Deciphering the Role of the Y221H Ω-loop Substitution in *Pseudomonas*-derived Cephalosporinase (PDC) in Cephalosporin Resistance

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INTRODUCTION

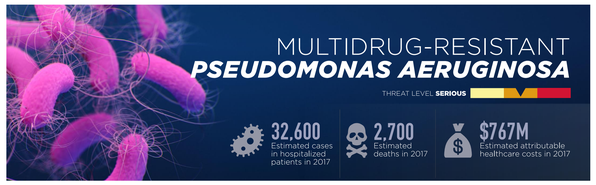

Figure 1: Updated CDC Threat Assessment for Multidrug-resistant *Pseudomonas aeruginosa*. (1)

***Pseudomonas aeruginosa* and β-lactamases**

- *Pseudomonas*-derived cephalosporinase (PDC) is the major class C β-lactamase produced by *P. aeruginosa* and is a major cause of antibiotic resistance.
- Clinically reported single amino-acid substitutions (including Y221H, a tyrosine to histidine substitution at position 221) in the PDC Ω-loop, a region contributing to positioning of substrate in the active site, increase antibiotic resistance. (2,3)

Ceftolozane-Tazobactam (TOL-TAZO) and Ceftazidime-Avibactam (CAZ-AVI)

- Combinations of a newer cephalosporin with an older β-lactamase inhibitor (TOL-TAZO) & older cephalosporin with newer β-lactamase inhibitor (CAZ-AVI).
- Increasingly used to treat otherwise resistant *P. aeruginosa* infections.
- Resistance is of particular concern as Ceftolozane was specifically developed to overcome the weaknesses of Ceftazidime, a preciously very important anti-pseudomonal cephalosporin. (4)
- Resistance to new treatments emerges quickly. Strains resistant to TOL-TAZO have been found in collections predating FDA approval and clinical introduction. (5)

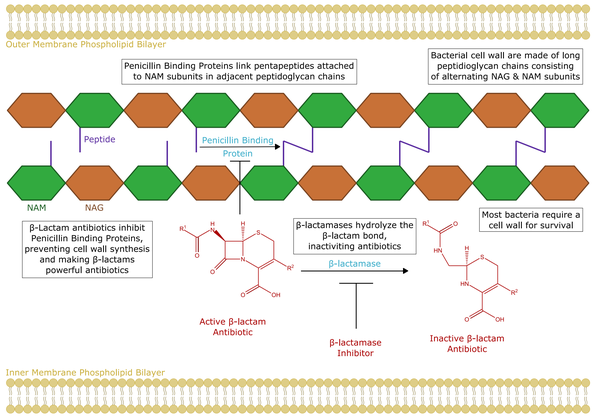

Figure 2: The role of the cell wall, β-lactam antibiotics, β-lactamase enzymes, and β-lactamase inhibitors in antibiotic treatment and resistance. β-lactams are the most commonly used class of antibiotics in the United States.

HYPOTHESIS

We hypothesize that specific amino acid substitutions in the Ω-loop of PDC, including Y221H, alter conformational flexibility and charge of the loop, leading to more efficient cephalosporin hydrolysis and increased antibiotic resistance.

RESULTS

The Y221H substitution increases cephalosporin resistance, which β–lactamase inhibitors overcome

Strain or Construct	TOL	TOL-TAZO	CAZ	CAZ-AVI
***P. aeruginosa* 18SH (PDC-3)**	8	8	64	2
***P. aeruginosa* PA01 (PDC-1)**	0.5	0.5	1	1
***E. coli* DH10B**	0.5	0.25	0.25	0.25
***E. coli* DH10B + pBC SK**	0.5	0.25	0.25	0.25
***E. coli* DH10B + pBC SK *bla*$_{PDC\text{-}1\ WT}$**	0.5	0.5	2	0.25
***E. coli* DH10B + pBC SK *bla*$_{PDC\text{-}3\ WT}$**	0.5	0.5	2	0.25
***E. coli* DH10B + pBC SK *bla*$_{PDC\text{-}3\ Y221A}$**	32	4	32	0.5
***E. coli* DH10B + pBC SK *bla*$_{PDC\text{-}3\ Y221H}$**	8	1	16	0.5

Table 1: Minimum inhibitory concentrations (MICs) for selected *P. aeruginosa* strains and isogenic *E. coli* expressing PDC β-lactamase constructs. Values in mg/L. CAZ = ceftazidime, TOL = ceftolozane, TAZO = tazobactam, AVI = avibactam. Y221A is a laboratory mutation. Data collected using agar dilution methods per CLSI guidelines and previously reported in (3).

The Y221H variant enables ceftazidime hydrolysis

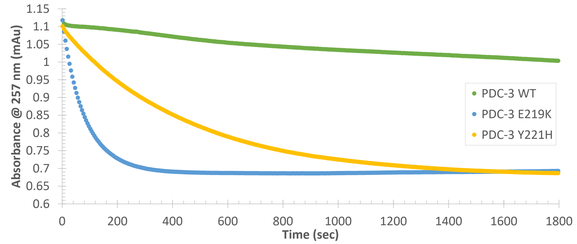

Figure 3: Spectrophotometric ceftazidime hydrolysis progress curves collected at 257 nm. PDC-3 Y221H hydrolyzes CAZ more efficiently than WT. PDC-3 E219K is included as a variant with especially strong CAZ hydrolytic abilities.

Catalytic Efficiency	PDC-3 WT	PDC-3 E219K	PDC-3 Y221H
k_{cat}/K_M (s^{-1} μM^{-1})	Not Determined	5.2 ± 0.8 x 10-2	5.8 ± 0.2 x 10-3

Table 2: Catalytic efficiency (k_{cat}/K_M) accounts for both turnover and substrate binding to calculate a how "good" a substrate is for an enzyme. PDC-3 E219K is a variant with especially strong CAZ hydrolytic abilities. No value can be calculated for PDC-3 WT due to the slow hydrolysis rate. Collected spectrophotometrically with nitrocefin (NCF) as a reporter substrate.

The Y221H substitution decreases thermal stability

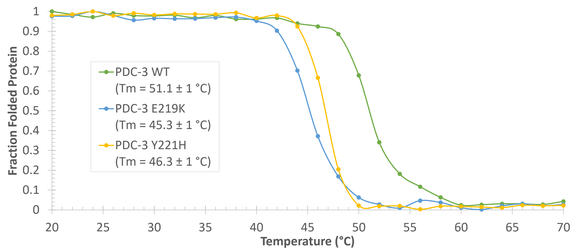

Figure 4: Thermal denaturation curves collected using circular dichroism spectrophotometry reveal a 4.8°C decrease in melting temperature of Y221H compared to PDC-3 WT, suggesting a decrease in protein stability. PDC-3 E219K is included as a an even less stable variant.

The impact of the Y221H substitution on inhibition by common β-lactamase inhibitors is minimal

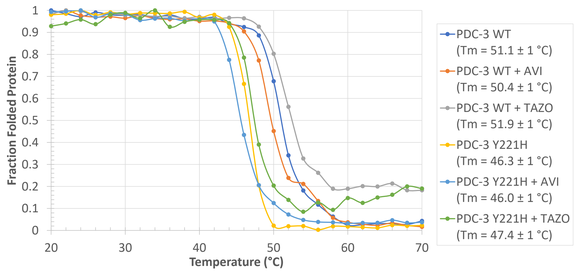

Figure 5: Thermal denaturation curves of PDC-3 WT and the Y221H substitution with AVI and TAZO. Tm changes are minimal and largely within the margin of error and both WT and Y221H exhibit similar changes to their denaturation curves with the addition of inhibitor. Curves were collected using circular dichroism spectrophotometry.

Inhibitor	PDC-3 WT K_i (nM)	PDC-3 Y221H K_i (nM)
Avibactam	19.4 ± 2	69.5 ± 7
Tazobactam	399.7 ± 40	37.5 ± 4

Table 3: The inhibitor constant (K_i) is the concentration of inhibitor needed to slow the reaction rate by half, with lower values generally indicating better or more effective inhibitors. Values were determined spectrophotometrically after a five-minute preincubation of inhibitor and enzyme.

The Y221H substitution appears to have substrate-specific effects

Substrate	PDC-3 WT K_M (μM)	PDC-3 Y221H K_M (μM)
Nitrocefin	24.7 ± 1.6	27.7 ± 2.1
Ceftazidime	3 ± 0.3	662 ± 70
Cefepime	241.3 ± 24	6.8 ± 0.7
Piperacillin	1.7 ± 0.2	5.1 ± 0.5

Table 4: Although not a binding constant, the Michaelis constant (K_M) varies in relation to substrate binding and suggests possible differences or changes in active site affinity. Data was collected spectrophotometrically. NCF was measured directly and others as an NCF inhibitor.

Construct	Cefepime	Piperacillin
***E. coli* DH10B + pBC SK**	<0.0625	2
***E. coli* DH10B + pBC SK *bla*$_{PDC\text{-}3\ WT}$**	0.0625	16
***E. coli* DH10B + pBC SK *bla*$_{PDC\text{-}3\ Y221H}$**	0.125	32

Table 5: MICs (in mg/L) for isogenic *E. coli* expressing PDC β-lactamase constructs. MICs to not seem to correlate with changes in K_M. Turnover (k_{cat}) data was not yet available for this poster.

WT-MetaDynamics simulations reveal a hidden pocket opened by the Y221H substitution

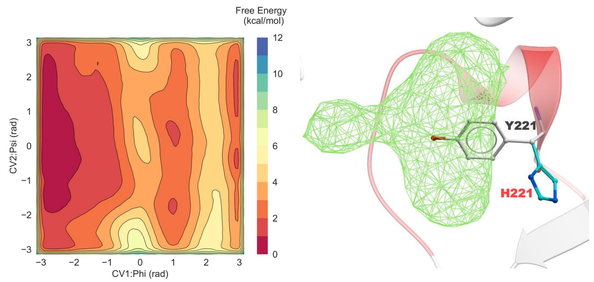

Figure 6: (Left) Enhanced sampling metadynamics simulations generate free-energy landscapes as a function of the dihedral angles of residue 221. This identifies the differences in the dynamics of the tyrosyl side chain in the wildtype Y221 and the imidazole ring of the H221 variant. Red basins represent energy minima containing more stable conformations. (Right) The rotation of the H221 side chain in one such stable conformation opens a cryptic pocket (green mesh), which is occluded in the wild type, potentially allowing for novel positioning of antibiotics in the active site. The Ω-loop is colored red.

CONCLUSIONS

- PDC-3 Y221H confers antibiotic resistance to both ceftolozane and ceftazidime and increases hydrolysis of ceftazidime, compared to PDC-3 WT.
- Differences in thermal stability and the conformation of the 221 amino acid suggest an important structure-activity relationship (SAR) mediates this phenotype, likely through the opening of a hidden pocket along the Ω-loop and near the active site.
- Understanding the SAR and mechanism of action Y221H and similar substitutions will advance our understanding on protein evolution and enzyme-substrate interactions and guide the rational design of future antibiotics and inhibitors.

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