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The OXA-class of β -lactamases

A structural view on antibiotic resistance

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List of Papers

- Paper I** B. A. Lund, H. K. S. Leiros, and G. E. Bjerga. “A high-throughput, restriction-free cloning and screening strategy based on *ccdB*-gene replacement”. *Microbial Cell Factories* 13.38 (2014)
- Paper II** B. A. Lund, T. Christopeit, Y. Guttormsen, A. Bayer, and H. K. S. Leiros. “Screening and Design of Inhibitor Scaffolds for the Antibiotic Resistance Oxacillinase-48 (OXA-48) through Surface Plasmon Resonance Screening”. *Journal of Medicinal Chemistry* 59 (2016), pp. 5542–54
- Paper III** S. Ahkter, B. A. Lund, M. Lange, A. Ismael, J. Isaksson, T. Christopeit, H. K. S. Leiros, and A. Bayer. “A focused fragment library targeting the antibiotic resistance enzyme - Oxacillinase-48: synthesis, structural evaluation and inhibitor design”. Manuscript, 2017
- Paper IV** B. A. Lund, A. M. Thomassen, T. J. O. Carlsen, and H. K. S. Leiros. “Structure activity and thermostability investigations of OXA-163, OXA-181 and OXA-245, using biochemical, crystal structures and differential scanning calorimetry analysis”. Manuscript, 2017
- Paper V** Ø. Samuelsen, F. Hansen, B. Aasnæs, H. Hasman, B. A. Lund, H. K. S. Leiros, B. Lilje, J. Janice, L. Jakobsen, P. Littauer, L. M. Søres, B. J. Holzknacht, L. P. Andersen, M. Stegger, P. S. Andersen, and A. M. Hammerum. “Dissemination and Characteristics of a Novel Plasmid-Encoded Carbapenem-Hydrolyzing Class D β -Lactamase, OXA-436 from Four Patients Involving Six Different Hospitals in Denmark”. Submitted to *Antimicrobial Agents and Chemotherapy*, 2017

Paper VI B. A. Lund, A. M. Thomassen, B. Nesheim, T. J. O. Carlsen, J. Isaksson, T. Christopeit, and H. K. S. Leiros. “Structural basis for OXA-48 dimerization”. Manuscript, 2017

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Acronyms

BL β -lactamase.

BLI β -lactamase inhibitor.

ccdB coupled cell division B.

CD circular dichroism.

DBL class D β -lactamase.

DBO diazabicyclooctane.

DMSO dimethyl sulfoxide.

DSC differential scanning calorimetry.

EMP exponential megaprimer PCR.

FBDD fragment-based drug discovery.

HTS high-throughput screening.

IS insertion sequence.

LE ligand efficiency.

MBL metallo- β -lactamase.

MD molecular dynamics.

MIC minimum inhibitory concentration.

MM molecular mechanics.

MRSA methicillin-resistant *Staphylococcus aureus*.

MS mass spectroscopy.

MST microscale thermophoresis.

NMR nuclear magnetic resonance.

ORF open reading frame.

OXA oxacillinase.

PAINS pan assay interference compounds.

PBP penicillin-binding proteins.

QM quantum mechanics.

RF restriction-free.

Ro3 Rule of Three.

Ro5 Rule of Five.

SAR structure activity relationships.

SAXS small angle X-ray scattering.

SBL serine β -lactamase.

SEC size exclusion chromatography.

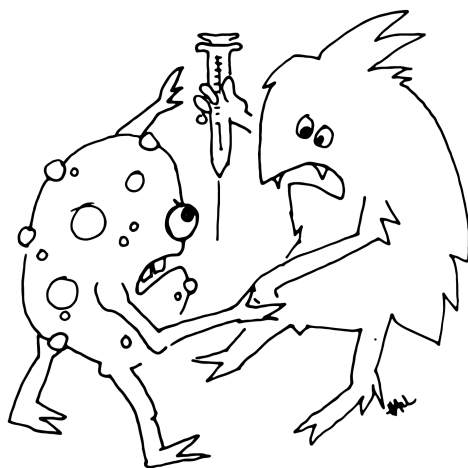
SGDD structure guided drug design.

SPR surface plasmon resonance.

Chapter 1

Background

1.1 Antibiotics and antibiotic resistance



Antibiotics kill or prevent the growth of bacteria, and are given to treat bacterial infections. Their use has increased our life expectancy and improved our quality of life. The access to working antibiotics makes invasive surgery and chemotherapy possible.¹

We have several classes of antibiotics available, including:¹

β -lactams that interfere with cell-wall synthesis

Macrolides that binds to the 50S subunit of bacterial ribosomes, preventing protein synthesis

Aminoglycosides that binds to the 30S subunit of bacterial ribosomes, preventing protein synthesis

Quinolones that inhibit topoisomerases, preventing DNA replication

However, bacteria adapt. From the beginning of time microorganisms have employed compounds very similar to our antibiotics as weapons against each other.² And bacteria have mechanisms to avoid the action of antibiotics. We refer to these mechanisms as antibiotic resistance.

There are four main mechanisms bacteria use to become resistant to antibiotics.¹

- Preventing access to target
- Mutations to prevent the antibiotic from interfering with target
- Protection of target by other mechanisms
- Modifications to the antibiotic

Bacteria have cell walls (Figure 1.1) that separate them from environment. If an antibiotic is to be effective against a cell, the antibiotic has to be able to cross the cell-wall of the organism. Some microorganisms are naturally non-susceptible to certain antimicrobial drugs.³ For example, the bacteria belonging to the *mycoplasma*-genus have no cell wall, and are obviously unaffected by any compound targeting the cell wall.⁴ Other bacteria contain efflux pumps that transport drugs out of the cell as quickly as they enter.⁵

Sulfonamides, an early class of antibiotics, worked by inhibiting the production of folic acid. Bacteria like *Staphylococcus aureus* countered these drugs by mutating the targeted enzyme, so that the drugs would not bind. The synthetic antibiotic linezolid targets the 23S rRNA ribosomal subunit of gram-positive bacteria. Bacteria, including *Staphylococcus aureus*,

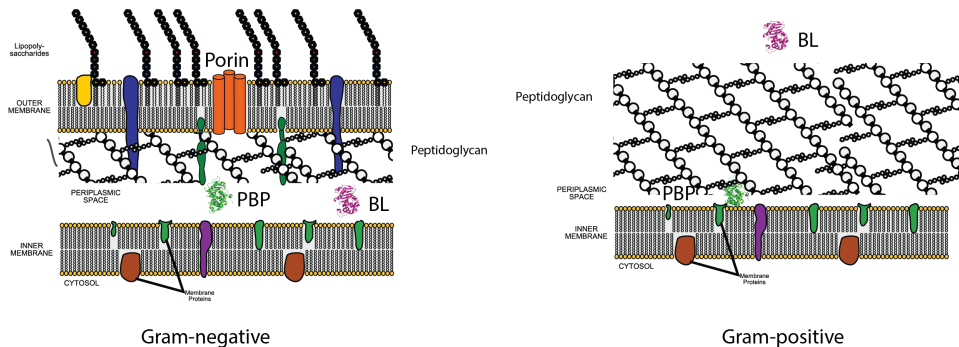


Figure 1.1. Bacteria uses cell membranes to keep nutrients and essential machinery inside, while fending off enemies. Bacteria are divided into two major groups, gram-negative and gram-positive, by the composition of their cell walls. Gram-positive have the simplest wall, with an inner membrane enclosed by a peptidoglycan layer. Gram-negative bacteria have a outer membrane and a lipopolysaccharide-layer which adds an additional layer filtering the diffusion of molecules in and out of the cell.⁶ penicillin-binding proteins (PBP) which modify the cell wall and β -lactamase (BL)-enzymes that protect the bacteria from β -lactam-antibiotics are localized to the periplasm of gram-negative bacteria. However, in gram positive bacteria the BL are excreted.⁷ Figure adapted from works of Mouagip and Jeff Dahl.

rapidly gain resistance to linezolid by selecting for mutated copies of the gene encoding for the subunit.¹

Bacteria may also use other enzymes to protect target proteins from antibiotics. For example, the macrolide erythromycin binds to bacterial ribosomes. An enzyme called erythromycin ribosome methylase methylates the antibiotic-binding site, and prevents erythromycin and other macrolides from killing the bacteria.¹

Finally, the bacteria may also modify the antibiotic itself to resist its action.¹ β -lactamases (BLs) inactivate β -lactam-antibiotics by hydrolysing the antibiotic. This mechanism will be the focus of this thesis.

1.2 β -lactamase-mediated β -lactam resistance

1.2.1 The β -lactam antibiotics

Few discoveries have been so important as Alexander Fleming's isolation of penicillin in 1929,⁸ with millions (if not billions) of lives considerably

lengthened. Antibiotics of the β -lactam-class act by binding to cell-wall-synthesis enzymes termed PBP.

The β -lactam antibiotics bind to PBPs because they are structurally similar to the natural D-ala-D-ala-substrate of the PBPs,⁹ and as a suicide-inhibitor the β -lactam-PBP-complex is irreversible and leaves the enzyme permanently blocked. Without functioning cell-wall synthesis enzymes, the bacteria are unable to divide and will die.

There are several different β -lactam drugs on the market. Common to all of them is the four-atom ring with the carbonyl, the β -lactam-ring. The penicillins, which have the β -lactam-ring fused to a thiazolidine ring, were the first to be developed. The penicillins ampicillin and oxacillin (Figure 1.2A-B) are two examples of important penicillins. There is significant strain to the fused penam ring, and this is what makes the carbonyl of the penicillin so reactive. A normal tertiary amide would form a resonance structure, however the ring strain prevents this.¹⁰

Monobactams like aztreonam (Figure 1.2C) do not have any ring fused to the β -lactam-ring. It is only active against gram-negative bacteria, but does have the advantage of not having cross-allergies with penicillins and cephalosporins.¹⁰

Cephalosporins has the β -lactam ring fused to a six membered dihydrothiazine ring. The larger ring has less strain, and the first cephalosporins were less potent than penicillin G.¹⁰ However, cephalosporins target a broader spectrum of gram-positive and gram-negative bacteria than the penicillins. Cephalosporins are often referred to by their generation. The first-generation are similar to the cephalosporin C that was first discovered in the 1940s. The second generation added a methoxy-substituent to the β -lactam-ring, which made cefoxitin more stable to the actions of BLs. Likely, the methoxy-group adds some steric hindrance which slows down the hydrolysis. The third generation has improved activity against gram-negative bacteria and higher potency against the PBPs with an added aminothiazole ring to the sidechain. Drugs such as ceftazidime belong to the third generation. Fourth-generation drugs such as cefepime (Figure 1.2D) are zwitterionic with a positive charge added.¹⁰ Finally, ceftobiprole and ceftaroline belong to the fifth generation of cephalosporins. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major clinical problem, and these fifth generation cephalosporins are ac-

tive against MRSA due to their 1,3-thiazole-ring.^{11,12} An oxime group is added for BL-resistance and a 1,2,4-thiadiazole-ring is added to increase activity against gram-negatives and for affinity against the transpeptidase.¹² However, the classification as a new generation is disputed as there is little activity against gram-negative bacteria.¹²

Finally, the carbapenems have the β -lactam-ring fused to an unsaturated five membered ring. The hydroxyethyl side-chain and the change in stereochemistry of the β -lactam-ring-substitution contributes to the increased stability of carbapenems from BLs.^{10,13} Thienamycin was the first carbapenem, with potency against both gram-positive and gram-negative bacteria. However, thienamycin is too unstable to be used in clinical applications.¹³ Imipenem (Figure 1.2E) was designed to be more stable. Yet, imipenem is hydrolysed by a human enzyme, dehydropeptidase, and the metabolites are toxic to the kidney. Merck, the company that made imipenem, developed the dehydropeptidase inhibitor cilastatin so that imipenem could be used in the clinic.¹⁰ Meropenem was developed later, and while the potency is lower against gram-positive bacteria, it is more potent against gram-negatives including *Pseudomonas aeruginosa*. The more bulky substitution in the 2-position protects meropenem from dehydropeptidases, and cilastatin is not necessary with meropenem.¹⁰ Ertapenem has a methyl group in the 1-position as well as a bulkier substitution in the 2-position, which makes it more stable against dehydropeptidases.¹⁴ The plasma binding also extends the biological half-life of ertapenem so that ertapenem can be given to the patient in a dosage of 1 gram per day, instead of multiple infusions.¹⁴

The timeline in Figure 1.3 shows how the landscape of β -lactam antibiotics gradually expanded in response to the challenges caused by BLs. However, no new classes of β -lactam-antibiotics has entered the clinic since the introduction of the monobactams.

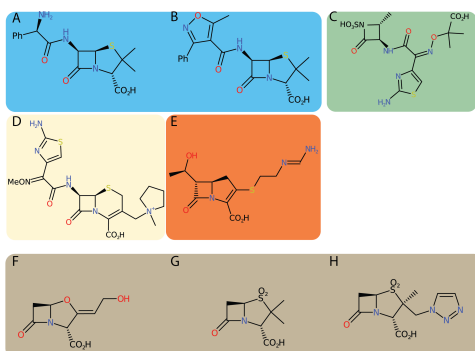


Figure 1.2. β -lactam containing molecules of different classes. The penicillins ampicillin (A) and the preferred substrate for class D β -lactamases (DBLs) oxacillin (B). The monobactam aztreonam (C). The cephalosporin cefepime (D). The carbapenem imipenem (E). The β -lactam-based β -lactamase-inhibitors clavulanic acid (F), sulbactam (G) and tazobactam (H).

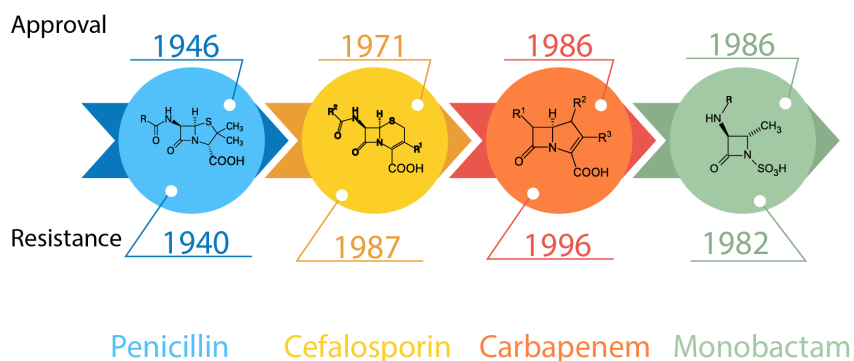


Figure 1.3. Timeline of the development of the β -lactam antibiotics, with dates of approval and year of described resistance.^{15,16}

1.2.2 Origin, classification and phylogenetics of β -lactamases

In December of 1940 Abraham and Chain published their discovery of a penicillinase from *Escherichia coli*.¹⁷ Since then, over 2000 enzymes¹ have been identified all over the world. In order to differentiate and classify it became necessary to divide the BL into classes based on amino acid sequences and conserved motifs.^{18–20} Eventually we ended up with the four Ambler classes.^{21,22}

Class A BLs are common serine β -lactamases (SBLs) like TEM, SHV and KPC with a serine-based hydrolytic mechanism.

Class B BLs have metal ions as key elements of their hydrolytic mechanism and are known as metallo- β -lactamases (MBLs).

Class C BLs are SBLs, and are represented by e.g. AmpC.

Class D BLs are atypical SBLs, the most known are the oxacillinases (OXAs).

In addition, we have the Bush-Jacoby-Medeiros classes based on substrate (penicillins, cephalosporins, carbapenems) and inhibitor (clavulanic acid/EDTA) profiles.²³

The sequence similarity is so low between the classes of BLs that it is not meaningful to use sequence-based methods for alignment. However, the three dimensional structure is more conserved and may be used to construct a rough phylogeny (Figure 1.4) of the serine BLs. It appears that the SBLs share a common ancestor, a PBP with transpeptidase activity that evolved to be more efficient in cleaving the β -lactam-ring. The MBLs have evolved separately from the other BLs and is an independent branch.²⁴

It is important to realize that BLs are ancient, and that their origin date to the time before the divergence between gram-positive and gram-negative bacteria based on phylogenetic analysis.^{24,25} This finding is supported by the presence of antibiotic resistance genes in bacterial samples from 5000 year old corpses²⁶ and from undisturbed localities,²⁷ including a cave believed to be isolated from the surface for over 4 million years.²⁸ While our reckless use of antibiotics is certainly a problem, it is not the cause of antibiotic resistance.

¹The BL-database (BLDB) at <http://bldb.eu> contains 2666 entries per 12.06.2017

Several BL-genes have been identified in *Shewanella*-species,²⁹ a marine bacteria known to be a part of the surface flora of fish.³⁰ It is not clear exactly how the genes are mobilized, but it is clear that *Shewanella*-bacteria are an important reservoir for antibiotic resistance genes.²⁹ BL-genes of all classes have also been identified in remote Alaskan soil, where they had no expectation of any human involvement.²⁷ This means that antibiotic resistance is all around us.

Of particular concern is the horizontal transfer of antibiotic resistance genes such as the OXAs to human pathogens such as *E. coli*,³¹ *Klebsiella pneumoniae*,^{32,33} *Pseudomonas aeruginosa*³⁴ or *Acinetobacter baumannii*.³⁵

BL genes have been shown to be carried by plasmids or transposons.³⁶ Most plasmids are circular and double-stranded DNA molecules, which contain from 2 to 400 genes. Plasmids can be described as small, auxiliary and dispensable chromosomes.³⁷ Resistance plasmids carry one or more genes encoding antibiotic resistance enzymes. Conjugative plasmids encode the functionality needed to transfer DNA to other bacteria by cell-to-cell coupling with a sex pilus.³⁷ Some conjugative plasmids have a broad host range, and may be transferred between many classes of bacteria, whereas other plasmids have a more limited host range.³⁷ Integrons, often called gene cassettes, are genetic structures for recruiting open reading frames (ORFs). The integron will contain a recombinase for inserting the ORF into the cassette directed by a integron-encoded recombination site.³⁸ The resulting gene cassettes, with one or several resistance genes, may then be transferred by a mobile genetic element such as a plasmid.³⁷

Insertion sequences (ISs) are small genetic elements capable of being inserted at multiple sites in the genome.³⁹ Sometimes, IS elements functions in pairs, and are then able to mobilize the genetic sequence between the ends of the pair. This kind of structure is called a composite transposon. Transposons are capable of mediating antibiotic resistance genes, unlike ISs that only encode ORFs needed to replicate themselves.⁴⁰ IS may still be important, as they may contain promotor-sequences important for the expression of BLs enzymes, which was shown for the DBL OXA-23.⁴¹

Most commonly the DBLs are referred to as OXAs for their preferential hydrolysis of the penicillin-like antibiotic oxacillin (Figure 1.2B). The OXA-enzymes are numbered sequentially after their discovery and characterization,

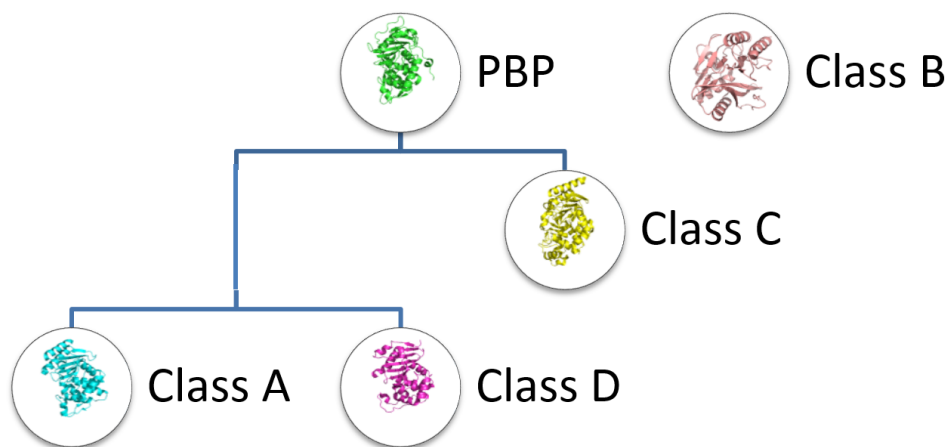


Figure 1.4. Structure based phylogeny of BLs showing the suggested relationships between the classes of SBLs and the PBPs.²⁵ Note that the class B MBLs does not derive from the PBPs.

so the number has no particular meaning. To add to the confusion sequencing errors have led to some duplications (OXA-24/40), and in other cases the enzymes were named before it was recognized that they in fact were OXAs (e.g. LCR-1).

1.2.3 Important subgroups of the class D β -lactamases

As Figure 1.5 shows there are several subgroups within the OXA-family. OXA-1 (also known as OXA-30 because of sequencing errors) has several known homologs. OXA-1 itself hydrolyses penicillins well, but there is also some activity against cephalosporins. All the OXA-1-like genes have been described has been on class 1 integrons.³⁶

OXA-2 only has 30 % identity to OXA-1, and constitutes its own subgroup. OXA-2 has a narrow spectrum of hydrolysis.³⁶ However, there are also reports of extended-spectrum activity for OXA-2-like enzymes.⁴² OXA-9 is another narrow spectrum DBL. It has the unusual property for a DBL of being inhibited by clavulanic acid.³⁶

OXA-10, formerly known as PSE-2, has activity against some cephalosporins as well as the monobactam aztreonam.³⁶ Laboratory mutants of

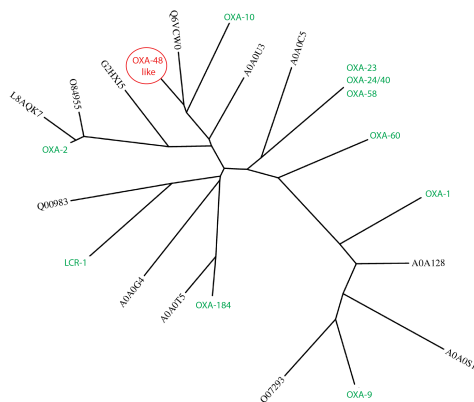


Figure 1.5. A phylogenetic tree on all OXAs registered in the UniProt database clustered by a sequence identity level of 50% (UniRef50). The tree shows the immense diversity within the class, with every branch having less than 50% identity. Alleles with known names are named, otherwise UniRef50-identifiers are used. Figure prepared using phylogeny.fr.⁴³ OXA-48, OXA-163, OXA-181, OXA-245 and OXA-436 fall within the red circle having more than 50 % identity to each other.

OXA-10, some of which have also been observed in clinical isolates, have been shown to give ceftazidime resistance in *P. aeruginosa*.⁴⁴

Interestingly, LCR-1 from *P. aeruginosa* was first believed to belong to its own class of BLs. However, based on the genetic sequence and a reanalysis of the kinetic parameters of LCR-1, it was determined that it belongs to class D.⁴⁵

OXA-23, also known as ARI-1,⁴⁶ was the first DBL to show carbapenemase activity. While it OXA-23 has weak carbapenemase activity, it does yield resistant bacteria. OXA-24, also known as OXA-40 due to sequencing errors, was first identified on a chromosome, but has been shown to be plasmid borne as well. It has been shown to mediate carbapenem-resistance in *Acinetobacter baumannii* and *P. aeruginosa*.³⁶ OXA-58 is yet another carbapenemase with some sequence identity to OXA-23, but OXA-58 has also been regarded as its own subgroup.

OXA-48, which will be the main topic of this PhD-thesis, was first identified in a carbapenem-resistant isolate of *Klebsiella pneumoniae* in Turkey.⁴⁷ This is an important observation as the other carbapenemase DBLs have been mostly limited to *A. baumannii*.⁴⁸ The gene for OXA-48 was shown to be plasmid-mediated, with IS1999 shown to be upstream of the gene.⁴⁷ The OXA-48 enzyme was shown to have activity against penicillins, some cephalosporins and carbapenems, with only weak inhibition by clavulanic acid, tazobactam or sulbactam.⁴⁷ When cloned into *E. coli* OXA-48 only yields modest increases in minimum inhibitory concentrations (MICs), however combined with porin-deficient strains the increase in MICs shows that OXA-48 is capable of conferring high levels of carbapenem resistance.⁴⁹ Many OXA-48-like enzymes have been described.⁵⁰ It appears that *Shewanella*-species may be the host genus of the OXA-48-like enzymes.^{29,51} OXA-163 is an interesting example because it only has one mutation and a four residue deletion that shifts the substrate hydrolysis profile from carbapenems towards cephalosporins, and also increases activity against the monobactam aztreonam.⁵² OXA-181 is another OXA-48-like carbapenemase that causes significant concern, as it has been shown to be co-expressed with the MBL NDM-5 (the "evil twins").⁵³

OXA-60 is a chromosomally encoded carbapenemase from the emerging pathogen *Ralstonia pickettii*. The production of OXA-60 appears to be

induced by β -lactams, and the genetic environment of the OXA-60 gene indicates that its expression is under the control of a transcription regulator and two other ORFs with unknown function.⁵⁴

Many OXAs are identified in metagenomic sequences and from uncultured and environmental bacteria. For many it is unclear whether they are active. Some DBLs like OXA-184 from *Campylobacter jejuni* appear to be inactive when tested in standard assays.⁵⁵ One could speculate that DBLs might have other functions in the bacteria as well.

Most DBLs have been identified in gram-negative bacteria (Figure 1.1) such as *E. coli* or *K. pneumoniae*, but DBLs has also been identified in gram-positive bacteria, especially within the *Bacillaceae*-family.⁷ In gram-negative bacteria the BLs are exported to the periplasm, but for gram-negative bacteria BLs are excreted into the media.⁵⁶

There has been significant confusion about the hydrolytic profiles of the different DBLs with the differences in the buffers used for *in vitro* characterization. Some studies include bicarbonate in order to provide a saturating amount of CO₂, as this more closely resembles the assumed biological condition with full carboxylation of Lys73 (which will be covered in subsection 1.2.4).

The choice of host is also important, and it has been shown that DBLs like OXA-2 and OXA-10 which in *E. coli* or *P. aeruginosa* do not cause resistance to carbapenems produce high levels of resistance in *A. baumannii*.⁵⁷

1.2.4 Biochemical and structural analysis of class D β -lactamases

Like other SBLs OXAs have a serine as the primary catalytic residue. This serine is residue number 70 according to the DBL-numbering⁵⁸ and it is responsible for the hydrolysis of the β -lactam-substrate.³⁶ The active site serine is part of the highly conserved ₇₀STFK₇₃-motif, which together with the motifs ₁₁₈SVV₁₂₀, ₁₄₄YGN₁₄₆ and ₂₀₈KTG₂₁₀ make out the key elements of the active site of OXAs.

Several residues has been shown to be critical for substrate hydrolysis to occur for the DBLs. Ser70 is essential for substrate hydrolysis and is conserved among all DBLs.⁵⁹ Lys73 is also strictly conserved, and quite unique because it is carboxylated in DBLs.⁶⁰⁻⁶² Carboxylation of lysines is a fairly uncommon post-translational modification, which occurs sponta-

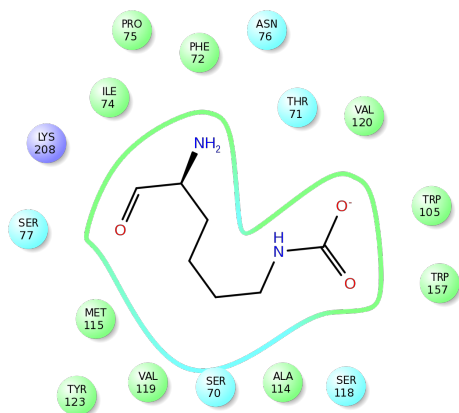


Figure 1.6. The surroundings of Lys73 are predominantly hydrophobic residues, stabilizing the unprotonated lysine allowing the entrance of CO₂

neously. The lysine has to be unprotonated for the carboxylation to happen, which limits the activity of OXA-enzymes at low pH.⁶³ Poisson-Boltzmann calculations show that the pK_a of the Lys73 side-chain is approximately 7, compared to 10 for the free amino acid. This reduction is probably due to the hydrophobic environment of Lys73 in DBLs as shown in Figure 1.6.⁶⁴ It has been estimated that approximately 1 % of large proteins contain a carboxylated lysine, however, carboxylated lysines are not observed with mass spectroscopy because acidic conditions release the carboxylate-group.⁶⁵ The carboxylation turns the positively charged lysine into an acidic mimetic of arginine, with the potential of coordinating metal ions and forming strong hydrogen bonds. Mutants of Lys73 have been shown to be inactive.^{61,62,66} Several residues have been shown to be important for the carboxylation of Lys73. Val120 provides a hydrophobic environment for Lys73. Interestingly in BlaR1 penicillin receptor protein, which does not deacylate after binding a penicillin, residue 120 is a threonine. Mutating Val120 to a threonine in OXA-10 significantly reduced the activity, and it was shown that the affinity for CO₂ was significantly weakened.⁶²

Trp157 which participates in hydrogen bonding with Lys73 has been shown to be important in OXA-10,⁶⁷ with reduced substrate hydrolysis rates and decreased stability for the mutants. Interestingly, the researchers were able to retain activity with a histidine in the position of Trp157, and the

activity could be rescued with the addition of sodium bicarbonate. Without a carboxylated lysine, there is a positive charge in the active site capable of binding chloride-ions. This indicates that the reports of chloride-inhibition of OXAs is due to the lack of sodium bicarbonate in the reaction buffers.⁶² Trp157 belongs to the ω -loop (residues 157-165), which is thought to be important for substrate recognition.⁶⁸

The main chain amides of Ser70 and Tyr211 create the oxyanion hole of the OXA-enzymes,⁶⁹ analogous to the oxyanion-holes in proteases.

The loop spanning residues 212-220, referred to in the literature as the $\beta 5 - \beta 6$ loop is essential for activity against carbapenems.⁷⁰ OXA-163, which is a homolog of the carbapenemase OXA-48, has a shortened $\beta 5 - \beta 6$ -loop, and has lost its carbapenemase-activity.⁷¹ However, mutations of OXA-10 to introduce carbapenemase-like $\beta 5 - \beta 6$ -loops were shown to yield activity against carbapenems.⁷² Arg214 in the $\beta 5 - \beta 6$ -loop forms an ionic bond with Asp159 from the ω -loop.⁶⁹

In OXA-24 the residues Tyr112 and Met223, which are not conserved in OXA-48, pack against each other forming a hydrophobic bridge that spans the active site.⁷³ This tunnel-like access is not found in OXA-48.⁶⁹ The affinity for carbapenems is weaker for OXA-48 than for OXA-24. This tunes the activity in such a way that for imipenem the catalytic efficiency (k_{cat}/K_m) is higher for OXA-48,⁷⁰ whereas for meropenem OXA-24 is more active than OXA-48.¹³

It realized early on that OXA enzymes forms dimers in solution.⁷⁴ OXA-10 was first crystallized with the divalent cation cobalt present in the crystallization condition. Glu227 and His203 (OXA-10 numbering) from one monomer and Glu190 from the other monomer forms a octahedral complex with a cobalt ion (Figure 1.7).

Each dimer thus binds two cobalt-ions in a symmetric fashion.⁷⁵ The dimerization was shown to be important for the enzyme activity as well as the thermal stability.⁷⁶ *In vivo* cellular concentrations of OXA enzymes have been determined to be in the micromolar range. This indicates that the dimer is likely to be the biologically relevant state of the enzymes.⁷⁶ OXA-29 is a OXA-1 homolog (38% identity) which forms stable dimers, with a K_d -value estimated to be lower than 20 nM.⁷⁸ Preliminary crystal structure analysis reveals that OXA-29 has an atypical dimer interface when compared

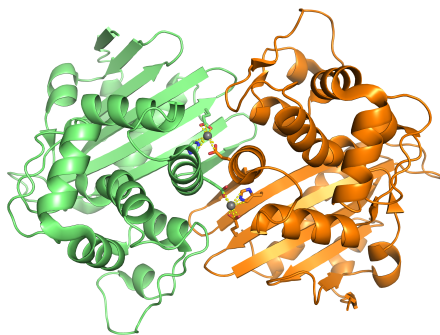


Figure 1.7. OXA-10 forms dimers in solution. Extensive hydrophobic interactions facilitate dimerization, however binding of cations such as cobalt promotes dimerization and lowers the K_d of dimerization significantly.^{75,76} Residues Glu190, His203 and Glu227 are shown as sticks, and the two (one per chain) cobalt-ions are shown as grey spheres. PDB-ID: 4s2o.⁷⁷

to OXA-10 and OXA-48.⁷⁹ Whereas the dimer interface in OXA-48 involves residues in the range 89-116 and 185-206,⁶⁹ OXA-29 forms dimers with residues from a β -sheet (₆₄RFA₆₆) preceding the STFK-motif and with a C-terminal helix (₂₆₇ASFRAKNETLNQ₂₇₉) that is not present in OXA-48. With this quaternary structure parts of the active site are covered with the other monomer.⁷⁹ Unexpectedly, cations such as copper appear to inhibit OXA-29, although no ions were observed in the dimer interface.^{78,79}

However, not all DBLs are dimers. OXA-1, OXA-23 and OXA-24 are examples of monomeric DBLs. OXA-1 for example is a monomer in solution,⁸⁰ but it is interesting to note that OXA-1 also has cysteines capable of forming stabilizing disulphide-bridges. One could speculate that in the absence of dimerization such a stabilizing covalent interaction could be necessary for other parts of the enzyme to be flexible enough for the catalytic action.⁸¹

OXA-23 has recently been shown to be especially interesting. By *in vivo* cross-linking mass spectroscopy (MS) OXA-23 was shown to interact with several proteins in *A. baumannii*.⁸² In particular, OXA-23 was shown to interact with porin-forming proteins, and it has been suggested that the OXA-23 BL could act as an inlet filter for the bacteria, hydrolysing any β -lactam entering the cell.⁸² *A. baumannii* has acquired the gene for OXA-23, indicating that these protein-protein interaction sites may be conserved and present for other DBLs.

1.2.5 Reaction mechanism of class D β -lactamases

DBLs are known to hydrolyse penicillins, especially oxacillin, from which the name derives. But cephalosporins and carbapenems are also common substrates. The scaffolds of representative β -lactam-containing drugs are shown in Figure 1.2. Some DBLs also have activities against monobactams.⁸³ The traditional β -lactam-based inhibitors are generally less efficient against the DBLs.⁸⁴

New reaction mechanisms for the DBLs are proposed rather frequently.^{66,75,85–88} However, the models have some features in common. The hydroxyl-group of Ser70 acts as a nucleophile, forming an acyl-complex with the carbonyl of the β -lactam-ring. The nitrogen of the β -lactam-ring departs, opening the ring. This is common for all SBLs. Cysteine can also act as a nucleophile, and a laboratory mutant of the class A SBL TEM with a cysteine substitution of the active site serine was shown to be active.⁸⁹

Unique to the DBLs is the carboxylated Lys73 acting as the general base. In the proposed models Lys73 has two roles: activating Ser70 for the initial nucleophilic attack and accepting the proton of a water molecule. The activated water molecule acts as a nucleophile and deacylates the acyl-enzyme complex releasing the enzyme to start over again with a new substrate.^{85,90} Conformational changes in Leu158 have been proposed to be a gatekeeper for the access of the hydrolytic water to the carboxylated Lys73.^{35,70}

Ser118 is often given the role of stabilizing the complex, with Lys208 contributing as either a proton acceptor or donor. In some structures it is observed in multiple conformations. In one conformation it is within hydrogen bonding distance to Lys208, and in the other conformation it is within hydrogen bonding distance to Ser70.^{69,75}

Other residues in the active site may contribute to the catalysis by trapping the substrate, like Arg250 which forms an ionic bond with the carboxylate group,⁷⁵ or by stabilizing transition states. Interestingly, the gram-positive DBLs BPU-1 from *Bacillus pumilus* does not have this conserved arginine, and as a result the binding mode of substrates is quite different (Figure 1.9).⁷ The activity is also lower, which may explain why these enzymes were discovered first in 2015.⁷

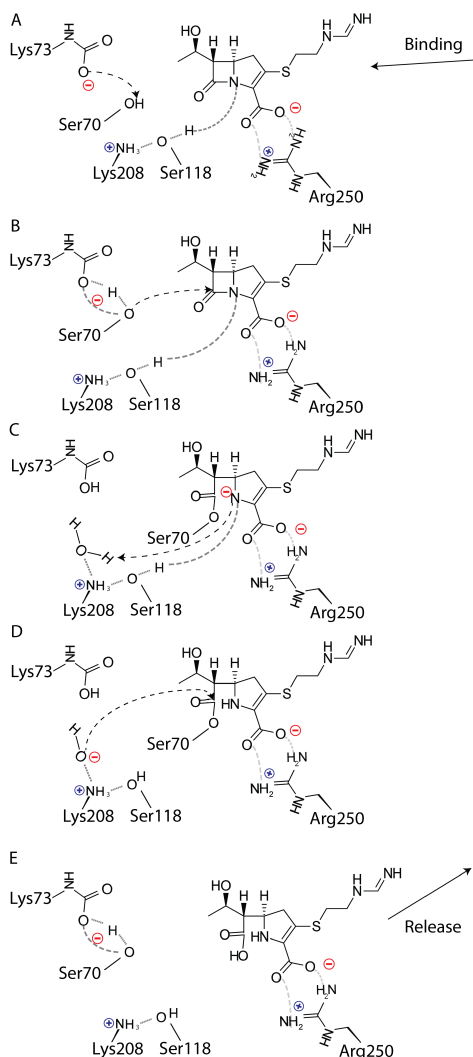


Figure 1.8. One of the proposed reaction mechanisms for DBLs based on quantum mechanics (QM)/molecular mechanics (MM)-calculations on OXA-23.⁸⁵ The pre-acyl complex is stabilized by an ionic bond of the carboxylate group to Arg250 (A), while Ser70 is activated by the carboxylated Lys73. The activated Ser70, a strong nucleophile, attacks the β -lactam-ring (B). The β -lactam-ring opens and a covalent acyl-complex is formed. Lys208 stabilizes a catalytic water, and the water donates a proton to the negatively charged nitrogen of the pyrroline-ring (C). The hydroxyl-ion, stabilized by Lys208, attacks the carbonyl-group of the acyl-complex (D), leading to the release of the substrate (E).

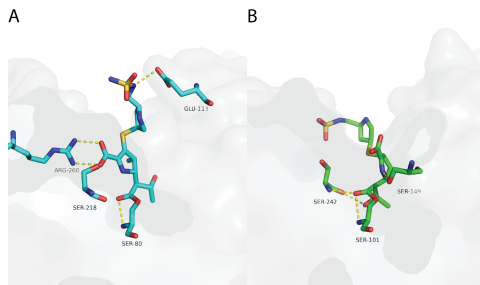


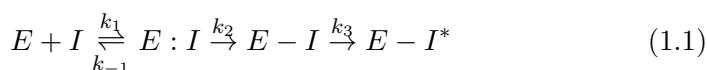
Figure 1.9. DBLs exist in both gram-negative and gram-positive bacteria. Some gram-positive DBLs does not have the conserved Arg250-residue for anchoring the substrate carboxylate. The lack of anchoring reduces the affinity and changes the binding pose significantly. OXA-51 (cyan, A) with doripenem shows the expected binding pose for carbapenems in the active site of a DBL,⁹¹ while BPU-1 (green, B) demonstrates a flip of the pyrroline ring and fewer polar interactions.⁷

1.2.6 Inhibitors of class D β -lactamases

To facilitate further use of β -lactam antibiotics there is a need for new β -lactamase inhibitors (BLIs) targeting DBLs.^{16,92} BLIs are used in combination therapies with β -lactam-antibiotics to treat infections, which would otherwise be resistant to the β -lactam-antibiotics.¹⁶

Classical β -lactam-based β -lactamase inhibitors

To extend the use of the existing β -lactam-antibiotics BLIs like clavulanic acid, sulbactam and tazobactam (Figure 1.2F-H) were developed. These β -lactams have little activity against PBPs, however they act as suicide inhibitors against several SBLs, the class A BLs being especially susceptible.⁸⁶ Equation 1.1 represents a general mechanism for irreversible inhibitors. First a non-covalent complex is formed (E:I), followed by a formation of a covalent bond (E-I). Finally, the reaction is terminated by some modification to the inhibitor-molecule which causes permanent enzyme inactivation ($E - I^*$).⁸⁶



Clavulanic acid (Figure 1.2F) was isolated from *Streptomyces clavuligerus* in the 1970s, and had little activity against PBPs, but showed synergistic

effects together with other β -lactam antibiotics.⁸⁶ Clavulanic acid is a clavam, meaning that it has a five membered ring where one of the atoms is an oxygen fused to the β -lactam-ring.¹⁶ SBLs belonging to class A are usually susceptible to inhibition by clavulanic acid.¹⁶

Penams, where the oxygen in the five membered ring is replaced with a sulfur, were investigated following the success of clavulanic acid. Sulbactam (Figure 1.2G) and tazobactam (Figure 1.2H) was prepared synthetically result of this process in 1978 and 1980 respectively.⁸⁶ Sulbactam and tazobactam are better against class C SBLs, and do not induce production of the chromosomally mediated AmpC BL.¹⁶

The heteroatom in the five membered ring is critical to the mechanism, as it functions as a leaving group.⁸⁶ After formation of the acyl-complex with the enzyme the five-membered ring opens, making the complex more stable.⁸⁶ Electrospray ionization MS with the SBL TEM-2 and clavulanic acid shows that several intermediates are produced in the inhibition process,⁹³ as expected from Equation 1.1.

Polycarboxylic acids

Based on the crystallographic observation of citrate binding in the active site of an unrelated SBL a group investigated analogues and homologs of citrate as inhibitors of OXAs. They were able to identify the lipophilic compound 2-aminopropane-1,3-di(benzyloxycarbonyl)-2-carboxylic acid shown in Figure 1.13A as a inhibitor of OXA-10 with a K_i of 20 ± 4 μ M. Based on the crystal structure of benzylpenicillin they believe that the aromatic rings are confined within the hydrophobic pocket.⁹⁴

Along the same lines, a tartrate molecule (Figure 1.13B) was observed in the crystal structure of OXA-46. H-bonds to Ser70, Lys73, Lys208, Thr209 and Arg250 were observed. The tartrate-molecule only bound to the non-carboxylated Lys73 according to the crystal structure. However, the orientation between subunits were different, and the concentration of tartrate was at least 400 mM in the crystallization condition.⁹⁵

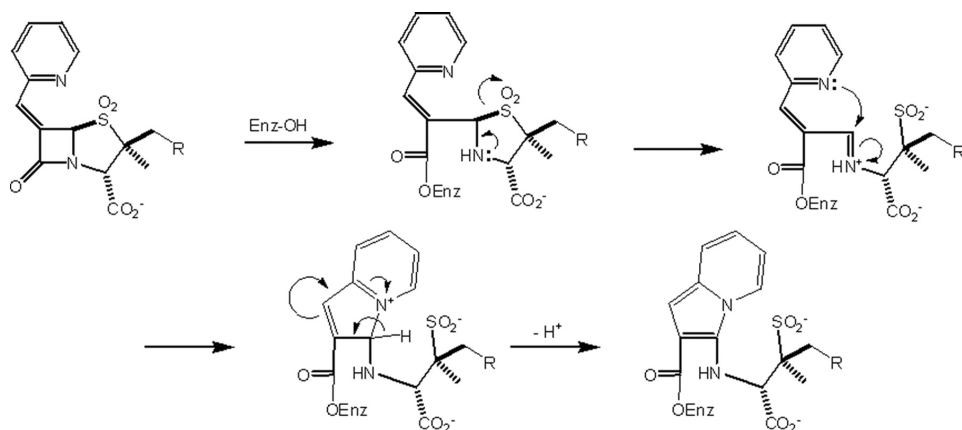


Figure 1.10. The proposed reorganization reaction mechanism for LN-1-255. Reused from Pattanaik et al. [97] with permission.

Penicillanic acid sulfones

Penicillanic acid sulfones such as LN-1-255 shown in Figure 1.13C have been shown to inhibit both class A and D SBLs. LN-1-255 belongs to the penam subclass of the β -lactam inhibitors together with tazobactam and sulbactam. LN-1-255 showed activity even at 4 mg/L towards *E. coli* and *K. pneumoniae* with OXA-48 in MIC-synergy assays. LN-1-255 contains a catechol-ring making it resemble a natural bacterial siderophore in order to utilize the iron uptake mechanism to cross the outer membrane.⁹⁶ Upon formation of the acyl-complex, the ring system is reorganized with the formation of a bicyclic aromatic intermediate (an indolizine) as shown in Figure 1.10. There is no crystal structure of LN-1-255 with any OXA at this time, however, docking studies suggest that LN-1-255 interacts with Arg250, Lys208, Tyr211 and Thr209. Both the carboxylate and the sulfinate group interacts with Arg250, the carboxylate group of the ester-linkage interacts with the amide of Tyr211 and one of the phenol-groups of the catechol hydrogen bonds to the amino group of Lys208.^{97,98}

6-hydroxyalkylpenicillanates

6-hydroxyalkylpenicillanates are another class of penam derivatives, and they were designed to probe the enzyme mechanism of BLs.⁹⁹ The compounds are designed to prevent the hydrolysis of the acyl-complex by pre-

venting the approach of the hydrolytic water, either from beneath the β -lactam-ring (α) or above (β). Whereas the class A BLs are inhibited by the α -hydroxyalkylpenicillanates and the class C BLs are inhibited by the β -hydroxyalkylpenicillanate shown in Figure 1.13D, OXAs are inhibited by both.¹⁰⁰ The ^{13}C nuclear magnetic resonance (NMR) spectra for OXA-10 show a signal for the carboxylated lysine. This peak is only slightly broadened with the α -hydroxyalkylpenicillanate, but the binding of a β -hydroxyalkylpenicillanate both broadens the signal significantly and shifted the peak upfield indicating a strong binding.¹⁰⁰ The crystal structure of a β -hydroxyalkylpenicillanate shows a covalent link to Ser70. Lys73 is carboxylated in the crystal structure. It appears that the 6 β -hydroxyisopropyl disrupts the canonical conformation of the penicillin-core in the active site, and the carboxylate-group is forced away from Arg250 and Lys208. The hydroxyl-group of the 6 β -hydroxyisopropyl appears to be hydrogen bonding to Ser70 and the carboxylated Lys73. The authors propose that the hydroxyl-group is in the position of the hydrolytic water in enzymatic reaction, and that this precludes the enzyme from deacylating the complex with the β -hydroxyalkylpenicillanate. The compounds were shown to be active both against OXA-10 and OXA-58.⁶⁶

Phosphonates

Phosphonates have been shown to be active against other SBLs. While the classical and acyl phosphates were ineffective, a dibenzoyl phosphate was demonstrated to be active against both OXA-1 and OXA-10.^{101,102} Against OXA-1, the compound shown in Figure 1.13E had activity comparable to clavulanic acid.^{101,102} The binding appeared to be covalent, but reversible. A challenge for this set of compounds is phosphodiesterases, which will cleave many phosphonates. Another challenge appears to be the binding of the best compound to a unidentified second site of the BL at high inhibitor concentrations. It is believed that the compounds form a stable anionic tetrahedral complex resembling the transition state of the enzymatic hydrolysis.¹⁰³

Since the binding of the phosphonates is likely to require a pentacoordinated intermediate, oxyanions with higher coordination numbers were investigated. ^{51}V NMR studies indicated that both penta and hexacoordinated vanadium complexes were possible for class C BL. Based on this

observation catechol-vanadate complexes as shown in Figure 1.13I were evaluated against OXA-1 and shown to be active on a μM scale.¹⁰³

Cyclic boronates

Cyclic boronates like the one shown in Figure 1.13G were designed to mimic the hypothetical shared tetrahedral intermediate of MBL and SBL-catalysed hydrolysis of β -lactams.¹⁰⁴ With 10 minutes of incubation these compounds show IC_{50} -values in the nanomolar range against OXA-23 and OXA-48. A strong shift of 6 ppm was observed for the ^{13}C NMR-peak for the carboxylated lysine upon binding of a cyclic boronate to OXA-10. This shift was significantly larger than the 0.4 ppm shift observed for the complex between OXA-10 and the β -hydroxyalkylpenicillanate shown in Figure 1.13D. The crystal structure with OXA-10 (Figure 1.11) reveals that the boron atom is covalently linked to Ser70, with a clear tetrahedral geometry in contrast to the planar geometry expected for acyl-complexes with β -lactams. The carboxylate group of the cyclic boronate is positioned towards Arg250, and the acetamido-group is positioned to form a hydrogen bond with the main-chain carbonyl of residue 211. The boronate ester oxygen, analogous to the β -lactam-nitrogen, appears to form a hydrogen bond with Ser118.¹⁰⁵ Vaborbactam from the Medicines Company is a cyclic boronate currently in Phase 3 in the USA.⁹²

Avibactam and diazabicyclooctanes

Avibactam, a diazabicyclooctane (DBO), was the first non- β -lactam BL-inhibitor to reach the market in 2015.¹⁰⁶ Its spectrum of activity includes SBLs from class A, C and D.¹⁰⁷ Avibactam binds covalently to OXA-enzymes including OXA-24 and OXA-48. The binding is reversible, but the deacylation occurs via recyclization, reforming the intact avibactam molecule ready to inhibit a new enzyme.¹⁰⁸ For the traditional BLIs there was a competition between hydrolysis and inhibition by the BLI, leading to a low efficiency where 10s-100s of molecules were necessary to inhibit each BL. However, for avibactam the hydrolysis in most cases leads to recyclization and only 1-5 molecules of avibactam are necessary to inhibit a BL-enzyme.¹⁰⁹ Acylation rates are faster for OXA-48 than for OXA-10, but the deacylation rates are

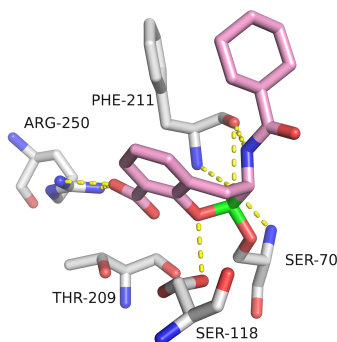


Figure 1.11. Cyclic boronates bind covalently to DBLs, with a tetrahedral complex for the boron-atom (green) mimicking the expected tetrahedral intermediate of β -lactam-hydrolysis. In this complex with OXA-10 the cyclic boronate 1C forms a salt-bridge with Arg250, hydrogen bonds with the side-chains of Ser70, Ser118 and Thr209 and with the main-chain of Ser70 and Phe211. PDB-id 5fq9¹⁰⁵

measured in days implying a stable complex.¹⁰⁷ In the crystal structure with OXA-48, avibactam is present in all the monomers of the asymmetric unit. However, Lys73 is only carboxylated in 2 of the 8 chains, with free CO₂ observed in 3 chains. This observation is somewhat surprising as the crystals are formed at pH 7.5, which in other instances has led to complete carboxylation of Lys73. The canonical deacylation water does appear to be present. The carbamate carbonyl goes into the oxyanion-hole. The sulfamate-group contributes with interactions with Ser118, Thr209, Lys208 and Arg250. The OXA-24 structure is somewhat different, as OXA-24 is a monomer. Also here is the Lys73 decarboxylated. OXA-24 has a "bridge" formed by Tyr112 and Met223 (OXA-24 numbering), spanning the active site, which interacts by van der Waals interactions with avibactam. It has been proposed that the presence of the polar moieties of the avibactam-molecule prevents the carboxylation of Lys73 by altering the pK_a . For the acylation (Figure 1.12A) it is suggested that the carboxylated Lys73 activates Ser70, while Ser118 acts as an acid donating a proton to the sulfamate-nitrogen assisted by the protonated Lys208. In reverse (Figure 1.12B), the carboxylated Lys73 has to donate a proton to Ser70 while Ser118 has to deprotonate the sulfamate-nitrogen in a concerted reaction with a deprotonated Lys208. Since Lys73 appears to be decarboxylated in a significant fraction of the enzyme monomers because of

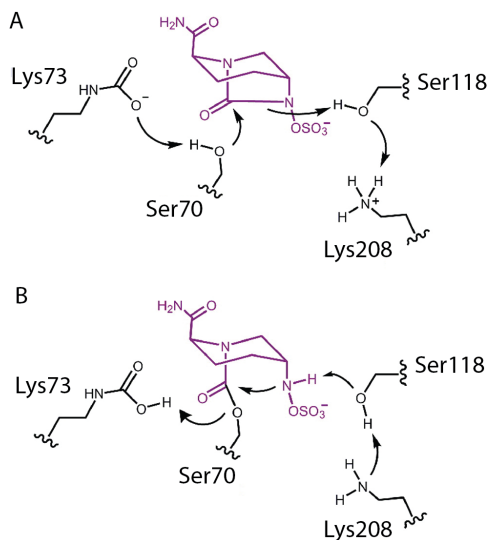


Figure 1.12. Proposed reaction mechanism for avibactam with DBLs. During the acylation (A) the carboxylated Lys73 activates Ser70, which makes a nucleophilic attack on the DBO-carbonyl. Ser118 acts as a proton shuttle. The deacylation (B) is basically the acylation in reverse. Reprinted with permission from Lahiri et al. [106]. Copyright 2015 American Chemical Society

the changes in electrostatics associated with avibactam binding, this mechanism will be slow.⁷⁷ Avibactam was approved for combination treatment with the third-generation cephalosporin ceftazidime. With other combinations under investigation to expand the spectrum of applicability,⁹² especially the aztreonam-avibactam combination that shows activity against *E. coli* and *K. pneumoniae* carrying both SBLs and MBLs.^{109,110} Mutants of both SHV and KPC SBLs have surfaced that are resistant to avibactam.^{111,112} And it was shown that some MBLs hydrolyze avibactam without being inhibited.¹¹³

After the approval of avibactam, several other DBOs were investigated. FPI-1465 is an avibactam derivative with an ether-linked pyrrolidine ring attached to the carboxamide-group. This compound and other derivatives show activity not only against BLs, but also against PBPs.¹¹⁴ This means that DBOs may turn out to be a novel class of antibiotics, not only BL-inhibitors. Merck also has a DBO named relebactam in Phase 3 in the USA.¹⁶

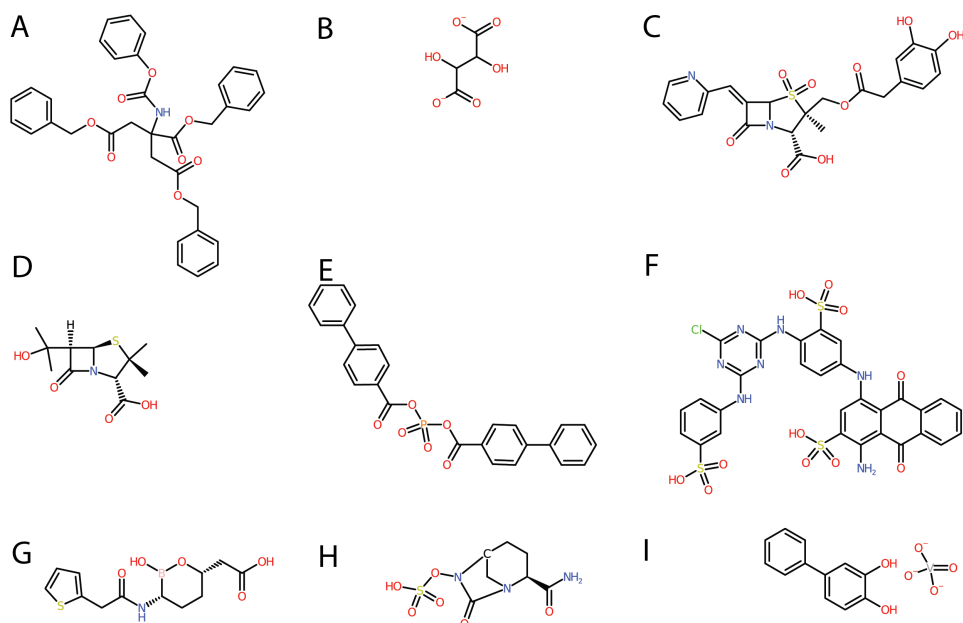


Figure 1.13. An overview of some of the reported inhibitors of OXA-enzymes. A) A lipophilic derivative of citrate. B) The polycarboxylate tartrate. C) The penicillanic acid sulfone derivative LN-1-255. D) A β -hydroxyalkylpenicillanate. E) A dibenzoyl phosphate. F) The anthraquinone dye Cibacron Blue 3G-A. G) A cyclic boronate. H) The DBO avibactam. I) A cathecol-vanadate complex.

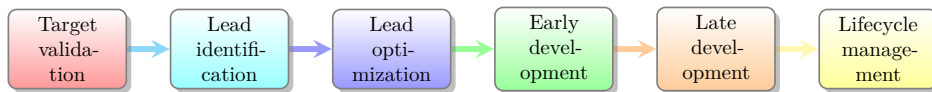


Figure 1.14. A summary of the drug development process, adapted from Scapin [117]

1.3 Drug Design

While the approval of ceftazidime/avibactam for complicated intra abdominal infections and complicated urinary tract infections was an important step forward, there is a continuous need for new treatment options. The first reports of enzymes resistant to avibactam have already been published.¹¹² Drugs are substances that cause a physiological change in the body, and in most cases drugs targets enzymes.¹¹⁵ For this reason we often refer to enzyme inhibitors as drugs, although there are drugs that have other targets than enzymes (e.g. cell membranes or DNA/RNA). Drug discovery is a multidimensional problem, often said to make rocket science look easy.¹¹⁶

The drug development process consists of several phases, and are often described with flowcharts like the one in Figure 1.14.

A successful drug has to have a good target. Validation of a drug target often involves mutating the gene of interest, and the drug target is considered valid if there is a relevant phenotypical change in the organism.¹¹⁸ This is a major challenge for new antibiotics. Not because it is hard to make the mutants, but because we need to ensure that the treatment is specific for the bacteria. The targets has to be unique to bacteria so that we can target bacterial enzymes, and avoid inhibiting our human enzymes. This is a solid argument for developing BLIs. It is known that PBP are a good drug target,¹⁰⁹ so if the BL is inhibited by a BLI then the β -lactams will still inhibit the PBPs.

After target validation comes lead identification. The lead compound needs to have potency against the target, it needs to be selective for the target, it should be water soluble, it has to get to where the target is, it needs to be stable for the time it takes to inhibit the target and it needs to do so with limited toxicity to the patient. This is a multidimensional problem as shown in Figure 1.15. Failure to deal with any of the factors is likely to cause problems in later stages. If the benefit of the treatment is large

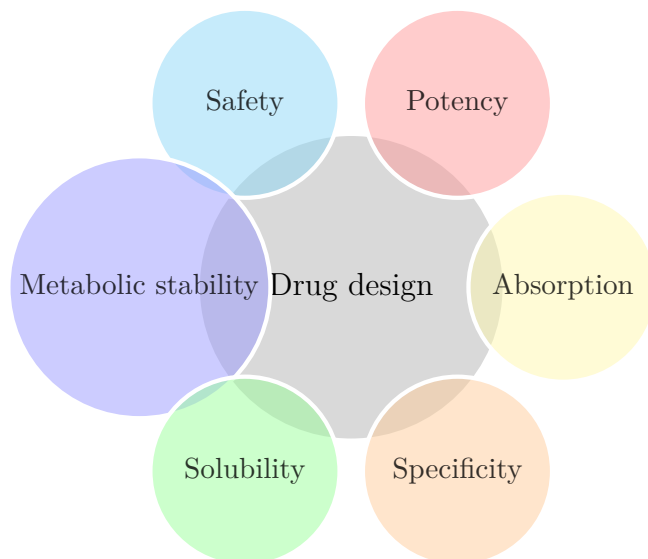


Figure 1.15. Some of the important factors in a drug design process.
Adapted from Shields et al. [112]

enough, it might be worthwhile to work around the problems. For example, imipenem is co-administered with cilastatin to prevent the hydrolysis by human dehydropeptidase-1.¹¹⁹ Cilastatin was developed for this purpose, as Merck saw the potential of imipenem.

While drug design is a multidimensional problem it is common that the lead identification is initiated by screening compound libraries for compounds with high potency. Compound libraries are usually huge libraries of drug like compounds or smaller fragments. Drug-like compounds are usually defined as compounds that follow Lipinski's Rule of Five (Ro5).¹²⁰ It is important to recognize the scope of applicability for the Ro5. A set of compounds that had passed Phase II-studies were investigated. The goal was primarily to identify the factors which would give good oral bioavailability. Many (active) drugs fall outside of the Ro5. Especially antibiotics seem to break these "rules", while still being important and successful drugs. IV-dosage is often accepted for antibiotics¹²¹ as infections are potentially life threatening, however, costs are much lower if the patient could be discharged from the hospital and receive the antibiotics orally. Antibiotics face the challenge of both being tolerated by humans and successfully penetrating bacterial

membranes. Their outer membrane is a barrier for amphipathic compounds, while the inner membrane restricts passage of hydrophilic substances. In addition, there are multidrug-resistant pumps that expel amphipathic compounds. Applying the Ro5 to antibiotic-leads may actually make this harder, by filtering out compounds that may have the ability to penetrate bacterial membranes¹²²

With that being said, the Ro5 are:

- Less than 5 hydrogen-bond donors
- Smaller than 500 Da
- LogP under 5
- Under 10 hydrogen bond acceptors

Every rule is a multiple of 5 giving the name. LogP is the octanol/water partition coefficient, indicating how hydrophobic a compound is by indicating how it would be distributed in a binary mixture of octanol and water.¹²³ If more than 2 of these "rules" are broken, there is a higher chance of poor absorption or permeability.¹²⁴ Fragments follow a very similar Rule of Three (Ro3) as described later in subsection 1.3.2.¹²⁵

Following the drug development process further takes you into the lead optimization phase where there often is a need for several groups to work together to ensure that all the factors in Figure 1.15 have been dealt with. It is often necessary to have several backup-compounds in case the lead compound falls out of the pipeline for any reason.¹¹⁷

After all this is done, the compound may be evaluated for safety. The early development (Figure 1.14), often termed the preclinical studies, will be performed on animals before the compound is tested on humans. This is important to determine approximately what the dosage should be in humans. While every country has its own rules, the late development(Figure 1.14) often occurs in three phases. Phase I tests the drugs on healthy volunteers to check for safety, in increasing doses. Phase II tests the drugs on a small group of patients to see if the drug has any efficacy, and to determine which dosage is necessary. Finally, in phase III the compound will be evaluated for its safety and efficacy in patients. The clinical work may take from 4 to 10 years, and is by far the most expensive part of the process. Since it

is so expensive to fail at this stage, avoiding obvious problems early on is important.

Even after the compound reaches the market it is necessary to monitor the safety and efficiency. There might be resistance to your drug that needs to be dealt with.

1.3.1 Natural products and derivatives

Penicillin, often hailed as a success of science, was indeed isolated from a natural source. The *penicillium*-strain that was used for the production of penicillin in the early years came from a mouldy cantaloupe melon.¹²⁶ A third of the drugs approved in the US between 1981 and 2010 were natural products or derivatives.¹²⁷ Natural products are often complex molecules and are often parts of complex mixtures. This leads to problems for the drug development process.¹²⁷ It is maybe for this reason the traditional phenotypical screening of natural products has diminished the last decades compared with the high-throughput screening (HTS)-campaigns launched against molecular targets.¹²⁷ What is being realized now is that the complexity of the natural products is beneficial.¹²⁷ Their complex stereochemistry allows natural products to target difficult targets.¹²⁷ Another benefit is that since they are natural, the transporter systems of the host might just deliver the compounds to their intracellular site of action.¹²⁷

Bioprospecting as a source for drug leads

A challenge with searching for drug leads from natural sources, bioprospecting, has been the complicated mixtures of compounds. The process may be simplified by including chromatography-steps that fractionate the extracts of interest.¹²⁸ This will also make it more likely to recover the responsible compound from the extract, aiding identification and structural elucidation. Advances in NMR and MS have been very important here.

For synthetic compounds there is always a concern whether the compound will be biologically relevant and active. Natural products are inherently biologically relevant, simplifying this process. This does not necessarily mean orally availability, but there are other modes of delivery. An antimicrobial peptide with poor uptake or metabolic stability, could be applied to

skin infections by a topical cream for example.¹²⁹

There are many environments in this world that have not been explored for novel compounds. Very important work has been done on growing "unculturable" bacteria,¹²² or by collecting organisms from extreme environments such as the cold oceans outside of Norway.¹³⁰

A technical challenge for natural product screening is reisolation. Often actives are found, but when the compound is identified it becomes clear that the compound was already known.¹³¹ Another challenge is that natural products are well known to any bacteria, and the bacteria has already had millions if not billions of years to develop resistance to the natural product.

1.3.2 Fragment based drug design

The opposite of natural product screening might be the fragment based methods. Libraries of carefully defined compounds are screened in a fragment-based drug discovery (FBDD)-campaign. A typical library will have between 500 and 25000 fragments.¹³² The compounds usually follow the Ro3, meaning:

- Molecular weight below 300 Da
- Equal or less than 3 hydrogen bond donors/acceptors
- CLogP below 3

These "rules" are the result of different FBDD-campaigns from Astex, and are only meant as guidelines..¹¹⁶

The overall goal of FBDD is to avoid "molecular obesity". Lipophilicity is often a quick way to high potency. However binding primarily driven by entropy often leads to unspecific binding, which in the case of drugs often means potentially dangerous side-effects.¹¹⁶ Yet, entropy-driven binding may be beneficial in some cases,¹³³ as the flexibility to reorient itself has been shown to be important for non-nucleoside HIV-1 reverse transcriptase inhibitors.¹³⁴

By starting out with the small fragments only efficient binders will be identified. Each atom of the fragment has to contribute to the binding for the fragment to be detected. This is actively used as a metric with the concept of ligand efficiency. Ligand efficiency (LE) is a simple metric

where the affinity K_d or the half maximal inhibitory concentration, IC_{50} , is expressed as the free energy (from Equation 1.3) of binding divided by the number of non-hydrogen atoms (Equation 1.3).¹³⁵

$$\Delta G = -RT \ln K_d \quad (1.2)$$

$$LE = \Delta G \div N_{\text{non-hydrogen-atoms}} \quad (1.3)$$

Higher LE-values are better, and it is recommended that hits should be in the range of 0.3-0.4 kcal/(mol \times atom).¹³⁵ There has been criticisms against the LE-concept, both on the mathematical validity and the practical use.¹³⁶ Several alternative metrics have been proposed to account for size, lipophilicity and other factors. Yet, the consensus appears to be that the concept of LE continues to be worthwhile. Traditional HTS-libraries are also often limited in their coverage of chemical space, biased against G-protein coupled receptor and kinase-like compounds, and emphasizing compounds that are easy to synthesize.¹³⁷ FBDD allows efficient sampling of chemical space with compounds that leave plenty of room for optimization without breaking the Ro5 for the lead compound which is to be made.

FBDD only became possible during the last years with the advances in technology enabling the detection of the often weak signals. Surface plasmon resonance (SPR)- and NMR-machines have become incredibly sensitive, synchrotrons have more sensitive and faster detectors and automation that allows rapid screening of crystals, and entirely new methods such as microscale thermophoresis (MST) have surfaced.^{137,138}

Another challenge with fragments is unspecific binding and pan assay interference compounds (PAINS). Using orthogonal assays, for example combining enzymatic assays with SPR, is a good way of avoiding these assay-specific problems.^{139,140}

Fragments rarely have the potency needed to be relevant as lead compounds, and need to be improved. If there exists crystal structures of the fragment-protein complex, and fragments binds in multiple adjacent binding sites, it may be possible to link the fragments. This is an incredibly powerful strategy as the free energy (??) of the linked fragment is the sum of the individual free energies plus the entropic gain of having one molecule instead of two as shown in Equation 1.4.¹⁴¹ If the binding mode of each fragment is

kept for two millimolar inhibitors, the resulting linked compound may have nanomolar potency.

$$\Delta G_{binding} = \Delta G_1 + \Delta G_2 + \Delta G_{linker} \quad (1.4)$$

There are several success stories with fragment linking. But often a combination of a focused library around the hits together with structure guided drug design (SGDD) is necessary to progress the hits into a lead compound. An example is the development of a novel covalent inhibitor of the MBL NDM-1 based on a fragment. In this case, screening of a focused library around one of the previous hits¹⁴² identified a novel covalent inhibitor with nanomolar inhibition.¹⁴³

1.3.3 Structure guided drug design

After the discovery of a new compound, either from HTS, natural sources or FBDD it may be very useful to use structural information to guide the design of the lead compound. In order to do so there are some requirements:

- You need a significant amount of protein crystals that diffract to better than 2.5 Å.¹⁴⁴
- The protein must crystallize in a biologically relevant conformation.¹⁴⁴
- Crystal contacts, other ligands or components from the crystallization condition must not interfere with the drug site.¹⁴⁵
- The crystals must be robust enough for soaking, or co-crystallize with the drug.¹⁴⁴
- The pH and ionic strength should preferably be close to physiological conditions to mimic the biologically relevant conditions.¹⁴⁴

Explaining crystallography is an endeavour worthy of a textbook, and luckily, those already exist, so no attempt will be made to write one here.¹⁴⁶ The process is summarized in Figure 1.16.

Having a structure of the protein-ligand complex ensures that the interactions of the compound are specific.¹⁴⁷ There might still be unspecific

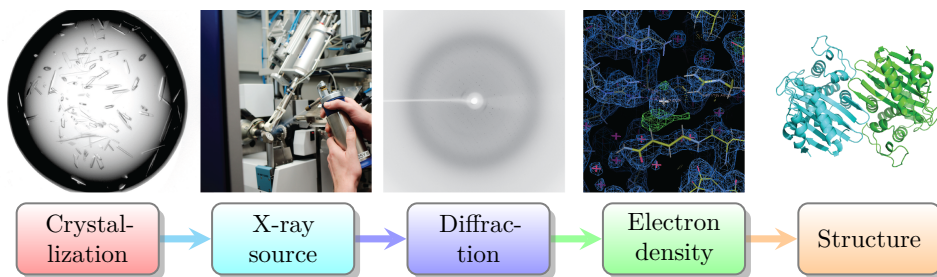


Figure 1.16. Overview of the process for solving structures X-ray crystallography. Protein crystals are rotated while exposed to a monochromatic X-ray beam. The resulting spot in the diffraction pattern represents spatial periodicities in the unit cell of the crystal. Reconstructing the sphere by rotating the crystal allows the reconstruction of the atomic contents of the unit cell, the repeating unit of a crystal, by the use of inverse Fourier transforms.¹⁴⁷ Higher resolution structures contains more information, and may allow the positioning of small molecules such as ions, cofactors, substrates or an inhibitor bound to the protein .

behaviour of the compound, but at least there is something specific to work with. The important part is that structural information shows what interactions any particular compound has with the protein. And perhaps as important, which interactions the compound does not engage in.

A case study with the development of the neuramidase inhibitor Tamiflu is a very good example of this. From a lead identification process compound A was identified (Figure 1.17). This compound had some potency, but not enough to reach the milestone of the development project. From the crystal structure the researchers realized that substituting the 4-hydroxyl group of the ring with a charged basic group could form interactions with a glutamic acid and a tryptophane. The resulting compound B was 5000 times more potent, and was launched as the first influenza drug Relenza. However, Relenza was not orally available and required a dry powder inhaler to get the drug to the lungs. The researchers realized that the basicity and high polarity prevented the oral uptake, and used the structural information to find compromises. Replacing the guanidine group with a primary amine, exchanging the pyran-scaffold with a cyclohexen and replacing the glycerol-tail with a 1-ethylpropoxy group made a smaller and somewhat less potent compound C that was orally available . This compound was launched on the marked as Tamiflu, and got enormous attention when the "swine flu"

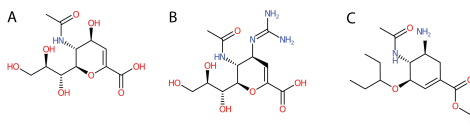


Figure 1.17. Compounds from a SGDD project on neuramidase inhibitors that ended up with the drug Tamiflu. A) A lead compound, B) The optimized compound that entered the market as Relenza. C) Oseltamivir with the trade name Tamiflu.

H1N1-influenza went pandemic. Not to say that this was easy, but the crystal structures were available to guide this process.¹⁴⁷

Chapter 2

Methods

2.1 Surface plasmon resonance for fragment based drug design

Surface plasmon resonance (SPR) is a biophysical method based on differences in refractive index caused by mass changes when two interaction partners bind (Figure 2.1A).¹³⁸ Often the protein is immobilized to a dextran matrix on the surface, with the most common tethering method being amine coupling to the surface. It is also possible to immobilize small molecules, or to use other coupling methods such as cystein-coupling or even hexahistidine-based non-covalent tethering.¹⁴⁸ The immobilized molecule is called the ligand in the SPR-terminology. Under continuous flow a binding partner referred to as the analyte is transported over the surface where the ligand is attached. If there is a binding event, the refractive index of the surface changes and this change is measured. The measured signal (response units) is plotted against time, yielding the sensorgram (Figure 2.1B).¹⁴⁹

The maximal signal (R_{max}) for a binding event with a saturated surface is determined by the molecular weights of the ligand and the analyte and the immobilization level of the ligand (Equation 2.1, $R_{protein}$).¹³⁸

$$R_{max} = \frac{MW_{analyte}}{MW_{ligand}} \times R_{protein} \quad (2.1)$$

This has previously limited the size of the analytes, however, with newer

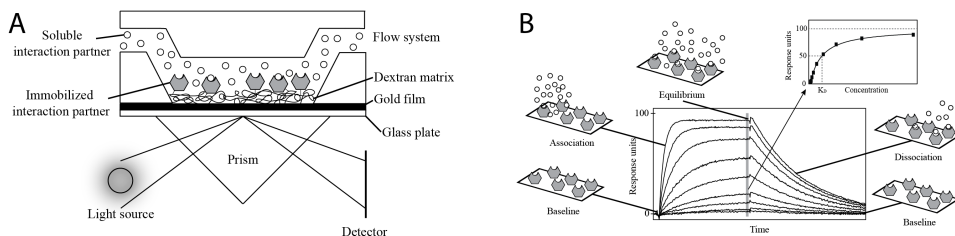


Figure 2.1. A schematic overview of the surface plasmon resonance-technology (A) which is based on the changes in refractive index based on mass changes.^{138,149,151} A binding event will increase the mass on the surface, changing the refractive index and giving rise to a signal, which is be plotted against time to give a sensorgram (B). The inset shows the corresponding steady-state plot used to determine affinities (K_d).¹³⁸ Reused with permission from Christopheit [152]

machines the noise level is low enough that this only causes problems when immobilization levels are low or the compounds are very small.^{138,150}

To analyse data there are some issues that need to be addressed. The analyte may bind to the surface or there might be differences in the refractive index of the buffers used. These events may also cause a measurable signal, which may obscure the actual binding response. To correct for the background response it is common to have a parallel flow cell with no ligand or an irrelevant ligand immobilized.¹⁴⁹ The signal from this flow cell is then subtracted from the sensorgram, a process that is called reference subtraction. It is also common to do blank subtractions, where the running buffer is injected over the surface as well for double referencing.¹⁴⁸

Another challenge is that the real signal is not a specific binding, but merely changes in refractive index. Solvents such as dimethyl sulfoxide (DMSO) gives a significant signal shift because of their refractive index is much higher than that of water. The experiments need to be carefully designed, and suitable controls should be included, to ensure that only the ligand-analyte interaction is measured.¹³⁸

SPR does offer real-time measurements that allow for kinetic parameters to be determined.¹⁵¹ The experimental data can be fitted to different models. The simplest model is the one-to-one interaction model where: $A + B \rightleftharpoons AB$. More complex models, accounting for heterogeneous ligand or analyte is also possible, but should be justified based on supporting evidence from other

methods.¹⁵³ However, fragments usually have low affinities, caused by rapid association and dissociation rates. As these rates are reaching the detection limits of the current instruments, affinities for fragments are determined from binding-levels at steady-state. Optimally the highest analyte concentration in an assay should be 10-fold or higher than the K_d . However, solvent concentration, unspecific binding at high concentrations and/or analyte solubility often prevents the use of higher concentrations. By using the theoretical R_{max} as shown in Equation 2.2 it is possible to get an estimate even from the nearly linear pre- K_d -range by simulating the expected signal at steady state (R_{ss} , Figure 2.1B) for a given analyte concentration ($[A]$).

$$R_{ss} = \frac{[A] \times R_{max}}{K_D + [A]} \quad (2.2)$$

This does however make an assumption about a specific one-to-one binding which is not necessarily correct especially for fragments.¹³⁸

As fragments are developed further by screening and synthesis of derivatives, affinities may be improved to a range that falls within the instrument specifications where kinetic parameters may be determined. It has been argued that compounds should be optimized for k_{off} -rates as it is concentration-independent and entirely dependent on the specific interactions between the compound and the protein.¹⁵⁴ Not all clinical indications calls for a long-lasting drug. Sleeping pills should only put you to sleep, not keep you drowsy the next day. Antibiotics on the other hand, should preferably be administered as few times as possible to maximize patient adherence and reduce safety concerns.^{154,155} This is a case where SPR excels, because the technology enables rapid determination of the kinetic parameters. Of course, drugs do also depend on other components and both pharmacodynamics and pharmacokinetics need to be taken into account. By varying the temperature, it is also possible to determine the thermodynamic parameters ($\Delta G, \Delta H, \Delta S$) from the van't Hoff equation, which is also known as the Arrhenius equation (Equation 2.4).^{148,156} The Arrhenius equation may be transformed to give a linear function, where the activation energy (E_a) is

determined from the slope (Equation 2.5).¹⁵⁷

$$k_{cat} = Ae^{\frac{-E_a}{k_b T}} \quad (2.3)$$

$$\ln k_{cat} = \ln A - \frac{E_a}{R(T^{-1})} \quad (2.4)$$

$$\Delta G^* = RT \times \left(\frac{\ln k_B T}{h} - \ln k_{cat} \right) \quad (2.5)$$

$$\Delta H^* = E_a - RT \quad (2.6)$$

$$\Delta S^* = \frac{\Delta H^* - \Delta G^*}{T} \quad (2.7)$$

R is the gas constant, k_b is the Boltzmann constant, h is the Planck constant and T is the temperature in Kelvin. It has been suggested that fragments that bind with predominately enthalpic energies (ΔH) are better starting points for optimization than more greasy compounds that bind with more entropic energies (ΔS).^{148,158}

SPR is increasingly used for fragment screening because of the low sample consumption and the relatively high throughput.^{148,159} While the throughput is lower than for biochemical HTS-screens, it is sufficiently high for fragment libraries of 1000-10000 compounds.

Fragment screening campaigns are often performed in several steps. The first step is the clean screen. This step confirms that the compounds do not bind nonspecifically to the control protein or a blank surface.^{159,160} The next step is the direct screen to identify compounds that bind to the protein of interest. After this step compounds may either be tested by an orthogonal assay (for example a biochemical assay) or tested in a competition experiment. Competition experiments may contain either reference compounds (with known binding mode) or other hits. Competition with reference compounds may indicate binding to the active site. Compounds that do not show competition with other hits are clear candidates for using fragment-linking approaches where two fragments are coupled using a linker to gain higher affinities as shown in Equation 1.4.¹³⁸

A major challenge with screening is promiscuous compounds. Promiscuous compounds bind non-specifically to the protein, and while this is not a problem in itself, these compounds will often bind non-specifically to other proteins as well, causing side-effects or even toxicity. These compounds

often form soluble or colloidal aggregates, with diameters from 30-400 nm, which may envelope the protein and inhibit the enzyme in a non-specific manner.^{161,162} Promiscuous compounds show changes in potency over time, detergent sensitivity or stoichiometric ratios of more than five to one for compound: enzyme binding.¹⁶¹ SPR is well-suited for identifying such binders. If the binding response significantly exceeds ($>5x$) the expected maximum binding level (Equation 2.1), the compound may be identified as a super-stoichiometric binder.¹⁶¹ It is of course possible for a compound, especially a fragment, to bind in multiple sites. However, higher than a five-to-one ratio is often an indication of problems. Many compounds show concentration dependent aggregation, where lower concentrations are well-behaved, but higher concentrations never reach saturation.¹⁶¹ Adding detergent often clears up this behaviour.¹⁶¹

2.2 Microscale thermophoresis (MST) for analysis of biomolecular interactions

MST uses the principle of thermophoresis, the movement of molecules in a thermal gradient analogous to electrophoresis, to quantify biomolecular interactions.¹⁶³⁻¹⁶⁵ The basic principle is schematically shown in Figure 2.2 The movement of biomolecules in an electric field only depends on size and charge, if not covered by sodium dodecylsulfate or similar. Movement in a thermal gradient (Equation 2.9, T is temperature) however, has been shown to be described by the Soret coefficient (S_T , Equation 2.9). The Soret coefficient depends on hydration entropy (Δs_{hyd}), size (A is the surface area), and charge.¹⁶⁴ The ionic contribution is complex and is described by the Debye-Huckel screening length λ_{DH} , the dielectric constant ϵ (and the vacuum dielectric constant ϵ_0) and the dielectric constants temperature derivate β .¹⁶⁴

$$\frac{c}{c_0} = \exp(-S_T \times (T - T_0)) \quad (2.8)$$

$$S_T = \frac{A}{kT} \left(-\Delta s_{hyd}(T) + \frac{\beta \sigma_{eff}^2}{4\epsilon \epsilon_0 T} \times \lambda_{DH} \right) \quad (2.9)$$

The movement of molecules is detected by change in fluorescence, either intrinsic fluorescence from tryptophanes or from fluorescent tags.

The experimental setup for MST (Figure 2.2A) includes an infrared laser responsible for creating a thermal gradient within the sample reflected by a IR-reflecting mirror, a excitation (and emission) beam and movable glass capillaries with a dilution series on a temperature controlled tray.¹⁶³ For each measurement (Figure 2.2B) a baseline fluorescence is measured (f_0) before the IR-laser is turned on. When the IR-laser is on, biomolecules will move in the thermal gradient depending on their size, hydration and charge. The difference in fluorescence when the IR-laser is on (f_1) may be plotted against the concentration to give a dose-response curve and the dissociation constant (K_d).¹⁶³

There is a wide variety of different labelling strategies for MST, including amine coupling¹⁶⁵ and His-tags. MST has successfully been used for sub micromolar binders,¹⁶³ and with the newer machines picomolar affinities are achievable.¹⁶⁶

There are two practical problems that often influence experiments. Adsorption, where the molecules stick to the glass capillaries, will often lower the fluorescence dramatically. Buffer additives or coated capillaries may help reduce adsorption. Another challenge is aggregation, which will cause bumps or waves in the MST-traces.¹⁶³ Sometimes aggregation may be prevented by spinning down the sample before the experiment, or by modifying the sample buffer.¹⁶⁵

One advantage of MST is that the experiments may be performed in complex biological liquids.¹⁶⁶ By using fluorescence fusion proteins such as mCherry or Green fluorescent protein, it is possible to analyze K_d -values without protein-purification.¹⁶⁶ This allows access to binding information from *in vivo*-like conditions.¹⁶⁶

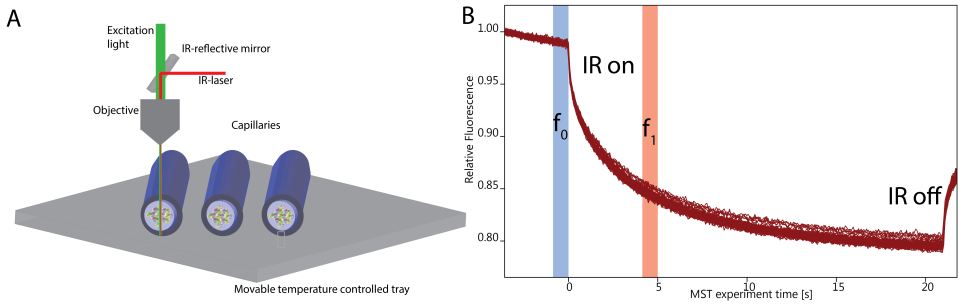


Figure 2.2. A schematic overview of the microscale thermophoresis-technology (A) with a typical series of MST-traces (B) shown.

Chapter 3

Aims of the studies

The purpose of the project was to investigate the structural basis for antibiotic resistance mediated by class D β -lactamases, and to develop new inhibitors of the antibiotic resistance enzyme OXA-48. A new inhibitor of the DBLs would restore the activity of β -lactam-antibiotics against bacteria carrying the genes for DBLs.

To accomplish the goals of the projects crystallography and surface plasmon resonance were key technologies.

The first objective was to characterize new DBLs through:

- Cloning of DBL-genes from clinical isolates into laboratory strains of *E. coli*
- Biochemical characterization and protein crystallographic studies of the new DBLs OXA-181, OXA-245 and OXA-436
- Mutations of possible key residues for the OXA-48 reaction mechanism and dimerization

The second objective was to identify new OXA-48 inhibitors by:

- Screening a fragment library to identify starting points for new inhibitors towards the clinically important OXA-48
- Using the fragment hits and OXA-48-fragment complex structures, in a structure based inhibitor design process to develop and biophysically characterize more potent inhibitors

Chapter 4

Summary of papers

4.1 Paper I

B. A. Lund, H. K. S. Leiros, and G. E. Bjerga. “A high-throughput, restriction-free cloning and screening strategy based on *ccdB*-gene replacement”. *Microbial Cell Factories* 13.38 (2014)

In Paper I we developed a novel cloning strategy to enable rapid cloning of β -lactamase (BL) genes. While restriction-free cloning is an established method, it usually requires the use of the restriction enzyme Dpn1 to digest the unmodified vector. This step prolongs the time needed for the experiment, and requires vector propagation in Dam⁺ strains. By using vectors containing the coupled cell division B (*ccdB*) gene, which is lethal for the bacteria if expressed, and designing the primers to replace the *ccdB* gene with the gene of interest we improved the clone screening process for restriction-free cloning protocols.

We also reported our successful cloning of OXA-48, OXA-181 and OXA-245, and demonstrated activity against meropenem for the purified OXA-48 enzyme.

As the first author to this paper, my contribution was the molecular cloning, recombinant expression and kinetics. I also participated in the design of the study and drafted the manuscript, figures and tables.

4.2 Paper II

B. A. Lund, T. Christopeit, Y. Guttormsen, A. Bayer, and H. K. S. Leiros. “Screening and Design of Inhibitor Scaffolds for the Antibiotic Resistance Oxacillinase-48 (OXA-48) through Surface Plasmon Resonance Screening”. *Journal of Medicinal Chemistry* 59 (2016), pp. 5542–54

In Paper II we presented the results of fragment screening for the OXA-48 BL and our first development of an improved compound based on crystallographic structures. We continued the work from Paper I, and produced a His-tagged construct of OXA-48 to aid the production of larger amounts of enzyme for structural studies.

We developed the first surface plasmon resonance (SPR)-assay for any class D β -lactamase (DBL), and used it to screen 490 fragments from Maybridge’s Rule of Three (Ro3)-library. The hits from the primary screen were validated by an orthogonal biochemical assay with the substrate nitrocefin. We were able to soak three fragments into OXA-48 crystals, and obtained the protein:inhibitor complexes. Based on the alternate conformations of one fragment, we designed an improved compound and synthesized it. The synthesized compound had ten times higher potency (IC_{50} : 18 μ M) than the starting fragment (IC_{50} : 250 μ M).

We also reported Michaelis-Menten kinetic parameters for OXA-48 with penicillins, cephalosporins and carbapenems, and verified that the His-tagged construct had equivalent activity.

My contribution as a first author of this paper was the cloning, recombinant expression, performing the SPR assays, adapting the enzymatic assays for OXA-48, crystal structure determination and the design of the novel compound. I also participated in the design of the study and drafted the manuscript, figures and tables.

4.3 Paper III

S. Ahkter, B. A. Lund, M. Lange, A. Ismael, J. Isaksson, T. Christopeit, H. K. S. Leiros, and A. Bayer. "A focused fragment library targeting the antibiotic resistance enzyme - Oxacillinase-48: synthesis, structural evaluation and inhibitor design". Manuscript, 2017

In Paper III we further developed the hits from Paper II into lead compounds, by modifying the compounds from Paper II.

51 compounds were synthesized in order to investigate potential hydrogen bond donors and acceptors. We set out to understand the structure activity relationships (SAR). We solved the crystal structure of 40 of these compounds in complex with OXA-48, and determined inhibition and binding constants from a competition experiment with nitrocefin and SPR respectively.

We also solved the crystal structure of the complex of OXA-48 with the substrate imipenem. We uncovered that the different orientations of our compounds in the active site corresponded to the side-chains of imipenem. We demonstrated that a ^{13}C nuclear magnetic resonance (NMR)-assay could be used to differentiate between compounds binding in the two different orientations.

Based on some of the best binders in the two major orientations we developed an asymmetric bisubstituted benzoic acid derivate with an IC_{50} -value of 3 μM . Unfortunately, we were not able to demonstrate cell based activity for these compounds. However, the SAR may guide new design-processes.

I was responsible for the enzyme-part of this paper, where we performed affinity and inhibition measurements on a library of compounds. I was also responsible for the crystallography that enabled the structure guided development of the bisubstituted compounds.

4.4 Paper IV

B. A. Lund, A. M. Thomassen, T. J. O. Carlsen, and H. K. S. Leiros. “Structure activity and thermostability investigations of OXA-163, OXA-181 and OXA-245, using biochemical, crystal structures and differential scanning calorimetry analysis”. Manuscript, 2017

In Manuscript IV we describe the OXA-48-like BLs OXA-163, OXA-181 and OXA-245. Continuing on the work from Paper I, we cloned, expressed and purified three OXA-48-like enzymes. The purified enzymes were used for enzyme kinetic studies, thermostability testing with differential scanning calorimetry (DSC) and X-ray crystallography.

We then used the crystal structures to explain the differences we could observe in enzyme hydrolysis and thermostability experiments. OXA-163, with the S212D substitution and 214-217 deletion, had the lowest melting point (T_m : 49.4°C). OXA-163 also had higher activity against ceftazidime, but diminished activity against the carbapenems and lowered against ampicillin. There already existed crystal structures of OXA-163; however, we found a new crystal form. The structure showed how the deletion disrupted two ionic bonds, and that the substitution partially compensated by introducing two new hydrogen bonds. The deletion removed R214 which is known to be important for carbapenem hydrolysis.

The OXA-181 enzyme differs from OXA-48 with four substitutions: T104A, N110D, E168Q and S171A. The melting point is lowered (T_m : 52.6°C), but the kinetic parameters are close to that of OXA-48. We reported the first crystal structure for OXA-181, and among the highlights were the disruption of an ionic network of D88-H90-E89 because of the formation of an ionic bond between D110 and H90.

OXA-245 only has a single substitution, E125Y. The activity and thermostability (T_m : 55.8°C) is nearly identical to OXA-48, as expected. The slight increase in stability was unexpected, as E125 in OXA-163 and OXA-181 forms an ionic bond and a hydrogen bond. However, Y125 participates in a $\pi - \pi$ -stacking network with F93 and F126, and also maintains the hydrogen bond that E125 made.

I performed the cloning of OXA-181 and OXA-245, and contributed to all the experimental work.

4.5 Paper V

Ø. Samuelsen, F. Hansen, B. Aasnæs, H. Hasman, B. A. Lund, H. K. S. Leiros, B. Lilje, J. Janice, L. Jakobsen, P. Littauer, L. M. Søres, B. J. Holzknrecht, L. P. Andersen, M. Stegger, P. S. Andersen, and A. M. Hammerum. “Dissemination and Characteristics of a Novel Plasmid-Encoded Carbapenem-Hydrolyzing Class D β -Lactamase, OXA-436 from Four Patients Involving Six Different Hospitals in Denmark”. Submitted to *Antimicrobial Agents and Chemotherapy*, 2017

In paper V we described the novel BL OXA-436 which was recently discovered in Denmark. OXA-436 was identified in four patients at different hospitals in Denmark. None of the patients had any recent history of travel, and were not admitted at the same time. While OXA-436 was discovered in an *Enterobacter asburiae* isolate, the same gene was also identified in both *Klebsiella pneumoniae* and *Citrobacter freundii*. Indicating that OXA-436 could also disseminate to other *Enterobacteriaceae*.

With 90% identity to OXA-48 it was not surprising that its activity profile was similar to OXA-48. OXA-436 has activity against penicillins and carbapenems, with some limited activity against cephalosporins. The diazabicyclooctane (DBO) avibactam did significantly reduce the minimum inhibitory concentration (MIC) for both temocillin and meropenem.

The genetic neighbourhood of the OXA-436-gene indicates that the origin of the gene was likely in a *Shewanella*-species. Replicon identification indicates that OXA-436 is located on an IncHI2/IncHI2a plasmid. It is interesting to note that there was an hypothetical enzyme encoded by the *sprT* upstream of *bla*_{OXA-436} and downstream there was a putative LysR-type transcriptional regulator, indicating that the expression may be regulated.

In this paper I was responsible for the cloning, expression and *in vitro* characterization of the enzyme.

4.6 Paper VI

B. A. Lund, A. M. Thomassen, B. Nesheim, T. J. O. Carlsen, J. Isaksson, T. Christopeit, and H. K. S. Leiros. “Structural basis for OXA-48 dimerization”. Manuscript, 2017

In Paper VI we investigated the dimerization of OXA-48. NMR showed that OXA-48 is a dimer in solution, and small angle X-ray scattering (SAXS) demonstrated that the crystallographic dimer is consistent with the biological assembly in solution. While several other OXAs have been shown to be dimers, their interaction has been shown to be facilitated by divalent cations such as cobalt, copper, zinc or cadmium. However, where OXA-10 has a histidine, OXA-48 has Arg206. And Arg206 coordinates a chloride ion.

Mutating Arg206 to an alanine destabilized the enzyme with nearly 9°C as determined by DSC. However, size exclusion chromatography (SEC) shows that the Arg206Ala is also a dimer in solution. The crystal structure of Arg206Ala verifies that the remaining dimer interaction site is maintained and is strong enough to keep the dimer associate.

As the Arg206Ala mutant alone did not prevent dimerization, *in situ* alanine scanning with BioLuminate were used to identify other residues that could be important for the dimerization. Several of the ionic residues in the dimer interface were identified as key residues. Performing SEC at an acidic pH showed OXA-48 and OXA-48 Arg206Ala migrating as a monomer, indicating that the ionic bonds from Asp229 and Glu89 in the dimer site might be essential to dimerization. We were able to determine the K_d of dimerization for the OXA-48 Arg206Ala mutant to be 700 ± 300 pM by using microscale thermophoresis (MST). Together with the destabilization (T_m :-9°C) observed for the mutant compared to the wild type, these results suggest that the wild type OXA-48 has an affinity for dimerization in the picomolar range.

In this paper I was responsible for the design of the mutant and the SEC-experiments. I performed the MST experiments and analysed the structural information from SAXS and X-ray diffraction experiments. I designed the DSC experiment.

Chapter 5

Results and discussion

DBLs like OXA-48 are able to hydrolyse the important antibiotics within the carbapenem class of β -lactams.^{50,90} While the β -lactamase inhibitor (BLI) avibactam is efficient against OXA-48, there are signs of resistance from other classes of BLs and other DBLs are naturally non-susceptible, so it is clear that new inhibitors would be useful.^{111–113} To guide the development of new inhibitors a clearer understanding of both the reaction mechanism and the diversity is needed, as well as novel chemistries. In this thesis a variety of different methods have been used to answer these questions. A new cloning procedure has been developed to ease the production of new OXA-48-like enzymes. Michalis-Menten kinetic parameters have been determined for several OXA-48-like enzymes, and their thermostability have been evaluated by DSC. Crystal structures of several enzymes and mutants, as well as 41 enzyme:ligand complexes have been determined. The biophysical SPR-technology was used for fragment-based drug discovery (FBDD) in an orthogonal assay with determination of IC₅₀-values in competition with a coloured substrate in a biochemical assay for validation. Hits from the screening were developed into potent inhibitors by a combination of fragment growing and fragment linking. The different enzymes and mutants, and the work that has been done on these enzymes are summarized in Table 5.1.

Table 5.1. A short summary of the enzymes, mutants, enzyme kinetics, crystal structure(s) and if inhibitor binding or biochemical characterization studies were performed in this thesis. Techniques for studying inhibitor or biochemical characterization and their abbreviations are: Half maximal inhibitory concentration (IC_{50}), surface plasmon resonance (SPR), protein X-ray crystallography (X-ray), differential scanning calorimetry (DSC), nuclear magnetic resonance spectroscopy (NMR), small angle X-ray scattering (SAXS), size exclusion chromatography (SEC), microscale thermophoresis (MST).

	Enzyme	Mutants relative to OXA-48	Restriction free cloning	No X-ray structure(s)	Enzyme kinetics	Inhibitor or biochemical technique(s)
Paper I	OXA-48		Y		Y	
	OXA-181	T104A, N110D, E168Q, S171A	Y			
	OXA-245	E125Y	Y			
Paper II	OXA-48		Y	4	Y	IC_{50} , SPR, X-ray
Paper III	OXA-48		Y	37		IC_{50} , SPR, X-ray, NMR
Paper IV	OXA-48		Y			DSC
	OXA-163	S212D		1		DSC, X-ray
		Δ 214-217 ^a				
	OXA-181	T104A, N110D, E168Q, S171A	Y	1	Y	DSC, X-ray
	OXA-245	E125Y	Y	1	Y	DSC, X-ray
Paper V	OXA-436	T365, S40T, K51T, N58D, T104A, N110D, V153L, E168Q, S171A, G201A	Y		Y	
Paper VI	OXA-48		Y			DSC, NMR, SAXS, SEC
	OXA-48 R206A	R206A		1	Y	X-ray, DSC, SEC, MST

^aResidues 214-217 are deleted compared to OXA-48.

5.1 A simplified and efficient cloning procedure

Restriction-free (RF)- and exponential megaprimering PCR (EMP)-cloning are widely used cloning strategies based on primers overlapping with both the vector and the insert of interest.^{167–170} The original protocol only requires a polymerase and the Dpn1 restriction enzyme to remove the parental DNA, and for the EMP-cloning it may be sufficient to do a single polymerase chain reaction (PCR). In paper I we adapted RF-cloning for rapid cloning of our BL-genes, and we successfully used our modified strategy for OXA-48, OXA-181, OXA-245 (paper I) and OXA-436 (paper V). We applied our protocol to vectors carrying the *ccdB*-gene, which when expressed is toxic to the bacterial cell. We designed the primers to replace the toxic *ccdB*-gene to ensure that no bacteria transformed with unmodified vectors would survive. The use of the *ccdB*-carrying vectors simplified clone screening, as most clones were positive, very few of the bacteria transformed with the *ccdB*-carrying vectors did survive. This strategy removed the need for DpnI-

cleavage. Based on our results we hypothesized that DpnI may cleave DNA unspecifically, reducing the likelihood of positive clones. By using our simpler protocol we observed higher number of clones.

The constructs produced by our cloning procedure was used in all the papers. The gene for OXA-163 was produced by gene synthesis because genomic DNA was not available.

5.2 Exploring the diversity of OXA-48-like enzymes

OXA-48 and the OXA-48-like DBLs are enzymes of major concern.⁵⁰ Members of the group have different hydrolytic features, from carbapenemase activity for OXA-48 to cephalosporinase activity for OXA-163.⁴⁹ In paper IV and paper V we investigated the OXA-48-like enzymes OXA-163, OXA-181, OXA-245 and OXA-436. We investigated the *in vitro* substrate hydrolysis profile and their thermostability, together with their three dimensional structure. These enzymes are spread across the phylogenetic tree of OXA-48-like enzymes (Figure 5.1) with sequence identities to OXA-48 from 91.3% for OXA-436 to 99.6% for OXA-245. OXA-436 is the most distant OXA-48-like DBL within the subgroup.

The enzyme kinetics of OXA-163 and OXA-181 have previously been characterized,^{33,71} and our observations are consistent with the previous works. OXA-245 has been reported previously,^{171,172} but has not been characterized at enzyme level and OXA-436 was first described in paper V. We were able to show that there are no significant differences in the kinetic parameters.

We were able to determine the first crystal structures of OXA-181 and OXA-245, and identified a new crystal form for OXA-163 in paper IV. New crystal forms are useful, as they may highlight differences due to crystal packing and might have other solvent channels.¹⁴⁴

It appears from the plasmid sequence we have collected for isolates positive for OXA-436 that the main difference between the OXA-48-like enzymes is the host origin. OXA-48 is believed to originate from *Shewanella oneidensis*⁵¹ and OXA-181 has been identified as a chromosomal gene in *Shewanella xiamenensis*.²⁹ Our results in paper V indicate that OXA-436 may originate from a new species of *Shewanella*. *Shewanella* are marine bacteria belong-

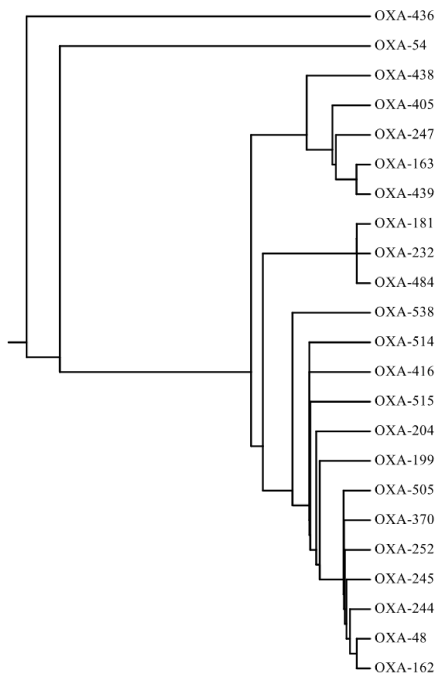


Figure 5.1. A phylogenetic tree of the OXA-48-like enzymes per 12.06.2017 from the Beta-Lactamase DataBase (BLDB).¹⁷⁴ Used with permission.

ing to the gram-negative class of gammaproteobacteria, which also contains the Enterobacteriales order including the pathogens *Escherichia coli* and *K. pneumoniae*.⁵⁷ Within the gammaproteobacteria family is the order of Pseudomonadales where *Pseudomonas aeruginosa* and *Acinetobacter baumannii* belong. And there have been reports of OXA-48-like enzymes for these bacteria as well.^{34,173}

We hypothesize that the competitive advantage of acquiring a BL is bigger than finding the optimal one with regards to the sequence and biochemical properties, and that different mutations of OXA-48 are tolerated.

5.3 Insights into the reaction mechanism of class D β -lactamases

As covered in subsection 1.2.5 there has been several attempts to describe the reaction mechanism of OXA-48.^{66,75,85–88} However, no comprehensive studies have fully explored the full reaction mechanism of the DBLs experimentally. While the reaction mechanism of the DBLs has not explicitly been covered in any of the papers, it has remained a significant interest.

5.3.1 Important residues for catalysis

In Paper II our SPR results (Figure 5.2) demonstrated that OXA-48 lacking the carboxylation of Lys73 was significantly slower in releasing the substrate meropenem than the carboxylated form. It turned out that the builtin degasser of the Biacore T200 lowers the amount of dissolved CO₂ in the buffer enough to disturb the equilibrium of the carboxylation. Including sodium bicarbonate in the running buffer alleviated the problem and reconstituted activity against meropenem. However, from the same results it does appear that the enzyme is able to form the acyl-complex without the carboxylated Lys73.

Previous work from our lab has shown that Ser118 and Arg250 are important, as both the Ser118Gly and Arg250Ala mutants had significantly slower hydrolysis of the tested substrates (Table 5.2).¹⁷⁵ Alternate conformations were observed for Ser118 in our set of crystal structures with OXA-48. This has previously been observed for OXA-1, and it was argued that this rotation is necessary for Ser118 to act as a proton shuffle.¹⁷⁶ Our observations of significantly decreased turnover could indicate that Ser118 indeed serves an important role in enzyme catalysis. However, the Arg250Ala mutant thought to merely destabilize the protein-substrate complex also reduces both K_m and k_{cat} to the same degree as the Ser118Gly mutant. This suggests that Ser118 may only serve to anchor the substrate instead of participating in proton shuffling.

The Arg250 mutant is interesting because this residue is not present in the gram-positive DBL BPU-1, which also have overall lower activity than other DBLs.⁷ However, against ampicillin and nitrocefin the overall efficiency

Table 5.2. Michaelis-Menten kinetics for OXA-48 and active site mutants. The results for the Ser118Gly and Arg250Ala were previously published in the master thesis of Nesheim [175]. For comparison the kinetic parameters for the Ser70Gly mutant OXA-48 are reproduced from Stojanoski et al. [59].

		Ampicillin	Imipenem	Nitrocefin
OXA-48	K_m (μM)	400	10	100
	k_{cat} (s^{-1})	700	5	1000
	$\frac{k_{cat}}{K_m}$ ($\mu M^{-1} s^{-1}$)	2	0.5	10
OXA-48 Ser118Gly	K_m (μM)	40	6000	250
	k_{cat} (s^{-1})	30	40	500
	$\frac{k_{cat}}{K_m}$ ($\mu M^{-1} s^{-1}$)	0.7	0.01	2
OXA-48 Arg250Ala	K_m (μM)	50	200	500
	k_{cat} (s^{-1})	20	2	750
	$\frac{k_{cat}}{K_m}$ ($\mu M^{-1} s^{-1}$)	0.4	0.01	1.5
OXA-48 Ser70Gly	K_m (μM)	1600		
	k_{cat} (s^{-1})	200		
	$\frac{k_{cat}}{K_m}$ ($\mu M^{-1} s^{-1}$)	0.12		

of BPU-1 is actually closer to the wildtype than to the OXA-48 Arg250Ala mutation, indicating that BPU-1 has some compensating mutations.

5.3.2 Crystal structure of OXA-48:imipenem complex

There has been several crystal structures of DBLs in complex with carbapenemases. However, many of the experiments have been done using non-carbapenemase DBLs such as OXA-13¹⁷⁷ or with a carbapenemase at a non-physiological pH such as for OXA-23.³⁵ In paper III we solved the structure of OXA-48 in complex with the carbapenem substrate imipenem. We hoped that this would help to elucidate the reaction mechanism of OXA-48, which does still appear to be incomplete. We were able to trap imipenem in a covalent intermediate with OXA-48. Continuous electron density was observed from the hydroxyl-group of Ser70 to the carbonyl of the opened β -lactam-ring. The main-chain nitrogens of Tyr211 and Ser70 forms the oxyanion-hole where the carbonyl-oxygen of the substrate is bound by hydrogen bonds. No other hydrogen bonds are observed, but there are many hydrophobic contacts shielding the substrate from the solvent. Only the

amidine-tail of imipenem appear solvent exposed. With the short soaking time and high substrate concentration it was not necessary to disable the enzyme activity either by mutations or change of pH, meaning that the enzyme is in a biologically relevant state. No water within a reasonable distance from the substrate carbonyl was observed in the electron density. Likewise, in the complex of OXA-1 with doripenem, resolved to 1.4 Å, no water was observed in the vicinity of the acyl-complex.¹⁷⁸ One could speculate that a rearrangement or conformational change is needed for the water to gain access, and that the disassociation proceeds rapidly after this change.

5.3.3 Thermodynamic characterization of enzyme catalysis

We were unable to find any thermodynamic parameters for the reaction of DBLs with carbapenems. With empirical valence bond (EVB)-calculations it is possible to validate proposed reaction mechanisms by comparing the calculated thermodynamic properties with the experimental values.¹⁷⁹ To aid this effort, we determined the thermodynamic properties of the reaction by monitoring the substrate hydrolysis at different temperatures by UV-spectroscopy. We chose the substrate imipenem, and performed the experiments at temperatures from 6-37°C (279 to 310 K) to determine k_{cat} -values from different temperatures. The Arrhenius-equation Equation 2.4 may be rearranged to yield Equation 2.5 which gives a linear function which we could fit to our experimental data (Figure 5.3).¹⁵⁷ From these we could calculate ΔG^* , ΔH^* , ΔS^* corresponding to the free energy of activation, the activation enthalpy and activation entropy. We calculated an activation energy (E_a) for the reaction of OXA-48 and imipenem to be 33 kJ. From experiments spanning temperatures from 279 to 310 K we calculated ΔG^* (Equation 2.6) to be 68 kJ/mol, ΔH^* (Equation 2.7) 31 kJ/mol and ΔS^* (Equation 2.7) -130 J/molK.

5.4 Investigating dimerization of OXA-48 and stability of OXA-48-like enzymes

OXA-48 is a dimer in solution,⁶⁹ and quaternary structure is known to be even more conserved than tertiary structure.¹⁸⁰ However, this does not

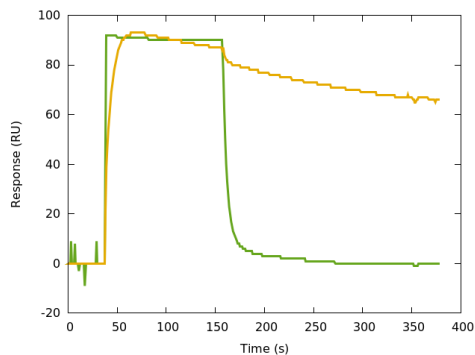


Figure 5.2. Sensorgrams for the interaction between meropenem and tOXA-48 in the presence (green) and absence (yellow) of 50 mM bicarbonate reveals that carboxylation of Lys73 is essential for the deacetylation part of the reaction mechanism with carbapenem substrates.

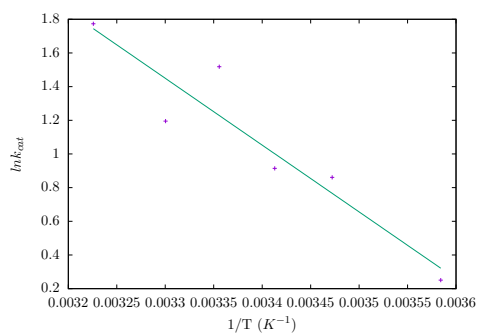


Figure 5.3. Arrhenius plot showing the linear relationship between the natural logarithm of the turnover rate and the inverse of the temperature. From the slope ($E_a \div R$) it is possible to calculate thermodynamic properties by using Equation 2.5.

explain why so many members of the DBLs form dimers. To better understand the role of the dimerization we performed the first described DSC-experiments for OXA-48, OXA-48 Arg206Ala, OXA-163, OXA-181, OXA-245 in paper IV. We were able to show that the T_m of these enzymes were similar, with $OXA-163 < OXA-181 < OXA-48 < OXA-245$. With our crystal structures we were able to highlight probable causes for the shifts in thermostability.

5.4.1 The impact of sulfate on thermostability

In paper VI we were able to reproduce the melting points observed by circular dichroism (CD) for OXA-48 by changing our buffer in our DSC experiments.⁵⁹ It appears that K_2SO_4 which is present in the DSC buffer we used in paper IV destabilizes OXA-48, which is an very interesting observation as K_2SO_4 is included in many experiments to avoid inhibition by chloride,⁴⁷ while still maintaining ionic strength to avoid adsorption.⁷⁶ It has later been shown that chloride inhibition, once hallmarked as a key property of DBLs, is an artefact of experimental setups.⁶² As it has been shown that DBLs are only sensitive to chloride if Lys73 is not carboxylated⁶² it seems clear that it is time to move away from buffers with K_2SO_4 . *In vivo* the pH and concentration of CO_2 is high enough that Lys73 is expected to be carboxylated at all times.⁶² The buffer used for enzyme kinetics contains 100 mM sodium phosphate,⁵⁷ which at pH 7 contributes significantly to ionic strength.¹⁸¹ Perhaps this buffer could be used for more purposes to avoid both chlorides and sulfate, however, many cations forms precipitation with phosphate and may complicate protein crystallography by the formation of salt crystals.¹⁴⁶ Our MST-, SAXS-, SEC- and SPR-experiments included K_2SO_4 in the buffers, which may have impacted the results.

5.4.2 Arg206 contributes significantly to stability

In the first crystal structure of OXA-48 a water was built in the electron density near Arg206.⁶⁹ However, in our work in paper II we realized that it was probably a chloride ion bound in this site based on B-factor analysis. We could also verify that the atom mediating the dimerization of OXA-48 was an anomalous scatterer (Figure 5.4), further strengthening our belief

that the atom is indeed a chloride which has a anomalous difference in scattering factor ($\delta f''$) of 0.7 electrons at the wavelength that was used in the experiment (1.54 Å).¹⁸² A buried chloride in a dimer interface appeared novel,¹⁸³ and we decided to investigate the site by site directed mutagenesis in paper VI.

We observed a significant decrease of 9°C in melting point for the OXA-48 Arg206Ala mutant in the sulfate-free buffer compared to the wild type. Interestingly, the difference was smaller in the sulfate-containing buffer, indicating that sulfates binding to Arg206 may be the source of destabilization of OXA-48. This indicates that dimerization is important for the stability of the enzymes. Oxacillinase (OXA)-enzymes are exported into the periplasm in gram negative bacteria. The periplasm is vulnerable to changes in the external environment, with abrupt changes in pH, temperature and osmolarity.¹⁸⁴ For this reason, proteins in the periplasm tend to have disulfide bonds to increase their stability.¹⁸⁴ Perhaps the absence of stabilizing disulfide bridges in OXA-48 is compensated for by the stabilization from dimerization. No stable monomeric intermediate was observed in the thermal unfolding curves.

Our OXA-48 Arg206Ala mutant was shown to have a K_d of dimerization of 0.7 nM by MST, and the enzyme kinetic experiments were performed with 1 nM of enzyme. At 1 nM concentration one would expect a equilibrium with both monomeric and dimeric OXA-48 Arg206Ala. If the monomer was significantly less or more active than the dimer, this should have influenced the results. However, there were differences between the buffers used for kinetics and the MST-experiments. The K_d -value we determined for the OXA-48 Arg206Ala by MST indicate that OXA-48 is a dimer *in vivo*. One or more additional mutations may be necessary to have OXA-48 behave as a monomer in solution at relevant concentration ranges. The sensitivity to changes in pH observed in the SEC experiments indicate that mutations to some of the charged residues would be effective. Glu89, Lys116, Arg189 and Asp229 were predicted by the *in situ* alanine scan to contribute even more than Arg206 to the dimerization and would be clear candidates for further site-directed mutagenesis. The crystal structure of the OXA-48 Arg206Ala had lower resolution than the other OXA-48 structures we solved, perhaps due to the increased disorder.¹⁸⁵ However, the structure clearly showed that

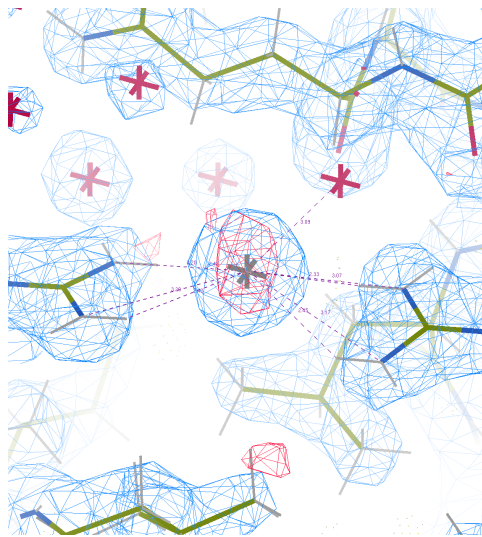


Figure 5.4. Electron density map ($2F_oF_c$, blue, contoured at 1.5σ) and anomalous map (red, contoured at 3σ) for OXA-48 S118G mutant (unpublished) collected with a wavelength of 1.54 \AA shows that the atom binding between Arg206 from each chain is an anomalous scatterer and together with the electron density this indicates that this atom is a chloride.

the remaining dimer interface was undisturbed and strong enough to keep the dimer associated. MST has not been used to study dimerization for other DBLs, however, results from SEC indicate that OXA-10, OXA-14 and OXA-29 has K_d -values in the nanomolar range.^{76,79} However, the cation-stabilized OXA-10 has a T_m of 78.4°C , where OXA-48 has a T_m of 59.1°C as determined by DSC, showing that there is not a clear correlation between thermostability and affinity for dimerization.

The dimer interface of OXA-48 contains a large percentage of charged amino acids, 47% according to the protein-protein-interface webserver 2P2I inspector v2.0.¹⁸⁶ An average dimer interface contains closer to 25% charged amino acids.¹⁸⁶ It is unclear why OXA-48 has such a charged dimer interface, but it is clearly contributing significantly to the overall affinity for dimerization.

5.5 Designing novel inhibitors for OXA-48

Avibactam, other DBOs, LN-1-255 and cyclic boronates have been shown to inhibit OXA-48.^{98,104,106,114} However, with such a diverse class of enzymes as the DBLs it seems inevitable for resistance to surface. And when resistance surfaces we need novel inhibitors.

A novel inhibitor of OXA-48 would not resemble a β -lactam, to avoid preexisting resistance mechanisms.¹⁸⁷

The story of the design of our new OXA-48 inhibitors turned out to very rewarding (Figure 5.5). We screened a library of 490 fragments from the Maybridge Ro3-library in paper II, the first published fragment screen against a DBL. The Maybridge library has previously been shown to be nearly free of promiscuous compounds.¹⁶⁰ With our orthogonal screening¹⁴⁰ approach, with both a biochemical and a biophysical screening method, we were confident in our hits. Then X-ray crystallography confirmed that our top hits bound in the active site of OXA-48. Using the alternate conformations from a single crystal structure turned into an example of single compound fragment linking. We were able to generate an interesting focused fragment library of 49 compounds probing the pockets more thoroughly than previously in Paper III. We designed five disubstituted inhibitors to validate the observed conformations for the monosubstituted fragments, and were able to reproduce the binding pose for two of the three we obtained crystal structures of. We believe that the structural information from our 41 protein-ligand complexes in Paper II and III will be useful for future projects developing inhibitors for OXA-48 and related enzymes. The interactions with Arg214 appear to be beneficial for ligand binding, and the structures indicate that there is a buried pocket in the vicinity of Arg214 that the compounds may be expanded to target. Both amides and tetrazoles showed interactions with Arg214, and it could be interesting to test a more acidic functional group to increase the strength of the interaction with the positively charged Arg214.

In retrospect, it is tempting to think that a bigger focus on diversity would have been good in Paper II. The best hits we obtained in Paper II all shared a common scaffold. A more diverse starting library could perhaps have revealed other scaffolds. Looking at the natural substrates of

Inhibitor design process for OXA-48

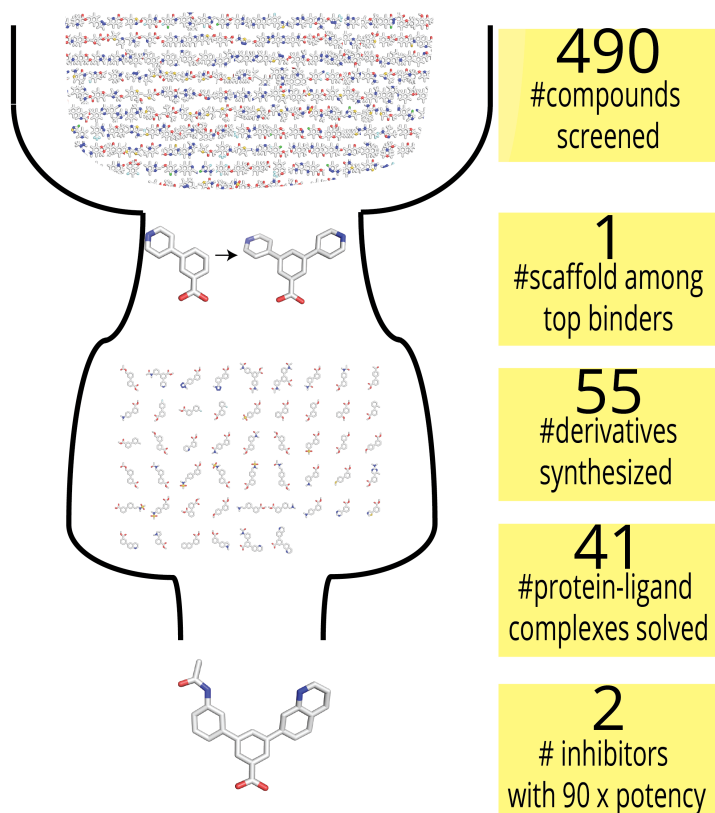


Figure 5.5. We screened a library of 490 fragments, and ended up with 10 hits with IC_{50} -values below 1 mM. The best hits with IC_{50} -values of 250 μ M shared a 3-benzoic acid scaffold, and one of these compounds showed alternate conformations. Merging the two conformations yielded 3,5-Di(4-pyridinyl)benzoic acid which had an IC_{50} -value of 18 μ M. To optimize the substitutions of the benzoic acid scaffold a focused fragment library was synthesized. The best compounds showed a IC_{50} -value of 2.9 μ M, a 90 \times improvement in IC_{50} .

OXA-48, there are several ring systems. Most of the substrates have non-aromatic rings, whereas our fragments have aromatic rings. The stiffness and hydrophobicity of aromatic rings together with the negative charge could make transport into gram-negative cells more challenging.^{10,188,189}

5.5.1 Anthraquinone dyes as inhibitors of OXA-48

Anthraquinone dyes such as Cibacron Blue have previously been shown to inhibit OXA-1 and OXA-2.^{87,190} We repeated chromatography-experiments that were performed previously with a semi-pure fraction of OXA-48. Our experiment with Blue Sepharose chromatography¹⁹⁰ showed that OXA-48 indeed binds to Cibacron Blue (the immobilized dye) with high affinity and that OXA-48 may be eluted by the addition of an antibiotic, in our case ampicillin (Figure 5.6A). A 1.0 M wash with NaCl was useful to elute strongly bound enzymes. This observation together with the previous results for OXA-1 and OXA-2 indicates that the binding of Cibacron Blue is localized to the active site of DBLs. To quantify the strength of the interaction we purchased the semi-pure dye and purified it with thin layer chromatography (TLC). An IC_{50} -value of 30 μ M was determined for Cibacron Blue 3GA in competition with nitrocefin against OXA-48 (Figure 5.6B) as described in Paper II and Paper III. However, Cibacron Blue 3GA showed a K_d -value > 1000 μ M in the SPR-assay (Figure 5.6C) indicating that at least parts of the binding to OXA-48 are unspecific and/or detergent sensitive. The SPR experiment could be repeated with higher concentrations of the dye, however, bulk effects limits the maximum concentration.

The molecular structure of Cibacron Blue shows multiple sulfonate-groups that may bind to either Arg250 or Arg214, as well as multiple aromatic rings that could form $\pi - \pi$ interactions with Tyr211 or Trp105 as we have observed for our compounds in paper II and III. However, we were unable to obtain crystal structures of OXA-48 in complex with Cibacron Blue, even in co-crystallization experiments. The docked pose (Figure 5.6D) is partially overlapping with our experimentally determined binding poses for 3-benzoic acid derivatives from paper II and III.

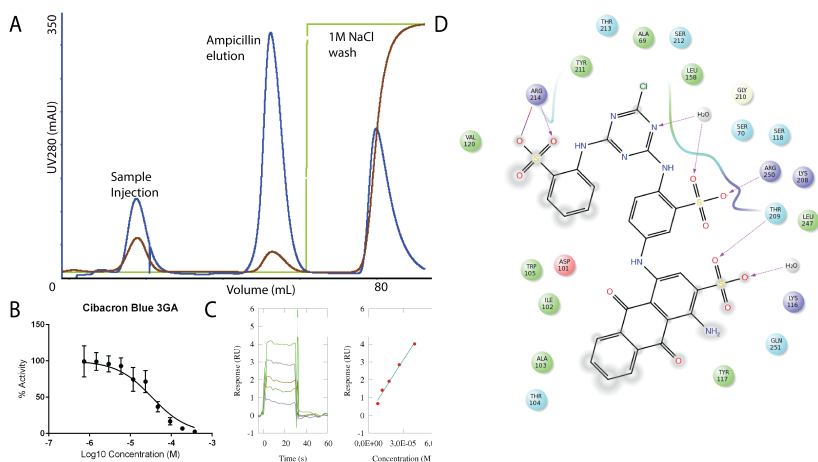


Figure 5.6. (A) Chromatogram for OXA-48 on Blue Sepharose column material showing that the majority of the loaded sample bound and eluted with injection of ampicillin as previously shown for OXA-2.¹⁹⁰ The brown line represents the conductivity of the buffer, the blue line the absorbance at 280 nm and the green line the concentration of buffer B containing 1 M NaCl. Buffer B was used for washing the column. Our biochemical inhibition assay with nitrocefin determined a 30 μM IC_{50} -value (B), in contrast SPR sensorgrams and steady-state plots shows that Cibacrom Blue 3GA has a K_d -value > 1000 μM (C). The docked pose of Cibacron Blue 3GA to OXA-48 shows ionic bonds to Arg214 and Arg250 as well as a hydrogen bond to Thr209 (D).

5.5.2 Membrane-penetration strategies

Several attempts were made to test our inhibitors against bacterial cells in synergy with β -lactam antibiotics. We cloned OXA-48 into a BL-free vector and transformed into a *E. coli* strain without BLs. We tested the most potent compounds against the chromogenic substrate nitrocefin in a spectroscopic assay and we evaluated compounds in a MIC-synergy assay with piperacillin. We also evaluated some of the most potent compounds, compound **26a**, **35** and **36** from paper III, against a clinical strain of *E. coli* carrying OXA-48 in a MIC-synergy assay with meropenem. The results were non-significant or negative in all cases, even though the concentrations (tested both at 100 and 1000 μ M) of the compounds should have been sufficient to saturate the binding site of OXA-48 based on our IC₅₀ results.

One could speculate that the compounds tested did not penetrate the gram-negative cell wall. Although, we did not actually test this hypothesis. Redoing the experiments with sublethal concentrations of Polymyxin B nonapeptide, or a similar membrane disrupting antibiotic could potentially disrupt the outer cell membrane enough for the compounds to enter.¹⁹¹ Many compounds enter bacteria exclusively through porins,¹⁰ perhaps such a strategy would be applicable for our compounds. For example, siderophores have been proposed as a Trojan horse to trick bacteria into taking up compounds.⁹⁶ The pharmaceutical companies Shionogi and GlaxoSmithKline have a joint discovery program on siderophores for β -lactam antibiotics, and their lead compound S-649266, a cephalosporin with an added catechol, have completed Phase 2 clinical studies.⁹² Based on our crystal structures, a hypothetical 3,5 disubstituted benzoic acid derivate with a catechol (as shown in Figure 5.7) in one of the positions might increase the transport into the gram negative bacteria. However, this is not a new idea, and bacteria may develop resistance to such an approach by mutations to the transport enzymes.¹⁹²

Investigating bioisosteres of the carboxylate-group could also be beneficial for transport over the gram-negative cell wall, as the negatively charged carboxylate prevents passive diffusion through the hydrophobic lipid bilayer.¹⁹³ If our compounds were able to enter the cells, there is still the possibility that efflux pumps actively export the compounds out of the

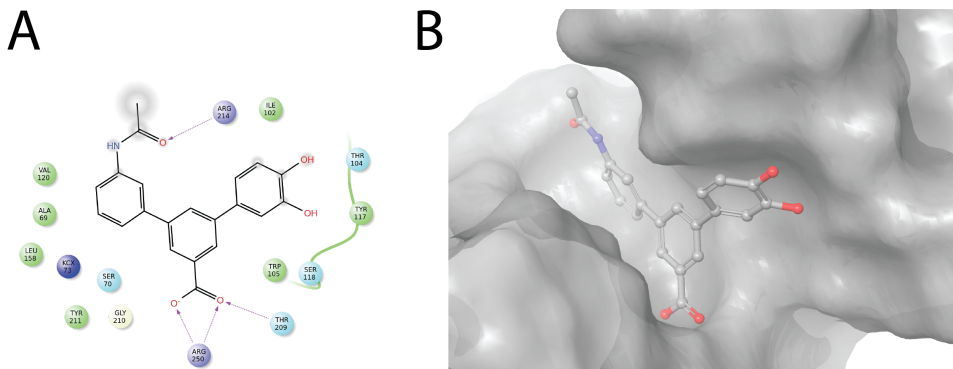


Figure 5.7. A docked structure with a hypothetical 3,5 disubstituted benzoic acid derivate with an added catechol-moiety that might help transport the compound into the gram negative cell showing (A) the interactions with OXA-48 in 2D and (B) the overall docking pose with OXA-48 in 3D.

cell.^{5,194} For this reason it would be interesting to evaluate efflux-pump deficient strains, for example a strain lacking TolC. TolC is a channel protein that participates in a larger pump complex, and strains with a deletion of TolC have been shown to be more sensitive to antibiotic compounds.¹⁹⁴

5.5.3 Druggability

There do not appear to be any other inhibitor projects against DBLs without a covalent warhead.^{187,195} A concern of covalent inhibitors is that they might not be as specific, as it might react with other activated serine residues.¹⁸⁷ It may be that the binding pocket is too shallow and exposed for non-covalent inhibitors to achieve high enough potency to show effect *in vivo*. In support of this hypothesis we have the results from the WaterMap-analysis (Figure 5.8) which shows that the active site is filled with water molecules that form favorable interactions with the enzyme.¹⁹⁶ Any potential inhibitor will have to compete with these waters, and water is present at 55 M in solution. There are some unfavorable water sites that might be exploitable, but overall the WaterMap-analysis suggest that the druggability of the OXA-48 active site is low.¹⁹⁷ However, a better approach to evaluate ligandability/druggability is to screen for compounds. If compounds do bind and show effect, the enzyme is druggable.^{194,198}

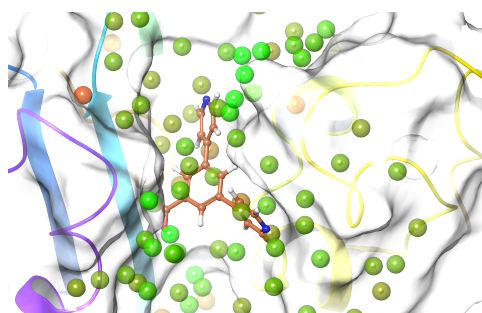


Figure 5.8. A watermap analysis of OXA-48. A 2 ns molecular dynamics (MD) simulation with restraints on the protein with solvation of the volume surrounding the active site. The analysis clusters water molecules and scores each water cluster by occupancy and interactions. Red waters bind in a disordered fashion, with favorable entropy. It is beneficial for ligands to displace these. Green waters bind specifically and in an ordered manner. Displacing these waters will be unfavourable unless the same interactions are satisfied with the ligand atoms.¹⁹⁶ It might be better to interact with these stable waters, using them as bridging waters. In the case of OXA-48 we see that the active site is filled with green waters, indicating that the druggability of the enzyme is low.

Chapter 6

Conclusions and perspectives on the future

We developed an improved methodology for RF- and EMP-cloning, by using negative selection with the *ccdB*-toxin system of Gateway cloning vectors. Our improvement lowers the hands-on time and removes the need for the Dpn1 restriction enzyme to remove paternal DNA.

We have contributed significantly to the knowledge on OXA-48 like DBLs with crystal structures of OXA-181 and OXA-245, and enzyme kinetic data on OXA-436. We also found a new crystal form of OXA-163 and characterized several point-mutants of OXA-48. Overall the mutations in the OXA-48-like enzymes appear to be tolerated well, and we were not able to identify any significant change in substrate preference or thermostability. Our active site mutants significantly lowered the activity of the enzyme. We determined the thermodynamic properties of the hydrolysis of imipenem, and hope to finish the EVB-calculations in the future to validate a reaction mechanism hypothesis for OXA-48.

We have shown that OXA-48 forms tight binding dimers, stronger than what has been reported previously for any DBL. Our R206A dimer, which we designed to disrupt the dimer site still had a K_d -value of 700 pM.

We performed the first published fragment screen against a DBL and established a SPR-assay shown to give reproducible results. With a comprehensive focused fragment library we have probed the active site of OXA-48 in search of new inhibitors. Over 40 crystal structures were determined

with different compounds in complex with OXA-48. The compounds sampled several different conformations within the active site with non-covalent interactions. We were able to use the structural information to design disubstituted benzoic acid derivatives, and our best disubstituted compounds had an IC_{50} -value of 3 μ M. While we could not show activity in cells, the data we have generated will certainly be valuable for new endeavours towards inhibitors of OXAs. It would be very interesting to evaluate different covalent warheads to gain higher potency against OXA-48. There has been several reports of resistance against ceftazidime-avibactam, and there is no reason to think that the DBLs will not develop resistance. When this happens, it is important to have new inhibitors based on a different scaffold in the pipeline.

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