Structural and functional characterization of new clinically relevant enzymes involved in antibiotic resistance

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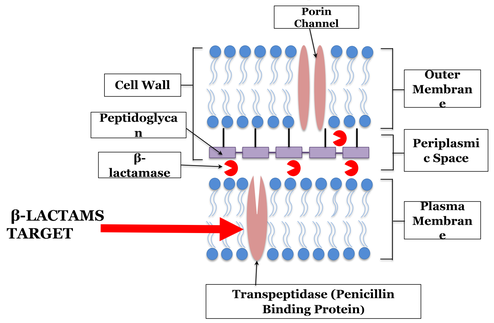
Abstract

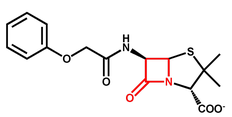
Resistance to β-lactam drugs, such as penicillins and cephalosporins, has become a worldwide health problem. The World Health Organization has listed multi-drug resistant *Acinetobacter baumannii* as requiring "top priority for new antibiotic development." [3] Such antibiotic resistance derives primarily from β-lactamases, including *Acinetobacter*-derived cephalosporinase (ADC), which bind and destroy antibiotics. A recent study of clinical *Acinetobacter baumannii* infections identified the most prevalent ADC β-lactamases conveying antibiotic resistance. This research study seeks to determine the structure and function of two clinical ADC variants: ADC-30 and ADC-162. By using X-ray crystallography and enzyme kinetics, we aim to relate the small changes in structure to their enhanced ability to turn over antibiotics. Characterizing the structure and function of clinical ADC variants will be critical to developing molecules that could inhibit ADCs, thereby restoring antibiotic effectiveness.

Introduction

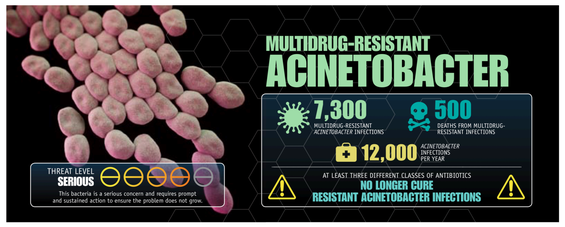
Mode of Action of β-lactam Antibiotics



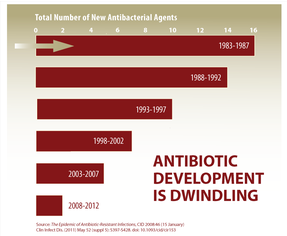
- β-lactam antibiotics bind and inhibit transpeptidase, an enzyme that maintains cell wall integrity.
- Transpeptidase inhibition interrupts peptidoglycan cross-linking, thus destabilizing the cell wall and resulting in eventual bacterial cell lysis.
- β-lactamases confer resistance to β-lactam antibiotics by hydrolyzing the key amide functional group.



Penicillin with its beta lactam ring shown in red

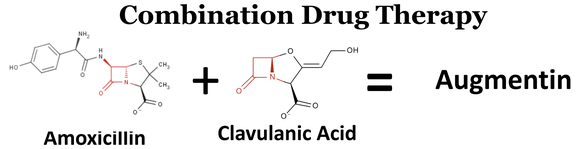


- β-lactamases found in *Acinetobacter baumannii's* provide resistance to a broad spectrum of cephalosporin antibiotics.
- *Acinetobacter baumannii* is classified as a "serious threat" by the World Health Organization, and due to it's multi-drug resistance, novel ways are needed to fight these infections.
- *Acinetobacter* is an opportunistic pathogen, infecting mostly patients in hospital care and others with higher susceptibility such as those in burn units. [3]



- As antibiotic resistance is causing decreased effective drug lifetimes, the investment financial returns are also decreased. Therefore, novel ways to fight bacterial infections are vital.
- One method is through a β - lactam inhibitor that binds to β-lactamases to counteract resistance

Combination Drug Therapy



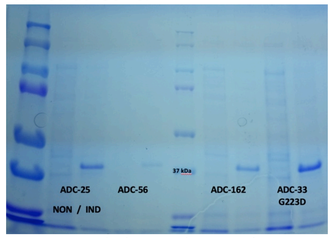
Methods

Expression and Purification of ADC-162

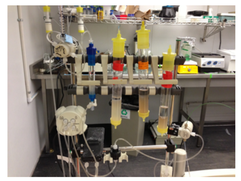
- A plasmid (pET28a) containing an *Acinetobacter baumanii* ADC gene was transformed into a non-pathenogenic *E.coli* strain.
- From these colonies, protein expression was tested in induced and non-induced conditions in LB-kanamycin.
- After confirming successful expression of ADC enzymes, larger growths (1 L) were done, and the the cell pellets was stored at -80 °C.

E.coli. containing ADC-162 plasmid

- Cell pellets were resuspended (25 mM MOPS pH 6.5), sonicated, centrifuged, and the resulting cell free extract was loaded onto a carboxy-methyl-cellulose column at 4 °C.



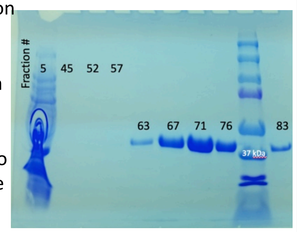
- Protein gel electrophoresis (SDS-PAGE) confirmed protein expression. Expression was controlled with IPTG. Lane 1 shows a molecular weight marker with a known band at 37kDa.

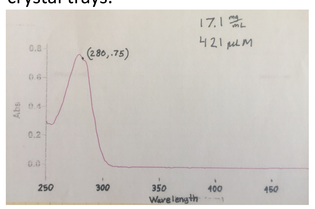


BioRad purification system with column

- Column was washed with 25mM MOPS, pH 6.5 then eluted with 25mM MOPS, pH 6.5, 500mM NaCl

- Dialysis reduced NaCl concentration from the elution buffer (10 kDa cutoff).
- Protein was concentrated using an Amicon centrifugal filter unit.
- UV-Vis Spectrophotometer measured absorbance at 280nm to be .75 which was used to calculate exact concentration of protein for crystal trays.

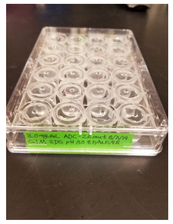




UV -Vis spectrum results for ADC-162

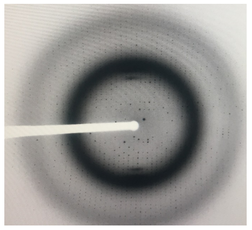
- After purification, gel electrophoresis (SDS-PAGE) confirmed the purified fractions containing ADC-162. A molecular weight marker is present in lane 9 to indicate that ADC-162 molecular weight is ~40 kDa.

- Purified ADC-162 was crystallized via hanging drop vapor diffusion in (0.1 M Succinate/ Phosphate/Glycine, pH 5.0).
- Crystals were grown at varying concentrations (3-4 mg/mL) at room temperature
- Some crystals were soaked with an antibiotic or inhibitor molecule
- Crystals were frozen in liquid N_2 and transported to the Advanced Photon Source at Argonne National Labs



Crystal tray for ADC-25

Argonne National Laboratories



Example of a diffraction pattern

- A high intensity X-ray beam focused on the crystal allowed for the collection of diffraction data through a 180° rotation.
- The diffraction patterns generated can be used to develop an electron density map of the protein.
- From the electron density map, an atomic model (ADC protein structure) can be determined.

Results

Successfully expressed plasmids for eight ADC clinical variants:

- ADC-25 A200D/P220L/A insert
- ADC-30
- ADC-33
- ADC-162 (ADC-30 A221E)
- ADC-56
- ADC-68
- ADC-25 (ADC-30 T317N)
- ADC-33 G223D

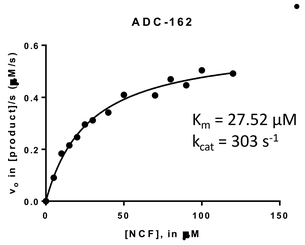
Four of these variants have been purified through a one-step CM-cellulose method resulting in 30-80mg of pure protein for each of the following:

- ADC-25 (A200D/P220L/A insert)
- ADC-30
- ADC-33
- ADC-162 (ADC-30 A221E)

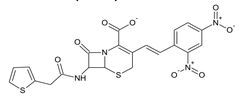
Three of these proteins were successfully crystallized. X-ray crystallography performed at Argonne National Laboratory resulted in structures between 1.25 – 1.8 Å resolution. The following six structures were obtained:

- ADC-30 apo and ADC-30 with MB076 bound
- ADC-33 apo and ADC-33 with MB076 bound
- ADC-162 apo and ADC-162 with MB076 bound

Steady State Kinetics

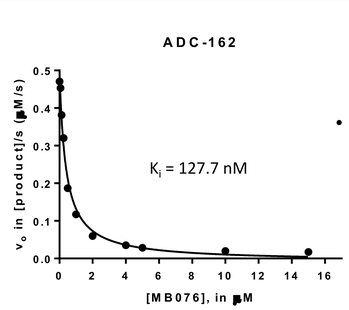


- The initial reaction rate for each purified ADC was measured from the inactivation of a cephalosporin antibiotic, nitrocefin (NCF).



Nitrocefin

- Kinetics for the turnover of variable concentrations of NCF by ADC-162 (2 nM) in 10 mM $NaPO_4$ buffer, pH 7.4 at room temperature, measured at 482 nm (follows the color of the hydrolyzed product).



- Competitive inhibition kinetics for ADC-162 (2nM) using 60 μM nitrocefin, 10 mM $NaPO_4$ buffer, pH 7.4, at room temperature. Inhibitor concentration was increased until nearly zero turnover of NCF occurred.
- Competitive inhibition kinetics allows for the determination of K_i, a measurement of the binding affinity of ADC-162 to the inhibitor MB076. The K_i in the nanomolar range indicates high affinity to the inhibitor and the ability to inhibit turnover of the substrate.

Conclusions

- Very small changes in the protein sequences of specific ADCs have enabled these enzymes to contribute to a broad spectrum of resistance to *Acinetobacter baumannii.*
- Characterization of these ADC variant proteins is an important step in determining how these small structural changes can be advantageous to *Acinetobacter baumannii* in resisting a broader range of antibiotics.
- Characterizing clinically relevant ADC variants is vital to developing effective inhibitors against these enzymes.

Future Directions

- Determine the crystal structures for the three ADC variants (ADC-25, ADC-30, ADC-33) with and without the inhibitor MB076.
- Investigate how the small sequence changes affect the protein structure and how it enables increased resistance to specific antibiotics.
- Determine kinetics of NCF turnover and K_i for all eight ADC variants.
- Identify ideal growing conditions for ADC-25 A200D/P220L/Ainsert crystals.
- Identify an inhibitor that will work in combination with β-lactam antibiotics to counteract class C β-lactamases.
- Our research ultimately will seek to discover an inhibitor for both class C and D β-lactamases.

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[3] Howard, A., O'Donoghue, M., Feeney, A., & Sleator, R. D. (2012). Acinetobacter baumannii: an emerging opportunistic pathogen. *Virulence*, *3*(3), 243–250. doi:10.4161/viru.19700