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Evolutionary adaptability of β-lactamase: a study of inhibitor susceptibility in various model systems

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Appendix

English summary

Nederlandse samenvatting

Curriculum vitae

List of publications

English summary

Millions of years of evolution has resulted in enzymes that are specialized in their function. β-Lactamases are enzymes that can break down β-lactam substrates, such as antibiotics, preventing the use of these antibiotics for the treatment of various infectious diseases. However, compounds have been developed that resemble the structure of β-lactam substrates or the transition state of the (de)acylation reaction, yet are not or not as easily hydrolyzed, the β-lactamase inhibitors. BlaC is the β-lactamase of *Mycobacterium tuberculosis* and is used as a model for protein evolution. **Chapter 1** provides an introduction to protein evolution, the enzyme BlaC, and β-lactamase inhibitors.

To understand if BlaC can develop resistance against inhibitors, laboratory evolution was performed, studying the evolutionary adaptability against the inhibitor sulbactam. Several amino acid substitutions in BlaC were identified that confer reduced sensitivity to sulbactam. The mutation G132S reduces the sensitivity for sulbactam inhibition and simultaneously causes a reduction in the rate of nitrocefin and ampicillin hydrolysis. Introduction of the side chain moiety of Ser132 causes the 104-105 peptide bond to assume the cis conformation. The side chain of Ser104 is rotated toward the sulbactam adduct, with which it forms a hydrogen bond that is not present in the wild-type enzyme. The gatekeeper residue Ile105 has moved relative to its position in the wild-type structure. These changes in the entrance of the active site can explain the decreased affinity of G132S BlaC for both substrates and sulbactam. Our results in **chapter 2** show that BlaC can easily acquire a reduced sensitivity for sulbactam, with a single amino acid mutation, which could hinder the use of combination therapies.

Conserved residues are often considered essential for function, and substitutions in such residues are expected to have a negative influence on the properties of a protein. However, mutations in a few highly conserved residues of BlaC were shown to have no or only limited negative effects on the enzyme. One such mutant, D179N, even conveyed increased ceftazidime resistance upon bacterial cells, while displaying good activity against penicillins. The crystal structures of BlaC D179N in resting state and in complex with sulbactam reveal subtle structural changes in the Ω-loop as compared to the structure of wild-type BlaC. The introduction of this mutation in four other β-lactamases, CTX-M-14, KPC-2, NMC-A and TEM-1, resulted in decreased antibiotic resistance for penicillins and meropenem. The results demonstrate that the Asp in position 179 is generally essential for class A β-lactamases but not for BlaC, which can be explained by the importance of the interaction with the side chain of Arg164 that is absent in BlaC. In **chapter 3** it is concluded that Asp179, though conserved, is not essential in BlaC, as a consequence of epistasis.

Epistasis is an important phenomenon in protein evolution that complicates the prediction of evolutionary pathways. The mutations I105F, G132S, and D179N, each have a different effect on BlaC, and we studied the effects of combining these mutations. Mutation I105F results in faster hydrolysis of ampicillin, while *Escherichia coli* cells producing BlaC D179N can grow on

higher concentrations of ampicillin and avibactam and the protein is more stable. The results show that combining I105F and D179N leads to a positive epistatic effect for ampicillin and results in a more thermostable enzyme, with a melting temperature 3 °C higher than that of the wild-type enzyme. Adding either I105F or D179N to BlaC G132S allows cells producing these variants to grow on higher concentrations of sulbactam, but mutations I105F and D179N are not able to compensate for the sensitivity of BlaC G132S to avibactam. In **chapter 4,** we discuss that an increase in the stability of BlaC can decrease the sensitivity of cells to sulbactam, but not to avibactam. Sulbactam is a slowly converted substrate and an increase in active protein will result in faster conversion, while avibactam acts as a stable reversible inhibitor. In BlaC G132S, the loop covering the active site resembles that of other class A β-lactamases that are sensitive to avibactam, such as KPC-2, indicating that structural changes are likely to be the cause of the increase in sensitivity.

E. coli bacteria are generally used to test the effects of BlaC variants in cells. In **chapter 5**, we tested the BlaC variants I105F, G132S, G132N, D172N, D179N, and K234R under more physiological conditions using the *M. marinum* infection model of zebrafish, which recapitulates hallmark features of tuberculosis, including the intracellular persistence of mycobacteria in macrophages and the induction of granuloma formation. To this end, the *M. tuberculosis blaC* gene was integrated into the chromosome of a *blaC* frameshift mutant of *M. marinum.* Subsequently, the resulting strains were used to infect zebrafish embryos in order to test the combinatorial effect of ampicillin and avibactam. The results show that embryos infected with an *M. marinum* strain producing BlaC show lower infection levels after treatment than untreated embryos and indicate that results obtained for *E. coli* can be extrapolated to the disease model. Additionally, embryos infected with the BlaC K234R-producing strain showed higher infection levels after treatment than those infected with bacteria producing the wild-type enzyme, demonstrating that the zebrafish host is less sensitive to the combinatorial therapy of β-lactam antibiotic and inhibitor for bacterial cells producing this variant. These findings are of interest for future development of combination therapies to treat tuberculosis.

Chapter 6 discusses the work presented in this thesis and presents an outlook on further research.