Structural and Functional Characterization of New Clinically Relevant Enzymes Involved in Antibiotic Resistance

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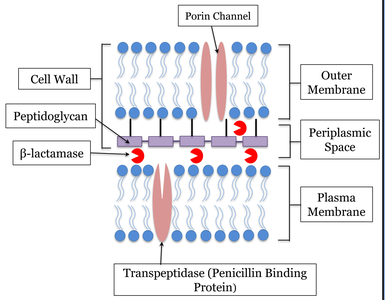
Abstract

The rise of antibiotic resistance is a global health threat requiring development of novel treatments. β-lactamase enzymes found in many multi-drug resistant bacteria contribute to resistance of β-lactam antibiotics. The enzyme cleaves the β-lactam ring, preventing the drug from reaching its cellular target. A class of β-lactamases, *Acinetobacter*-derived cephalosporinases (ADCs) are found in the multi-drug resistant bacteria *Acinetobacter baumannii*. Specific ADCs were selected from a group of antibiotic-resistant infections. With no known structures of these newly identified ADCs, it is key to discern how minor differences in sequences between ADCs contribute to drug resistance. In this project, structural and kinetics characterization of ADCs will aim to develop a relationship on how small structural variations can affect the enzyme's ability to bind and destroy antibiotics. These results will help identify potential inhibitors against enzymes that contribute to antibiotic resistance.

Introduction

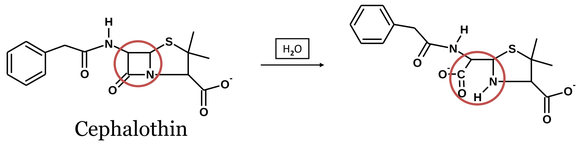
Process of β-lactam Antibiotic Function

- β-lactam antibiotics work by binding to the enzyme transpeptidase, which is found in the cell wall of the bacteria
- The binding of the antibiotic causes the bacterial cell to lyse open from osmotic pressure as the antibiotic inhibits cell wall biosynthesis



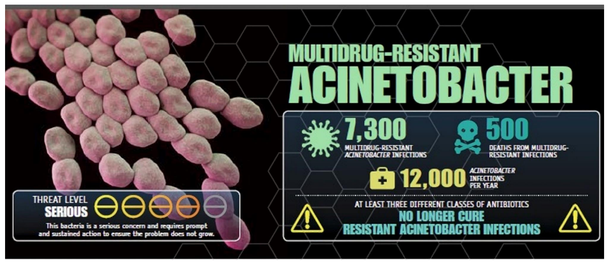
Adapted from Goodman & Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition

β-lactamase Enzymes and β-lactam Resistance



Cephalothin

- β-lactamases serve as one mechanism of bacteria that contribute towards antibiotic resistance
- β-lactamase enzymes bind and hydrolyze β-lactam antibiotics, cleaving the β-lactam ring and making them inactive
- The antibiotic is now unable to inhibit cell wall biosynthesis



- Antibiotic resistance is a growing concern and viewed as a global health problem, necessitating the development of novel alternative treatments to combat bacterial resistance towards antibiotics.
- Antibiotic resistance occurs in the bacteria *Acinetobacter baumannii*, which is an opportunistic bacterial pathogen. The bacteria is labeled as a top priority by the World Health Organization and the Centers for Disease Control. The bacteria infects mainly at the hospital level in critical care and burn units, particularly immunocompromised patients [1-2].

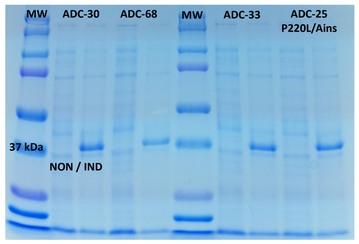
Acinetobacter-Derived Cephalosporinases (ADCs)

- *A. baumannii* contain class C β-lactamases called **A**cinetobacter-**D**erived **C**ephalosporinases.
- New ADCs have been isolated from recently infected individuals, some of which have shown resistance to carbapenems.
- These new variants have slightly different sequences that may be responsible for structural changes in the enzyme, possibly providing the ADC enzyme with an improved ability to bind and inactivate antibiotics.

Methods

E. coli colonies plated on LB-Kanamycin

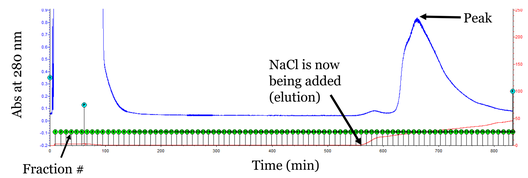
- A plasmid containing various ADC genes were inserted into a non-pathogenetic strain of *E. coli*.
- From these resulting colonies, protein expression was tested with non-induced and induced conditions.
- Confirm protein expression by gel electrophoresis (SDS-PAGE), molecular weight (MW) of ADC protein is ~ 40 kDa



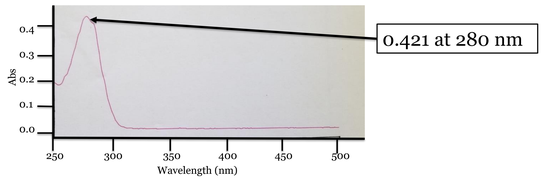
Growth and Purification With Ion Exchange Chromatography

- 1 L LB-Kanamycin cultures were grown and induced with IPTG
- Cultures were centrifuged and resulting cell pellet was stored at -80 °C.
- Resuspended cell pellets (25 mM MOPS, pH 6.5) were sonicated, centrifuged, and the supernatant was loaded on a carboxymethyl (CM)-cellulose column at 4 °C.
- The column was washed with 25 mM MOPS, pH 6.5 and eluted with 25 mM MOPS, pH 6.5, 500 mM NaCl, leading to fractions containing the purified ADC protein.

Elution profile of ADC purification by CM-cellulose chromatography

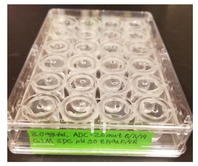


Quantitation of ADC concentration



UV-Vis spectrum from ADC-30 concentration

- NaCl from elution buffer was removed through dialysis (10 kDa cutoff) and concentrated using an Amicon centrifugal filter unit.
- Using UV-Vis spectrophotometry, the absorbance at 280 nm was measured to allow for [ADC] quantitation for calculations needed for crystal trays and steady state kinetics.



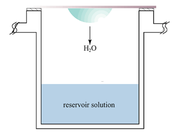
Crystal tray for ADC-25

Crystal Tray Preparation

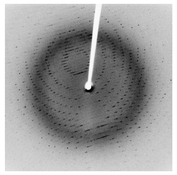
- Crystals are grown at varying concentrations to determine if ADC variants crystallize in the same known conditions as ADC-7
- Crystals were grown to be used in X-ray crystallography

Crystallization: Hanging Drop Technique

- Crystallize protein using hanging drop vapor diffusion (0.1 M Succinate/ Phosphate/Glycine. pH 5.0)
- Crystals were soaked in a solution containing drug/inhibitor amd frozen using liquid N_2



X-ray Crystallography



Example of a diffraction pattern [3]

Argonne National Laboratory

- After focused an X-ray beam hits the crystal, diffraction patterns result from the electron density in the protein crystal.
- The diffraction patterns can be used to calculate a map of the electron density of the protein (and allow for structure determination).

Results

Successful expression of plasmids for 8 ADC 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

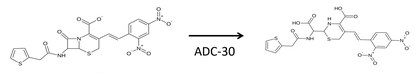
Four of the variants have been purified through a one step CM-cellulose method, resulting in about 50-80 mg of pure protein for each of the following variants.

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

Numerous crystal trays were set up for all variants and were in successful in growing crystals in three of them.

- ADC-30
- ADC-33
- ADC-162 (ADC-30 A221E)

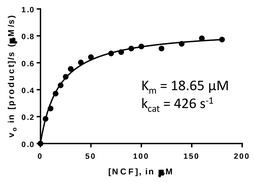
Steady State Kinetics



Nitrocefin (yellow)

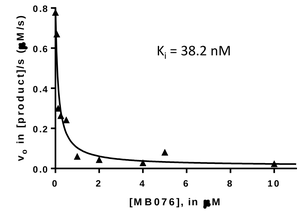
Hydrolyzed product (red, 482 nm)

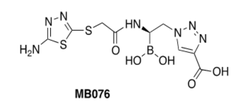
ADC-30



The reaction rate was determined for ADC inactivating a specific cephalosporin antibiotic, nitrocefin (NCF). Assay conditions: 2 nM ADC-30, 10 mM $NaPO_4$, pH 7.4, variable concentrations of NCF.

ADC-30





MB076

Competition kinetics were performed to determine the K_i values for the ADC variants with MB076. This graph shows that MB076 binds and prevents ADC-30 from inactivating nitrocefin. Assay conditions: 2 nM ADC-30, 10 mM $NaPO_4$, pH 7.4, 60 μM NCF, variable concentrations of MB076.

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

Conclusions and Future Directions

Conclusions

The slight changes between the genetic sequences of ADC-7 and the variants may have lead to changes in the ADC variant structure that allows them to bind to a wider range of antibiotics. Thus, it is crucial to characterize the structures that were obtained and determine what structural differences exist between ADC-7 and the variants. Identifying these differences is necessary as it ensure that potential inhibitors are developed for enzymes that have been found in recently infected individuals and will be applicable for treatment.

Future Directions

- Determine the crystal structures of the ADC variants and investigate how these differences enable bacteria to be resistant to more types of antibiotics.
- Complete kinetics to measure the K_i values for all ADC variants.
- Attempt to identify ideal crystal growing conditions for ADC-25 A200D/P220L/Ainsert crystals.
- The end goal of the project is to identify potential Boronic Acid Transition State Inhibitors (BATSIs), such as MB076, that can be used in tandem with β-lactam antibiotics to work against bacteria.

Acknowledgements

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References

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[2] 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

[3] Lorch, M. (2014, February 3). Explainer: What is X-ray crystallography? Retrieved from http://theconversation.com/explainer-what-is-x-ray-crystallography-22143