Poster # C-162

Structures of the Carbapenemase OXA-24 in Complex with the β-lactamase Inhibitor Avibactam

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Abstract

Avibactam is a non-beta-lactam beta-lactamase inhibitor in phase III clinical development that is active against class A, C and some class D enzymes. Its spectrum of activity is wider than the beta-lactamase inhibitors tazobactam, sulbactam and clavulanic acid. About 400 class D beta-lactamases have been identified so far. These display a great variety of sequence and substrate specificity including carbapenemase activity for enzymes such as OXA-24/40 and -48 which are respectively expressed by clinically important *A. baumannii* and *K. pneumoniae* strains. The efficacy of avibactam on class D beta-lactamases shows large fluctuations that cannot yet be explained partly due to the lack of structural data.

In this study, we measured the inhibition of the carbapenemase OXA-24 and the cephalosporinase OXA-163 by avibactam, showing a significantly higher susceptibility for the latter. In addition, we determined the structure of the OXA-24:avibactam complex by x-ray crystallography at a 3.1A resolution. The ring-opened avibactam molecule is covalently bound to the active Ser81 and the first motif Lys84 is partially carboxylated. The conformation of the inhibitor is similar to the one observed in the class A and C beta-lactamases complexes. The main difference is the absence of a hydrogen bond with the asparagine of the second conserved motif which is replaced by a valine in class D enzymes. The bond between the sulfate part of avibactam and the rest of the molecule seems to be cleaved as proposed by Ehmann *et al.* but stays bound to Arg261 and to Lys218 and Ser219 from the third conserved motif.

From the available data, the lower susceptibility of OXA-24 to avibactam compared to OXA-48 and -163 could not be attributed to a single structural feature. Several factors are therefore likely responsible

Introduction

Penicillin Binding Proteins (PBPs) are the target of the widely used antibiotics of the β-lactam family. They belong to 2 groups, the high molecular mass PBPs which are multimodular and involved in the peptidoglycan polymerization (class A and B) and the low molecular mass PBPs (class C) involved in peptidoglycan maturation and degradation

One of the most efficient ways developed by bacteria to resist β-lactam antibiotics is the expression of proteins (β-lactamases) capable of hydrolyzing them. These enzymes belong to four classes (A-D) depending on their sequence similarity and catalytic mechanism.

Inhibiting both families of enzymes simultaneously is a very efficient antibacterial strategy. However, resistance mechanisms arise more and more frequently and represent a growing threat for patients in hospitals, emphasizing the continuous need for new efficient molecules. Avibactam is a non-β-lactam β-lactamase inhibitor in phase III clinical development that is active against class A, C and some class D enzymes.

In this work, we present the structure of the class D β-lactamase OXA-24 and the class C PBP with DD-peptidase activity from *Actinomadura R39* (R39 DD-peptidase) in complex with avibactam, The acylation rates of OXA-24, OXA-163 and the R39 DD-peptidase for this compound were also determined.

Methods

Protein expression, purification and crystallization. The OXA-24 β-lactamase and the R39 DD-peptidase were expressed and purified as described previously (**1**, **2**). Crystals were grown at 20 °C by hanging drop vapor diffusion. For **OXA-24**, 2 µl of 10 mg/mL protein solution (20 mM Tris pH8) were mixed with 2 µl of a 1M ammonium sulfate and 0.1 M bis-tris at pH 6.5 solution. The crystal was soaked for 25 minutes by adding 0.5µL of a 90mM avibactam solution. For the **R39 DD-peptidase**, 2.5 µL of a 25 mg/mL protein solution (also containing 5 mM MgCl2 and 20 mM Tris, pH 8) were mixed with 2 µL of well solution (2.5M ammonium sulfate and 0.1 M MES, pH 6) and 0.5 µL of 0.1 M CoCl2 solution. Crystals were soaked in a solution consisting of 6 µL of 3M ammonium sulfate and 0.1MMES, pH 6, and 0.5 µL of 90mM avibactam for 35 min before data collection.

Structure determination. Data were collected on Proxima 1 beamline of the Soleil Synchrotron (Saint Aubin, France). Intensities were indexed, integrated and scaled using XDS (**3**). Refinement and building were carried out using REFMAC5 (**4**) and Coot (**5**). Data and refinement statistics are summarized in Table 1.

Enzymatic experiments. The acylation rates of OXA-24, OXA-163 and the R39 DD-peptidase for avibactam were obtained from inactivation experiments using the reporter substrates nitrocefin, ceftazidime and thiolester S2d.

Acknowledgment

We acknowledge the staff of the Proxima 1 beamline of the Soleil Synchrotron (Saint Aubin, France) for excellent technical assistance. This research was funded in part by AstraZeneca (Waltham, Mass., USA).

Structure of the OXA-24:avibactam complex

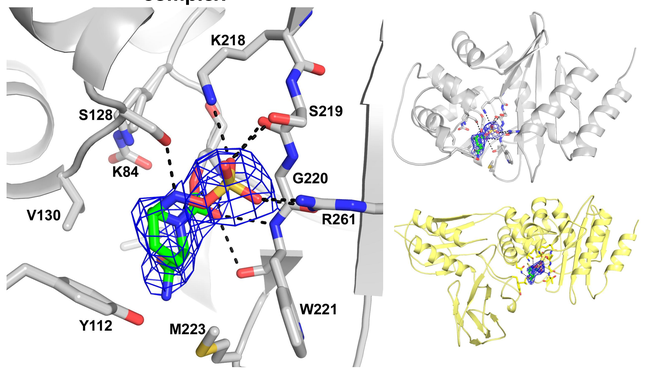


Figure 1. Structure at a 3.1Å resolution of the avibactam molecule covalently bound to the active Ser 81 of OXA-24. The corresponding 2Fo-Fc electron density map is shown at a 1σ level and dashed lines denote hydrogen bonds between avibactam and the protein. The first motif Lys84 is only partially carboxylated (approximately 50%). The bond between the sulfate part of avibactam and the rest of the molecule seems to be cleaved as proposed by Ehmann *et al.* (**6**) but stays bound to Arg261 and to Lys218 and Ser219 from the third conserved motif
This structure is similar to the one presented at the 2011 ECCMID conference by Docquier *et al.* (**7**).

Acylation rates

Enzyme	k_2/K (M^{-1} s^{-1})
OXA-24	55 ± 5
OXA-163	1720 ± 75
R39- DD-peptidase	27 ± 1
OXA-10 *	11 ± 1
OXA-48 *	1400 ± 100

* Values taken from Ehmann *et al.* (**6**) for comparison

Table 1. Data and refinement statistics

	OXA-24	R39
Data Collection		
Space group	$P4_12_12_1$	P1 21 1
a, b, c (Å)	103.18, 103.18, 87.69	103.67, 92.08, 107.16
α, β, γ (°)	90, 90, 90	90, 94.36, 90
Resolution range (Å)	46.1-3.10 (3.27 - 3.10)	46.2-2.2 (2.32-2.2)
No. of unique reflections	9014 (1278)	99905 (14438)
Rmerge (%)	22.2 (83.1)	6.3 (49.6)
Rpim (%)	6 (22.5)	3.7 (28.7)
<I>/<σI>	11.2 (5,4)	12.8 (2.7)
Completeness (%)	100 (100)	98.1 (97,9)
Redundancy	14.1 (14,3)	3.8 (3,9)
Refinement		
Resolution range (Å)	46.1-3.10 (3.18 – 3.1)	46.2-2.2 (2.26-2.2)
R work (%)	19,22 (24.9)	18,07 (23.8)
R free (%)	22,95 (36.3)	22,59 (27.2)
No. of non hydrogen atoms	1969	14306
Number of water molecules	12	655
Mean B factor (all atoms) ($Å^2$)	39.2	41.5
RMS deviations from ideal stereochemistry:		
Bond lengths (Å)	0,0099	0,0103
Bond angles (°)	1,22	1,19
Ramachandran plot:		
Favoured region (%)	95,8	96,8
Allowed regions (%)	4.2	3
Outlier regions (%)	0	0,2

R39 DD-peptidase (class C PBP) in complex with avibactam

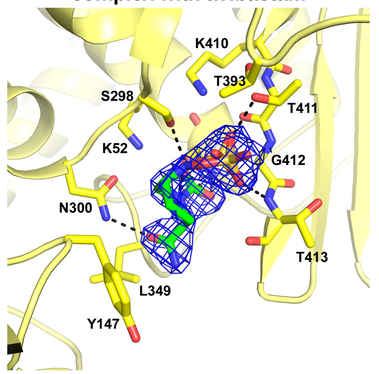
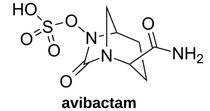


Figure 2. Crystals of the R39 DD-peptidase contain 4 molecules in the asymmetric unit (**8**). In each of them the catalytic S49 is partially acylated by avibactam (about 70% occupancy, only monomer A is represented). The corresponding 2Fo-Fc electron density map is shown at a 1σ level and dashed lines denote hydrogen bonds between avibactam and the protein. The sulfate moiety interacts with T411 and T413 of the 3rd conserved motif and no breakage is observed with the rest of the compound which interacts with the 2nd conserved motif N300.
This is the first structure of a complex between avibactam and a PBP.

Conclusions

- The acylation rates of OXA-10 and -24 are similar to that of MtBlaC (k_2/K = 15 ± 0.5)(**10**). This can potentially be correlated with the absence of an Asn residue binding the avibactam in the second conserved motif.
- OXA-48 and-163 which only differ by a 4 aa deletion in the loop following the 3rd motif known to be critical for the carbapenemase activity (**11**) have a yet unidentified structural feature that compensates for the lower affinity for avibactam due to the absence of a Asn in the second conserved motif.
- As expected from the structures, the loop following the 3rd conserved motif in class D β-lactamases is unimportant for avibactam recognition.
- The R39-avibactam structure is the first structure of avibactam in complex with a PBP, showing similarities to the β-lactamase complexes.
- The R39 DD-peptidase has a low affinity for avibactam (similar to OXA-10 and OXA-24). despite the interaction with N300 of the 2nd conserved motif.



avibactam

Comparison of OXA-24:avibactam complex with the class A and C β-lactamases and PBP complexes

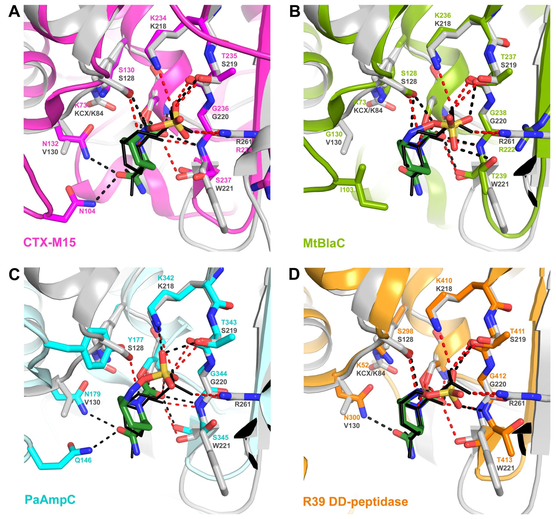


Figure 3. Superposition of the OXA-24:avibactam (grey sticks and ribbons for the protein, black lines for avibactam and red dashed lines for hydrogen bonds) with the available avibactam complexes : **A,** the class A β-lactamase CTX-M15 (magenta, and black dashed lines for H-bonds) (**9**), **B,** the class A β-lactamase BlaC from *Mycobacterium tuberculosis* (green) (**10**), **C,** the class C β-lactamase AmpC from *Pseudomonas aeruginosa* (cyan) (**9**) and **D**, the class C PBP R39 DD-peptidase (orange). The avibactam molecules are found in very similar orientations in all cases but H-bonds networks slightly differ.

- Despite the presence of an Arg residue covering the 3rd conserved motif in class A and D β-lactamase complexes, only R261 of OXA-24 interacts with avibactam.
- OXA-24 is similar to MtBlaC in its absence of interaction with the 2nd conserved motif

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