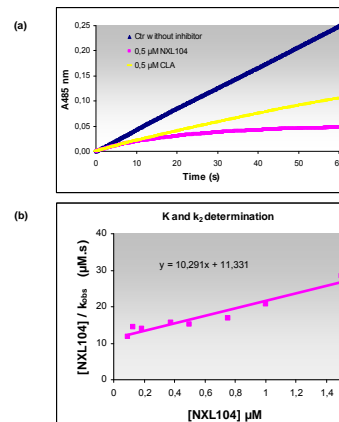


Table 1: Kinetic parameters of TEM-1 inactivation by NXL104 and clavulanate

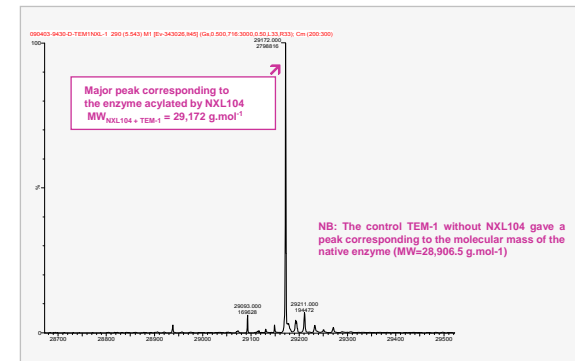
	Partition ratio (r)	K ( $\mu\text{M}$ )	$k_2$ ( $\text{s}^{-1}$ )	$T_{1/2}$ acylation** (s)	$k_2/K$ ( $\text{M}^{-1}\text{s}^{-1}$ )	Deacylation*
NXL104	0	$0.3 \pm 0.1$	$0.105 \pm 0.022$	7	340 000	5-10% after 15 min 5-10% after 24 h
Clavulanate	> 100	$0.5 \pm 0.2$	$0.027 \pm 0.005$	26	53 000	40% after 15 min 55% after 24 h

\* Biphasic progress curves were observed: <10% and 40% enzymatic activity was recovered at 15 min for NXL104 and clavulanate respectively, then little or no deacylation during several days for both inhibitors  
\*\* Calculated for saturating inactivator concentration

NXL104 behaves as an irreversible inactivator of TEM-1:

- Only 1 mol of NXL104 is required to inactivate 1 mol TEM-1
- High inactivation efficiency ( $k_2/K$ ) was found with a low dissociation constant (K) and a fast inactivation ( $k_2$ )
- Near irreversibility of inactivation by NXL104 was demonstrated. The acylenzyme complex was stable for several days.

Figure 4: Production of acyl-enzyme demonstrated by mass spectrometry



The increase in mass indicates that TEM-1 is covalently linked to NXL104 (266 Da) forming the acyl-enzyme complex

## CONCLUSION

NXL104 was shown to be a remarkably potent inhibitor of TEM-1.

- Only one mol of NXL104 was needed to completely inactivate one mol of TEM-1 (partition ratio of zero), whereas >100 moles of clavulanate were required under the same conditions.
- Efficiency of NXL104 was indicated by the high affinity of NXL104 binding, and the fast rate of inactivation (large  $k_2/K$  value).
- The quasi-permanence of the inactive enzyme complex (EI\*) was demonstrated by ESI-Q-ToF MS and by deacylation kinetics.