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Functionality of TEM β-lactamases

A computational study of inhibitor interactions with TEM-1 and TEM-1 mutants

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Abstract

Antibiotic resistance is one of the biggest threats to global health today. Although extensive studies regarding β-lactamases and β-lactamase inhibitors have been performed in the past, the knowledge regarding detailed molecular interactions is still limited. This project aims to use computational chemistry to further understand the interactions between a set of inhibitors and TEM-1 as well as TEM-1 mutants. Molecular docking and molecular dynamic (MD) simulations were performed for Avibactam, Sulbactam, Tazobactam, Clavulanic acid as well as Penicillin G in TEM-1 (PDB 3GMW). Binding free energy calculations were performed with the linear interaction energy (LIE) method. Based on statistical and trajectory analysis of MD simulations, important amino acid residues in the active site of TEM-1 could be identified. Ligand interaction with Lys234, Ser70 and Ala237 are responsible for ligand duration, while Ser130, Lys73 and Arg243 are responsible for ligand rotation and position. A proposed defense mechanism caused by Arg243 can also be observed. Binding free energies were calculated for Avibactam, Sulbactam and Tazobactam with values of 1.78, 2.14 and 6.6 kcal/mol respectively. The functional effects of K234A mutant were examined through MD simulations, in hopes of understanding the interactional differences between inhibitors and β-lactam antibiotics. The simulations showed a decreased ligand stability and duration. However, instances where the ligand remained indicates a linked interaction network with compensational properties.

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Populärvetenskaplig sammanfattning

Bakterier är orsaken till hundratals olika sjukdomar som drabbat mänskligheten historiskt, men även idag. Behandlingen för denna typ av sjukdomar sker med hjälp av antibiotika och har under århundraden varit begränsad och riskfylld vilket har resulterat i en mängd dödsfall. Det var först år 1928 som upptäckten av penicillin gav modern medicin en möjlighet att riskfritt bota bakterieinducerade sjukdomar. Denna tids begränsade restriktioner och uppmanade användning av penicillin ledde till att bakterierna utvecklade ett försvar, och blev resistenta. Detta motstånd mot antibiotika har existerat under lång tid, men har vuxit avsevärt under de senaste årtionden. Världshälsoorganisationen (WHO) rapporterade 2019 att över en miljon dödsfall är en direkt konsekvens av antibiotikaresistens. Utöver detta leder även den växande resistensen till ökad vårdkostnad och sjukhusvistelse för patienter.

Antibiotikaresistensen uppkommer från bakteriens olika sätt att försvara sig mot antibiotika. En av dessa försvarsmekanismer sker med hjälp av enzymer. Enzymer är proteiner som har möjlighet att skynda på diverse kemiska reaktioner och är essentiella för alla biologiska varelser. Inuti bakterien finns de specifika enzymer som ansvarar för att bryta ner antibiotika innan den hinner utöva sin effekt. Detta sker genom att antibiotikan sätter sig i en öppen bindingsfickan i enzymet, likt en nyckel i ett lås. Det har under en lång tid varit en stor kamp mellan utvecklingen av nya läkemedel och bakteriernas försvar. Detta är till stor del på grund av bakteriers förmåga att anpassa sig via mutationer. Uppbyggnaden av dessa enzymer har förändrats genom åren för att anpassa sig till nya typer av antibiotika. Den senaste upptäckten för att bekämpa bakterierna är hämmare av de tidigare nämnda enzymerna. Hämmarna verkar genom att blockera enzymet från att kunna utöva sin primära effekt. Detta är möjligt eftersom hämmare ofta strukturellt efterliknar antibiotika, utan att själva kunna brytas ned.

Att få en djupare förståelse för hur dessa hämmare interagerar med enzymet på en detaljerad nivå är kritiskt för att effektivt kunna utveckla framtidens läkemedel mot bakterier. Det är även relevant att försöka få en inblick i hur enzymerna kan tänkas utvecklas, vilket kan leda till att förebyggande åtgärder kan tas. I denna studie används datorbaserade simuleringar för att undersöka interaktionerna i bindingsfickan på en molekylär nivå. Simuleringarna är utformade för att efterlikna en biologisk miljö och ger information som inte kan observeras i en laborativ miljö. Simuleringarna utförs dynamiskt, vilket innebär att både hämmaren och enzymets rörelse tas hänsyn till. Även potentiella evolutionära förändringar undersöks samt vilken effekt de har på interaktionen mellan enzym och hämmare.

Resultatet från dessa simuleringar visade på en rad nyckelinteraktioner som kunde identifieras. Dessa är interaktioner med specifika segment av enzymet, och noggrann analys av simuleringar kan ge förslag på möjliga funktioner av respektive segment. De primära segmenten som ansvarar för varaktigheten och positionen av hämmare i bindingsfickan har identifierats. Utöver detta har ett segment som ansvarar för avlägsnandet av hämmaren kunnat pekas ut. Baserat på datorsimuleringarna har även styrkan av interaktionen mellan hämmare och enzym kunnat beräknas med hjälp av kemiska beräkningsmetoder.

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1. Introduction

1.1 Background

As of today, an estimate of 700,000 deaths per year occurs as a direct result of resistant bacteria and the number is increasing every year. [1] The concept of resistant bacteria is not new. However, due to the increased usage and limited restrictions of antibiotics the resistance has vastly increased over the last decades. The overuse, in addition to bacteria's relative ease to spread resistance also resulted in multidrug resistant (MDR) strands of bacteria. [2] The overall increase in antibiotic resistance is one of the biggest threats to global health. A wide array of infections become harder to treat due to less effective treatments resulting in an increase in mortality, medical costs, and hospital stays. [3]

Antibiotics have been used since early 1900s to treat a wide array of infections. During the last hundred years multiple different antibiotics have been discovered and developed, working in various ways. The main mechanisms of actions include inhibition of the bacterial cell wall synthesis, nucleic acid synthesis as well as protein synthesis. [4,5] The most commonly used group of antibiotics are the β-lactams. As the name suggests they consists of a β-lactam ring which is crucial for the mechanism. Bacteria cell walls consist of peptidoglycan (PGN) which is a polymer from sugar and amino acids. A specific segment of the peptide (D-Ala-D-Ala) is specifically important as the dipeptide addition is the last step of the PGN synthesis. [5] To achieve stability PGN undergoes cross-linking, catalyzed by transpeptidase which interacts with the D-Ala-D-Ala segment. β-lactam antibiotics binds to transpeptidase, through the carbonyl segment of the β-lactam ring, by mimicking the D-Ala-D-Ala portion. The irreversible inhibition of the enzyme hinders the construction of the cell wall and results in cell lysis. [6]

The growing problem revolving around antibiotic resistance is mainly due to the various ways the bacteria can defend itself. These defense mechanisms can target the drugs transportation, alter the target structure, or interact with the drug itself. [3] β-lactamases are enzymes that interact and break down β-lactam antibiotics. The enzyme catalyzes the hydrolysis of the β-lactam structure rendering the antibiotic ineffective. [5] The existence of these enzymes was identified as early as the first antibiotic and has since then undergone various structural changes for adaptability purposes, which have been closely investigated in the past couple of decades. [7] β-lactamases can be categorized into four different classes (A, B, C, D) depending on structural properties and mechanism of action. Class B utilizes metal cations to catalyze the hydrolysis. Classes A, C, and D are referred to as serine class β-lactamase due to the role the reactive serine residue in the active site plays in the mechanism. [8]

TEM β-lactamases belong to class A and are one of the most researched groups of β-lactamases. TEM-1 was the first variant to be identified and has since then been heavily researched. Hundreds of sequential and structural alterations have been identified and the differential impacts have been mapped. Despite this profound research, a deeper insight regarding molecular interactions is still lacking. [9] The general mechanism of class A β-lactamases and the involved amino acid residues have been proposed numerous times in literature. [8] The leading theory is that the hydrolysis is performed by a two-step acylation-deacylation reaction. Ser70 is activated through base mediation. This activation is often caused by Lys73 acting as a base, resulting in Ser70 as a nucleophile. Through a nucleophilic attack on the carbonyl carbon in the β-lactam ring an acyl-enzyme intermediate is formed. The deacylating water performs a second nucleophilic attack, resulting in the hydrolyzed β-lactam product. [10]

To compete with antibiotic resistant bacteria, β-lactamase inhibitors were developed and are today co-administrated with β-lactam antibiotics. [11] Comparatively to the different classes of β-lactamases, the inhibitors act through various mechanisms, and many are still not fully understood. However, the general mechanism consists of imitating structural components of β-lactam antibiotics to irreversibly or reversibly interact with the main motifs in the active site. [12,13] Since the development of Clavulanic acid as an irreversible inhibitor of class A enzymes, a wide variety of inhibitors (shown in figure 1) were developed for combination treatment with different kinds of penicillin. [14] These inhibitors are limited to class A enzymes which also limited the clinical use. At the same time, imperceptible β-lactamases increased, and the need for better treatment options emerged. The most important advancement were the diazobicyclooctanones of which Avibactam was the first to reach clinical use. Avibactam is a non-β-lactam β-lactamase inhibitor, which is able to acylate Ser70 in the active site in a reversible manner. [15] Over the years, the affinity of these inhibitors towards TEM-1 have been determined by various in vitro studies, with IC_{50}, K_I and MIC values. [16,17]

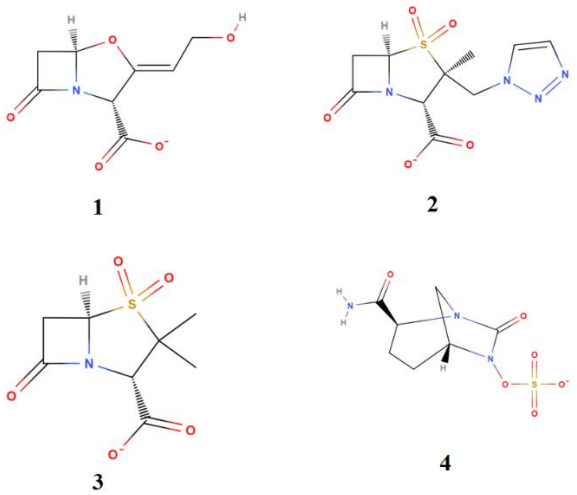

Figure 1. β-lactamase inhibitors in clinical use. 1) Clavulanic acid 2) Tazobactam 3) Sulbactam 4) Avibactam

The rate at which bacteria are able to evolutionary mutate in order to adapt to humanity's medical advancements results in an everlasting competition. Historically, many different mutations and their impact have been shown regarding TEM-1. A mutation of three amino acids caused TEM-1 to evolve to TEM-52 which resulted in resistance towards 2nd generation antibiotics. [18] In a similar manner there is no reason to doubt that bacteria will also obtain resistance towards inhibitors. TEM have been vastly studied with respect to sequence alterations and their impact on resistance development. Although this resistance is limited towards 1st and 2nd generation antibiotics, TEM can be used to research future evolutionary response and preventive actions. Preparation for this response will not only grant more information regarding the inner workings of bacterial resistance but also potentially save money, time, and lives in future drug development.

1.2 Aim

The aim of this thesis is to use computational chemistry to further understand the molecular interactions between a set of inhibitors and TEM-1 β-lactamase. Certain mutations will also be investigated to understand the mutational effects on protein functionality in hopes of preventing evolutionary response in future drug design.

2. Experimental

2.1 Computational chemistry

Over the last couple of years, computational chemistry has gained a dramatic increase in applicability. Due to the rapid increase of computational power, various methods allow for investigation of structural and functional properties of biomolecules such as proteins. The traditional computational methods are based on quantum mechanics with the subsequent addition of molecular mechanics (MM). MM are based on more traditional mechanics where each atom is represented by a sphere with varying mass, charge, and radius. These atoms later form molecules using potentials to unite them. The potential energies can be divided into a bonding term and a non-bonding term. The bonding term accounts for bond lengths, bond angles and torsion angles, while the non-bonding term accounts for van der Waals and electrostatic interactions. The combination of these terms and respective parameters is summarized in equations referred to as molecular force fields. [19]

Virtual screening methods based on MM are consistently used in order to perceive ligand-protein interaction. A commonly used method is molecular docking which identifies and generates the best ligand-protein binding mode. This is done by implementing a search method and scoring functions. The search methods are described as docking algorithms and can be either systematic or stochastic, which introduces a deterministic and random element respectively. There are multiple scoring functions available based on various force fields and grants an internal ranking of the generated binding poses. For molecular docking to be possible certain requirements are put on the ligand and docking target. During the docking process, ligands are treated as flexible. To account for this, the ligand structure is prepared to generate the correct conformation and state at relevant pH. The docking target is often obtained through a crystal structure and needs to be prepared. This preparation includes addition of missing hydrogens, removal of excess protein chains as well as generation of the correct heteroatomic states. [20]

Choosing a relevant ligand to the protein target is essential to evaluate the results. When using TEM-1 as a protein target there are many suitable ligands that can be used. Although various types of β-lactamase inhibitors are the primary ligands used, a β-lactam antibiotic will also be investigated in this study. Penicillin G has shown great affinity for TEM-1 and can be used to observe relevant interactions responsible for the enzymes function. Including Penicillin G as a ligand also highlights any interaction differences between β-lactam antibiotics and β-lactamase inhibitors. Clavulanic acid, Sulbactam and Tazobactam are all inhibitors with similar structure. Clavulanic acid is originally an antibiotic containing a β-lactam ring. However, it differentiates from Penicillin G in the second ring structure being an oxazolidine instead of a thiazolidine. The result is an interaction with the active site without enabling enzymatic activity. Sulbactam and Tazobactam are semi-synthetic inhibitors based on Clavulanic acid, both of which irreversible binds to TEM-1 at the active site, effectively inhibiting it. Avibactam is considered an azabicycloalkane and does not contain a β-lactam ring as opposed to the previously mentioned inhibitors. As a result, the mechanism of inhibition is different, and consists of a reversible acylation in the active site. [21] By including all of these as ligands, a deeper understanding regarding vital interactions can be obtained.

Although molecular docking provides an input in ligand-protein interactions, these only consider single ligand poses. Due to proteins being flexible, the dynamic often plays a crucial role in the functionality. Molecular dynamics (MD) can generate a more overall view of the stability, functionality as well as key ligand interactions. MD can be used to simulate time-

based movements of molecules under thermal fluctuations which more realistically resembles a biological environment. The atom and molecule representations follow that of MM mentioned above, making it possible to calculate potential energies as an ensemble over the timespan. [22] The motion of the molecules follows Newtons law of motion, with acceleration for each atom following Newtons 2nd law. [19] To further resemble a biological environment a solvation model with polar solvent is applied, in addition to a boundary condition. The boundary condition ensures a physically realistic system, and for homogenous systems a periodic boundary condition (PBC) is traditional. Various software can utilize various boundary conditions and when calculating binding affinities an accurate representation of the symmetrical solvent field is important. The implementation of spherical boundary conditions (SBC) proves advantageous compared to the traditional PBC in these situations. Positional restraints are applied at the boundaries, and the simulation occurs in the center of the sphere in the active site. In this part of the sphere there are no restraints, allowing for free movement. The middle layer is partly constrained to maintain solvent density in the system. The solvent molecules are conditioned by a polarization restraint according to the surface constraint all atom solvent (SCAAS) model in order to replicate the behavior of an infinite system. [23]

There are multiple methods available for estimating binding affinities between ligands and proteins based on free energy calculations. The linear interaction energy method (LIE) is used to estimate the absolute binding affinities and is based on comparing the ligand interaction energies in bound and free states. This is possible by implementing simulations of the ligand and ligand-protein complex solvated in water. Based on these simulations the averages of polar and non-polar potentials can be used to calculate the free energy from equation 1.

$$\Delta G_{bind,calc} = \alpha \left(\langle U_{l-s}^{vdw} \rangle_{bound} - \langle U_{l-s}^{vdw} \rangle_{free} \right) + \beta \left(\langle U_{l-s}^{el} \rangle_{bound} - \langle U_{l-s}^{el} \rangle_{free} \right) + \gamma \qquad (1)$$

Here $U_{l\text{-}s}$ is the ligand-surroundings sampled potential energies, α is a non-polar scaling factor, β is a polar scaling factor and γ is the offset parameter. α is unique for each ligand-protein complex but has been empirically proved to be used as the constant 0.18 in various systems with great success. β describes the electrostatic polar interactions and is constant for charged ligands with the value of 0.5 according to linear response approximation. γ is a parameter that accounts for scaling differences between experimental and calculated values and is constant for each unique protein. [24] When implementing SBC in the simulation the charge of residues is manually set. Depending on the position of the residues in comparison with the sphere, their charge is set to either on or off. In this case an electrostatic correction term is used to account for any long-range electrostatic interaction between the ligand and the residues that have their charge set to off. The correction term accounts for the formal charge of the residue (q_p), the partial charge of the ligand (q_l), the distance between the two (r_{pl}) and is calculated according to equation 2.

$$\Delta G_{correction} = \sum_p \sum_l \frac{q_p q_l}{\varepsilon r_{pl}} \qquad (2)$$

A method used for calculating differences of binding free energy is free energy perturbation (FEP) and can be used to obtain relative binding affinities. This is achieved by comparing the energies of an initial and final state. For the calculations to converge the difference between the two states is limited. The perturbation is therefore divided into intermediate states with associated potential energy functions. The generated energy functions for each intermediate state are linear combinations of the function representing the final state. The sum of free energy

difference between all intermediate states are then used to generate the total free energy difference between the initial and final state. By considering a primary amino acid residue as the initial state and the mutational alteration as the final state, the mutations effect on binding affinity can be determined. [25]

2.2 Method

Crystal structures of TEM-1 β-lactamase were obtained from RCSB protein data bank. Various crystal structures of TEM-1 were used, including 1BTL, 4RVA, 6APA and 3GMW. [26-29] 1BTL and 3GMW are classified as wildtype TEM-1 where no mutations have been performed. 4RVA and 6APA contain minor mutations for stability purposes. All crystal structures were determined by x-ray diffraction with resolutions varying between 1.44-2.1 Å. All structures were determined in the apo form, meaning no ligand in bound state was included in the structure.

The protein structures were prepared for docking using Schrödinger Maestros protein preparation wizard. Hydrogens were added and heteroatom states were generated at pH 7.0 ± 2.0. The structures were modified by removing excess chains and heteroatoms. In the case for all obtained crystal structures, chain A constitutes TEM-1. Hydrogen bond assignment were optimized, and the energy was minimized using an OPLS4 forcefield. Avibactam, Tazobactam, Sulbactam, Clavulanic acid and Penicillin G were used as ligands. Each ligand structure was prepared from 2D sketch drawn ligands and SDF files obtained from PubChem. Ligands were then prepared with Schrödinger Maestro using the OPLS4 force field while generating possible states at pH 7.0 ± 2.0. In each case the chirality was retained, and the generated clinically relevant conformation was identified for further use.

The automated molecular docking was performed using GLIDE. The receptor grid was generated by using coordinates of C_β of the Ser70 residue with a docking search volume restricted to 10 Å. In the initial phase of docking 5000 poses were kept, with the best 400 being kept for energy minimization. The docking was performed with standard precision using flexible ligand sampling and the best 10 poses per ligand were generated after post docking minimization. The most suited pose for molecular dynamics simulations were chosen based on previously known relevant interactions in addition to relative docking score.

Molecular dynamics simulation was performed using Desmond and the OPLS4 force field. The system setup was done with a TIP3P solvent model. The boundary condition box shape was a truncated octahedron, and the box size was calculated using a 10Å buffer region, with a minimized volume. 5 sodium ions were added to neutralize the system. The simulations were performed with timespans varying from 10 to 200 ns with 1000 frames collected in respective timespan. 10 ns were used for studying ligand protein interaction, while 100 and 200 ns were used while analyzing stability. The ensemble class used was NPT, with a Nose-Hoover chain thermostat method using 1 ps relaxation time at 300K and a Martyna-Tobias Klein barostat method and isotopic coupling with a 2 ps relaxation time at 1 atm pressure. For each ligand-protein complex pose, 5 replicate simulations were performed using the same parameters with randomized initial velocities.

Binding free energies were calculated using the Linear interaction energy method (LIE). This was done by performing MD simulations in Q for the two ligand states, i.e., free and bound. The average interaction energies were extracted from the simulations. One simulation was performed with just water as solvent, and one with the ligand-protein complex. The simulations were performed with the OPLS-2005 force field and SBC. The sphere radius was set to 30Å

with an inner layer radius of 20Å. The sphere was centered at the ligand and all residues in the outer layer were neutralized. The charge in the inner layer was determined to be -7. Residues in the middle layer were neutralized to stabilize the charge. The structural files were prepared in a manner fitting for Q and the simulation was performed. Electrostatic and van der Waals energies were treated separately with different scaling factors. α was set to 0.18 and β was set to 0.5 for all charged ligands.

3. Results and Discussion

3.1 Protein stability

Although multiple crystal structures of TEM-1 are available in various forms, the applicability in MD simulations is uncertain. The two aspects that conditions an analysis of molecular interactions between ligand and protein is the ligand remaining in the active site in addition to a stable protein structure. The ligand duration can be easily observed while the stability of the protein is determined by its convergence during the simulation. This convergence is calculated based on the root-mean-square deviation (RMSD) values for alpha carbons as well as the sidechain residues. Comparing the difference between average RMSD with the standard deviation grants an insight in protein convergence. Side chain residues have a lower chance of converging due to the increased flexibility compared to the main chain, however smaller fluctuations are tolerated as long as the difference does not occur in the active site where it might alter the ligand-protein interactions. Multiple of the crystal structures used failed to fulfill at least one of these conditions. There is a high probability that this is due to the stabilizing mutations that have been performed in the crystal structure. One of the wildtype crystal structures (3GMW) managed to uphold the requirements and is therefore suited for interaction analysis. The apo structure was simulated for 200 ns showing full convergence of the protein during the extended time. Figure 2 shows the protein movement over the course of the simulation, and clearly shows small fluctuations in flexible loops separated from the active site. Due to this the rest of the simulations could be carried out without having to consider the structural stability of the protein.

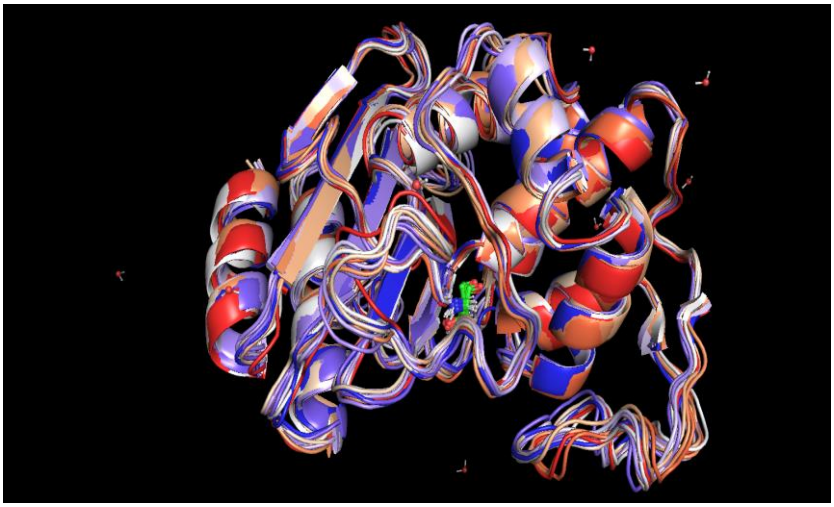

Figure 2. Overlay of snapshots from 200 ns simulation of Avibactam in TEM-1.

3.2 MD simulations

Trajectory analysis of the MD simulation can be used in order to determine relevant interactions between the β-lactamase inhibitors and TEM-1. Since all simulations were performed in

replicates of 5, a minimum of 5000 frames were generated for each ligand. The collective data displays a similar interactive network across all inhibitors built from the same amino acid residues. Common to all 4 inhibitors, the interactions shown are similar to those in the molecular docking results, with slight alterations and difference in frequency, further reinforcing the need for molecular dynamics. Although the main interactions responsible for keeping the ligands in the binding pocket are the same, alterations in occupied positions occur. By identifying the interaction and observing the following positional alterations a deeper insight in the importance and functionality of the residues in the active site can be obtained.

3.2.1 Avibactam

Figure 3 shows the most commonly occurring pose of Avibactam from the 10ns simulations. The interactions showcased can be observed throughout all replicates. The main interactions are formed between Avibactam and residues Ala237, Ser70 and Lys234. Ala237 contributes with a backbone hydrogen bond to the carbonyl oxygen of avibactam. Ser70 tend to form a hydrogen bond to the same carbonyl oxygen through either the backbone or residual hydroxyl group. Lys234 forms a salt bridge with the sulfonate part of Avibactam. Simulation of Avibactam in other crystal structures often resulted in the ligand leaving the active site. In these cases, at least one of these interactions was missing, indicating that these interactions are responsible for ligand stability and keeping the ligand in the binding pocket. In agreement with interaction strength, the hydrogen bonds are alternating while the salt bridge is constant through the entirety of the simulation. Hydrogen bonds to Ala237 and Ser70 are not exclusively present due to the presence of a polar solvent in the system.

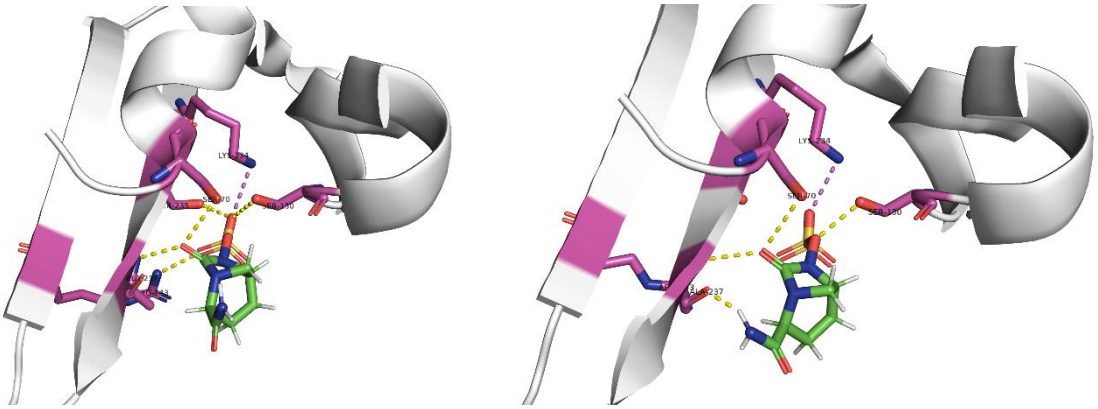

Figure 3. 10ns simulation of avibactam in TEM-1. Snapshot of frame 1 and 400.

In addition to the residues responsible for keeping the ligand in place, there are other residues in the active site that indicates an important role. These are residues that show interaction in conjunction with a rotation or shift in ligand pose. These are rarely caused by a single residue interaction and are often assisted by solvent interactions. However, the main residue responsible for the rotation can in most cases be identified. Figure 4 shows a series of snapshots showcasing the rotation of Avibactam in the active site. The position of O_{12}, which bridges the β-lactam ring and sulfonate, allows for an interaction with Ser130. As a result of this, avibactam is slightly shifted towards the residue which allows for interaction with the carbonyl. This interaction is sometimes assisted by Lys73, and in a solvent assisted motion it allows for a rotation of the ligand. The resulting position is one where the carbonyl carbon is orthogonal relative to Ser70.

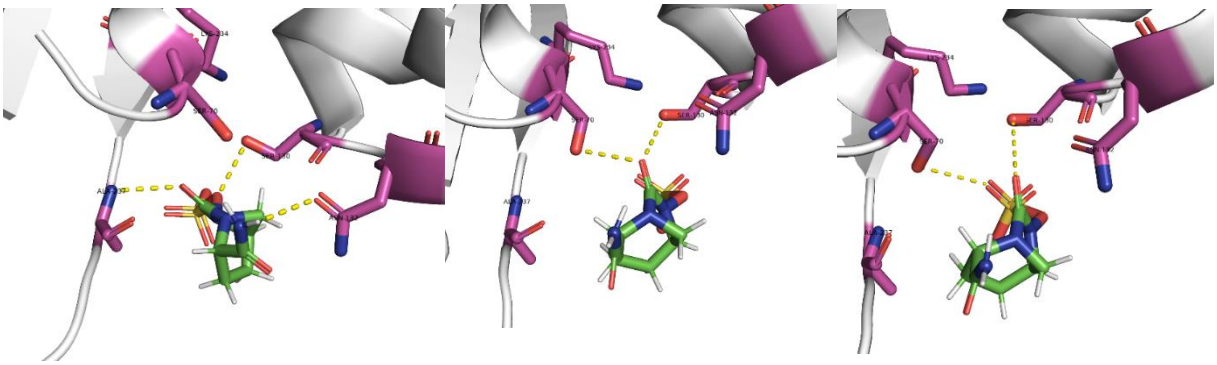

Figure 4. 10ns MD simulation of Avibactam in TEM-1. Series of snapshot showcasing interaction during a rotation.

In a similar manner additional rotations can occur, although more rarely. One of these observed rotations is the result of an interaction with Arg243. Considering all of this, three distinct poses of Avibactam in TEM-1 can be showcased (fig 5). The first pose is the most occupied indicating the highest stability. The second pose occurs after the rotation caused by Ser130.

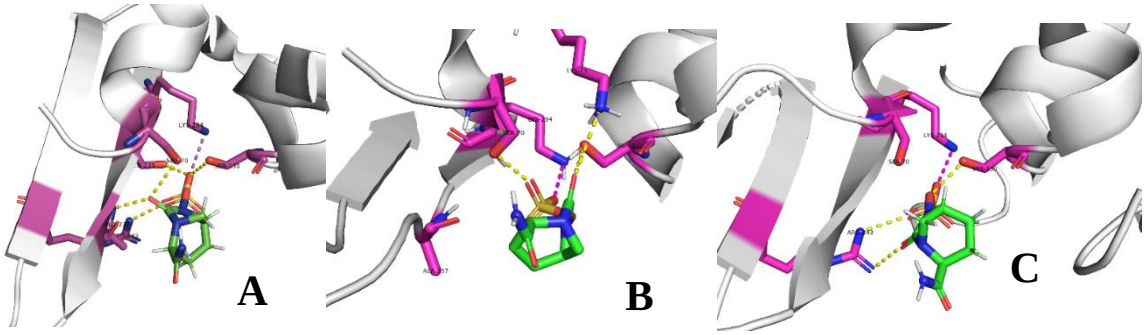

Figure 5. Avibactam poses. A) Interaction with backbone Ala237. Carbonyl positioned parallel with Ser70. B) Interaction with Ser130. Carbonyl positioned orthogonal to Ser70. C) Rotation caused by Arg243. Carbonyl positioned orthogonal to Ser70

The third pose (C, fig5) is commonly the result of an interaction with Arg243. The residue tends to interact either with the oxygen in carbonyl or the sulfonate, replacing existing interactions, causing the rotation. This pose is overall uncommon in the 10ns simulations but occurs more often in the longer 100ns simulation. The 100 ns simulations yield ambiguous results, indicating varying stability of the ligand in the active site. All replicates indicate stability during the first 20 ns where avibactam can be found in one of the three previously described poses. However, after approximately 30 ns Avibactam tends to exit the binding site which limits the stability of the system. In all these cases, avibactam is positioned in the third pose before leaving the pocket. The assumption that this is caused by Arg243 can be made, which is an indication that this residue is part of some defense mechanism against inhibitors.

3.2.2 Sulbactam

Figure 6 shows one of two frequently occurring poses of Sulbactam in TEM-1. The interactions of relevance are comparable to those of Avibactam. The same interactions with Ala237, Ser70,

Lys234 and Ser130 can be observed throughout the simulations. Although there are structural differences between the Sulbactam and Avibactam, the functionality of the groups remains the same. The carbonyl carbon in the β-lactam ring is able to form hydrogen bonds towards the backbone Ala237 and Ser70. The carboxylic acid contributes with a salt bridge in a similar manner as the sulfonate group would. Worth noting is the hydrogen bond shown between the carboxylic acid and Arg243. This interaction occurs when the ligand consists of a carboxylic acid instead of a sulfonate due to the increased reactivity of the functional group. One of the replicates resulted in Sulbactam leaving the active site at the end of the simulation. In this case, the carboxylic acid formed a salt bridge with Arg243, resulting in the ligand being dragged out of the binding pocket. This further strengthens the theorized role of Arg243.

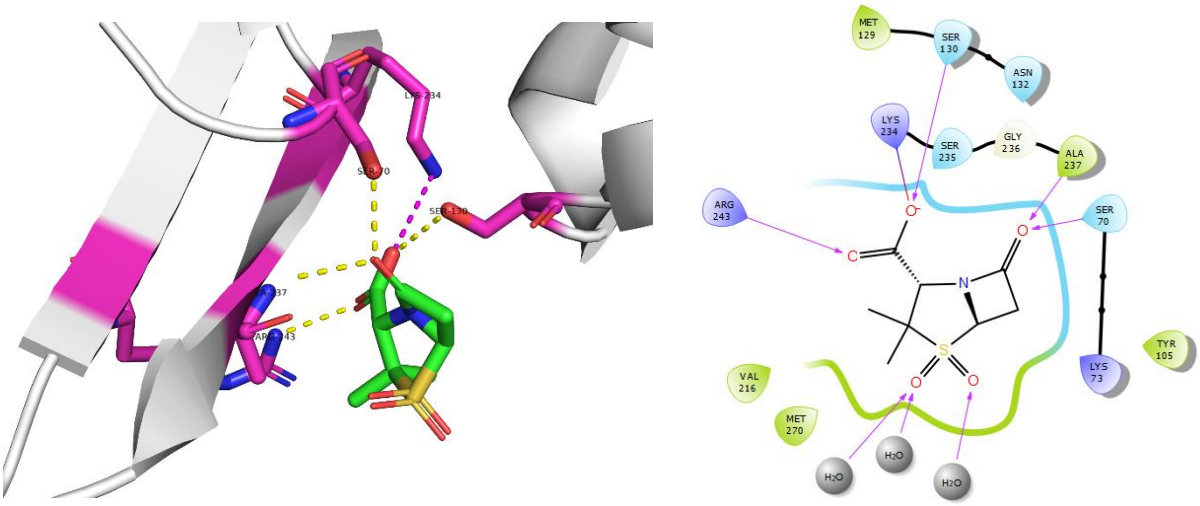

Figure 6. 10 ns simulation of Sulbactam in TEM-1 with interactions shown in 2D sketch.

Figure 7 shows two separate poses of Sulbactam in TEM-1. Based on the five replicates of the simulation, these are the two main poses occurring. Judging by the carbonylic carbon in the β-lactam ring, the positions are comparable to the poses observed in Avibactam. In the same way, the rotation causing the positions can be linked to the interaction with Ser130. The first pose shows backbone interaction with Ala237 resulting in the carbonyl being positioned parallel to Ser70. In the other position, the carbonyl shows interaction with Lys73, and is positioned orthogonal to Ser70.

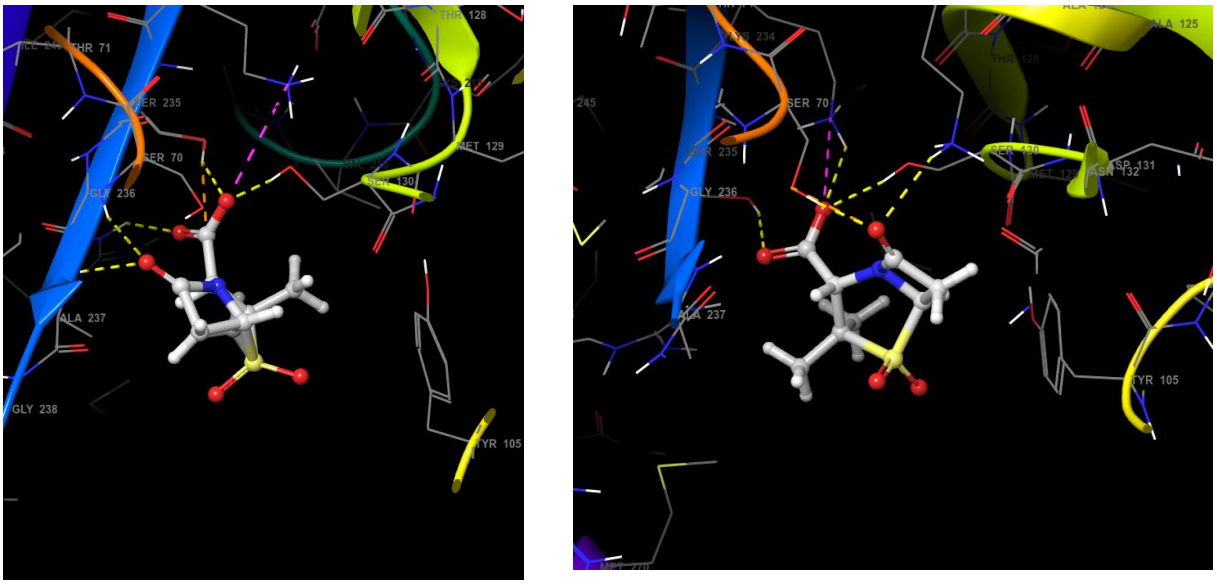

Figure 7. 2 identified poses of Sulbactam in TEM-1.

3.2.3 Tazobactam

In the case of Tazobactam, all the previously discussed interactions can be observed. However, the frequency of certain interaction is far lower and there are greater fluctuations in ligand position compared to the previous inhibitors. Figure 8 shows a plot of RMSD values for the protein and ligand over the course of the simulation. In the case of the previous inhibitors, the ligand RMSD was stable and indicated more distinct poses. In the case of Tazobactam there is more ligand movement which is shown by the RMSD as well as the simulation trajectories. Because of this increased movement, no separate poses can be determined for Tazobactam. Throughout the simulations there is only one constant interaction, being the salt bridge formed to Lys234. The remainder of the interactions are constantly alternating between Ala237, Ser70 and Ser130. Despite this, the ligand always stays in the active site through the entirety of the simulations, indicating that Lys234 is the most important residue for ligand duration.

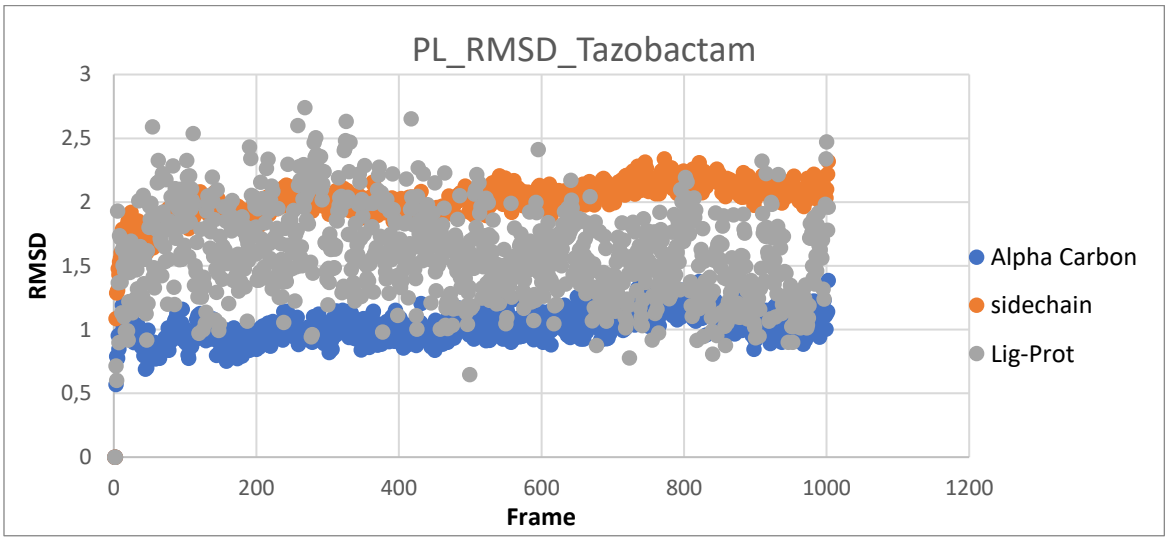

Figure 8. Plot of RMSD values through a 10ns simulation of Tazobactam in TEM-1

3.2.4 Clavulanic acid

When studying the interactions between clavulanic acid and TEM-1 the simulations produced less robust results. The residues interacting with the ligand are the same as for previously tested inhibitors with interactions towards Lys234, Ser70 and Ser130. However, the interacting part of the ligand is different. The carboxylic acid tends to be responsible for all enzyme interactions, including those to Ser70 and Ser130 (shown in figure 9). Throughout the simulations the carbonyl in the β-lactam structure is coordinated with the solvent molecules, which hinder interactions with the active site of TEM-1. Without this interacting segment clavulanic acid does not stabilize, and a higher degree of ligand rotation occurs, which disfavors ligand duration. The instability will also lead to a higher variance between replicates and produce less reliable results. When considering the structural similarity to Sulbactam and Tazobactam, the interactions with TEM-1 are expected to be similar. The most likely explanation for the instability of clavulanic acid is the MD simulation of an incorrect docking pose, and further simulations of other poses are needed for a reliable insight into clavulanic acids interaction with TEM-1.

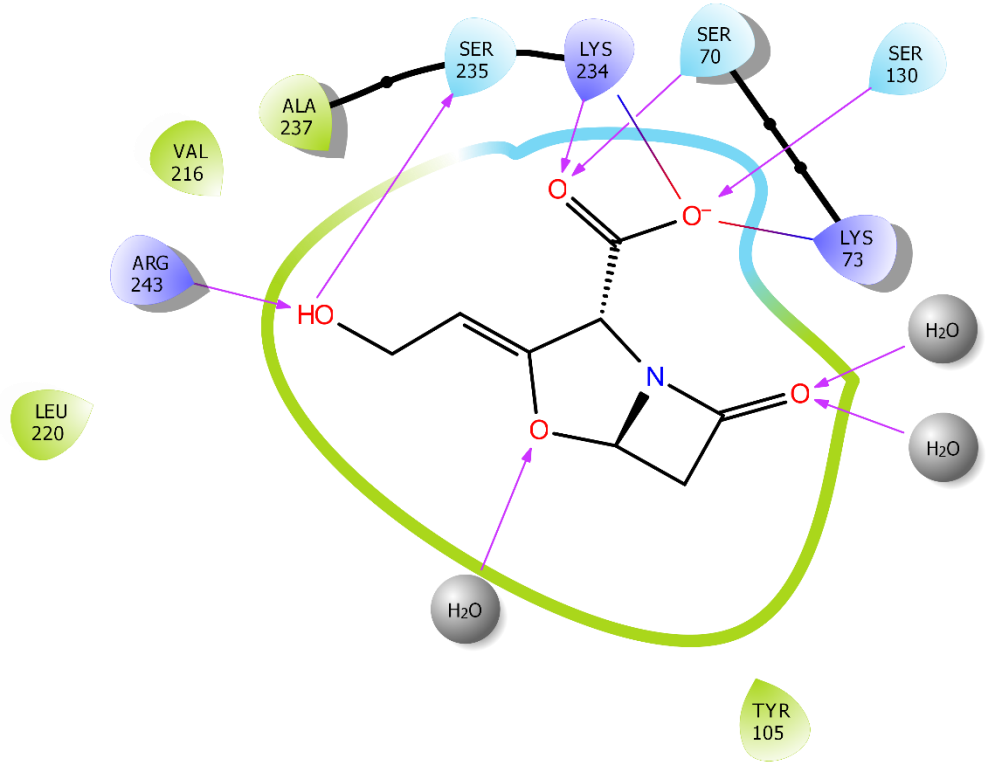

Figure 9. Interactions between Clavulanic acid and TEM-1.

Based on the observed interactions and the following effect on ligand pose there seems to be 3 groups of residues responsible for various operations. The interactions can be seen with all of the inhibitors and the role of each interaction is consistent across all simulations The first group constitutes the residues responsible for keeping the ligand in place and consists of Lys234, Ser70, and Ala237. Interactions with these residues are commonly occurring through each simulation where the ligand remains in the active site. The second group includes residues responsible for ligand rotation and resulting pose and consist of Ser130, Arg243 and Lys73. The varying poses identified for Avibactam and Sulbactam are a direct result of interactions

with these residues. The final group seems to be responsible for the ligand leaving the active site and is so far restricted to Arg243. Although Arg243 also promotes ligand rotation, a direct interaction with Arg243 can be observed in each case prior to the ligand leaving the active site

3.2.5 Penicillin G

Due to the structural similarities between β-lactam antibiotics and previously mentioned inhibitors most of the consequential residues are expected to be the same, which is the overall case. Key interactions with Ser70, Ala237, Ser130, and Lys73 are showcased throughout the simulation. However, the important salt bridge is formed towards another residue. For the inhibitors, Lys234 was responsible for forming this salt bridge which strongly contributed to the overall stability of the ligand position. In the case of penicillin G this salt bridge is instead created between the ligand and Arg243. The significance of this difference is further highlighted when considering the potential functionality of Arg243 in the inhibitor simulations. In the case of Avibactam and Sulbactam, this interactional shift is consequentially followed by a dispatch of the ligand. The reverse functionality of Arg243 for the 2 types of ligands can be used to determine a mutation point. Since the interaction is advantageous for β-lactam antibiotics and observed disadvantageous for inhibitors, the probability of evolutionary change to this residue is unlikely. By shifting focus to Lys234, the remaining salt bridge forming residue, a more realistic mutation point can be found. Removing this possible interaction may result in two different outcomes. Either the inhibitors are unable to stay in the active site and become unstable, or another residue replaces the interaction currently formed to Lys234.

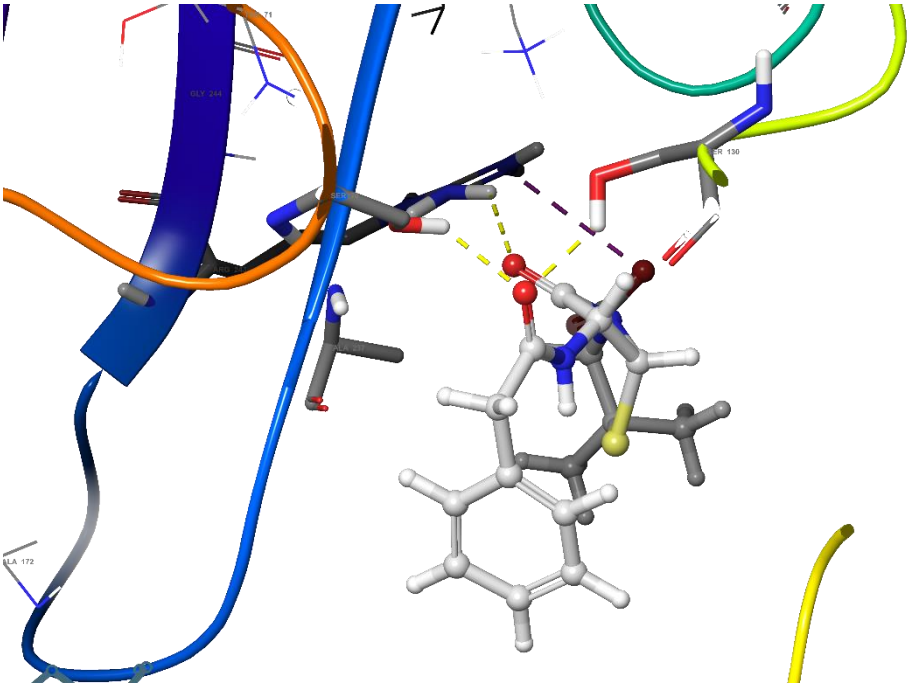

Figure 10. Interaction between Penicillin G and TEM-1.

3.3 Mutation of Lys234

By analyzing the extensive MD simulations performed on the different ligands, various mutation points of interest could be observed. These are residues in the vicinity of the active site suspected to have a functional effect on ligand binding. Considering the purpose and requirements needed for a mutation to prove evolutionary advantageous, Lys234 is one of the most susceptible to mutation. This is due to the most plausible mutation being one that does not impair the binding affinity of β-lactam antibiotics, while promoting the defense against inhibitors. Lys234 was mutated into Ala, to ensure no functional similarities emerged. MD simulation of the K234A mutant were performed with Avibactam. The majority of the simulations generated unstable results with varying ligand duration. By the end of the 10ns simulation Avibactam had exited the active site in all 5 replicates. However, two of the replicates indicate ligand duration for the first 7 ns. Figure 11 shows the occurring interactions in this case. In addition to the expected interactions with Ser70, Ala237 and Ser130, a salt bridge is formed towards Arg243, replacing the previously observed interaction towards Lys234. The decrease in ligand duration as a result of the mutation is in parts expected when considering the previously proven relevance of Lys234. Although the ligand-protein interaction appears to be less stable, the remain of Avibactam indicates that the interaction network is more linked than previously thought. The remaining residues appear to be able to compensate for the missing interaction.

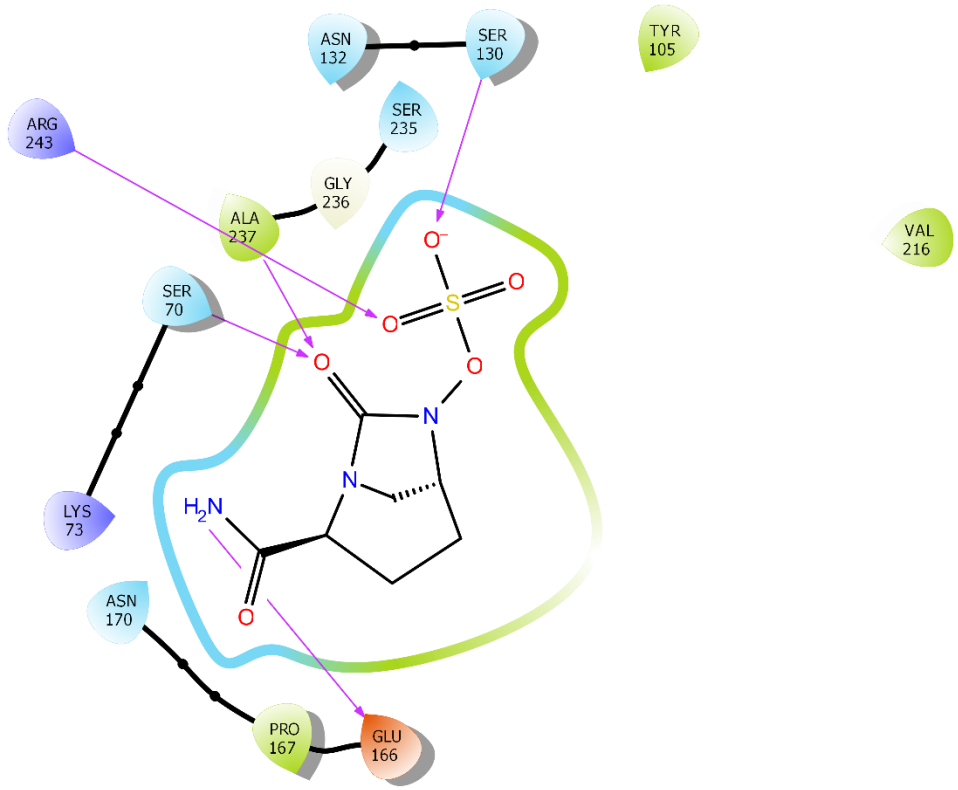

Figure 11. Avibactam interaction with K234A TEM-1 mutant.

3.4 Binding affinity

The binding free energy was calculated using the LIE method. By using a relatively static part of the ligand that is optimally located in the binding pocked, the obtained energies become more comparable across ligands. In order to do so, a stable ligand-protein interaction is needed, with little ligand movement. The instability of clavulanic acid discussed in section 3.2.4, justifies disregarding it when performing LIE calculations. The calculation of binding free energy is therefore limited to Avibactam, Sulbactam and Tazobactam. Total averages of potential energies in free and bound state were obtained in 5 replicates, and the binding free energy was calculated according to equation 1. Table 1 shows the average ΔG_{calc} for each replicate in addition to the average as well as the experimental value.

*Table 1. ΔG_{calc} determined with LIE for each inhibitor. Expressed individually for each replicate in addition to average of all replicates. * Replicate 2 of Avibactam is an outlier and not included in the average value.*

Inhibitor	ΔG_{calc} Replicate 1 (kcal/ mol)	ΔG_{calc} Replicate 2 (kcal/ mol)	ΔG_{calc} Replicate 3 (kcal/ mol)	ΔG_{calc} Replicate 4 (kcal/ mol)	ΔG_{calc} Replicate 5 (kcal/ mol)	ΔG_{calc} Average (kcal/ mol)	ΔG_{exp} (kcal/ mol)
Avibactam	0.84	9.29*	4.65	3.10	-1.47	1.78	-11.68
Sulbactam	5.06	2.52	1.42	-2.28	3.99	2.14	-7.92
Tazobactam	7.22	6.15	5.06	7.06	7.86	6.6	-10.08

The electrostatic correction term was calculated according to equation 2. During the application of SBC, the same residues were neutralized for each inhibitor. The term can therefore be expressed as an average and takes the value of $\Delta G_{corr}^{calc} = -1.13$. γ is a fitting parameter that is determined to achieve the best fitting between calculated and experimental values. Even though this term is specific for each protein regardless of ligand, one singular value is not applicable in this case. The γ term is instead determined individually for each inhibitor with $\gamma_{Avibactam}$ = -11.68, $\gamma_{Sulbactam}$ = -7.92 and $\gamma_{Tazobactam}$ = -15.55. To obtain a more comprehensive γ further calculations originating from one replicate is needed. The reason for this is the variation observed in between replicates. Disregarding the outliers, both Avibactam and Sulbactam shows values in two different ranges. These are due to the different poses the inhibitors can adopt in the binding pocket. In the case of Tazobactam, where no distinct poses were observed, the values are more consistent between replicates.

Due to time constraints, FEP calculation of mutated states have not produced results available for analysis.

4. Conclusion and future work

This study provides a detailed analysis of various ligand-protein interactions, where computational chemistry and MD simulations have been proved successful in granting an insight in the interaction between a set of β-lactamase inhibitors and TEM-1. The most relevant and frequent interactions with Avibactam, Sulbactam, Tazobactam, Clavulanic acid and Penicillin G have been showcased on a molecular level which is hard to observe through other means. In the case of Avibactam and Sulbactam separate distinct ligand poses in TEM-1 have been shown. These are poses with varying tendencies and discrete binding energies.

Three separate groups of amino acid residues in TEM-1 have been identified in accordance with their functional role during ligand interaction. Ligand interaction with these residues have been observed for all simulated ligands, and function is consistent in each case. Residues Lys234, Ser70 and Ala237 constitutes the group responsible for ligand duration. Ser130, Lys73 and Arg243 have been shown to be responsible for rotation and ligand pose. The third group consists of Arg243 and is proposedly responsible for ligand ejection and part of the TEM-1 defense system.

The functional effect of K234A mutation in TEM-1 has been examined. Although Lys234 is vital to ligand duration in the case of inhibitors, trajectory analysis of MD simulations concluded that Avibactam potentially remained in the active site of TEM-1 despite the mutation. Although the ligand-protein interaction appears less stable and the ligand duration is decreased, the remain of Avibactam indicates a highly linked interaction network capable of compensating for altered interactions. However, to fully understand the functional impact of these types of mutations, continued studies and further work is needed.

In future work additional energy calculations of various poses may prove advantageous when considering the reliability of the results. Further LIE calculations based on a satisfactory pose might also grant more consistent results in alignment with the experimental values. In addition, further investigation of the K234A mutant might prove the most important. A complete understanding of the role of Arg243 and Lys234 has the potential to predict a mutational response towards inhibitors, and consequentially aid in preventing bacterial evolutionary defense. A first step towards understanding these residues is through FEP calculations of the K234A mutant which can assist in understanding the interactional differences between inhibitors and β-lactam antibiotics.

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References

[1] Tagliabue, A., & Rappuoli, R. (2018). Changing Priorities in Vaccinology: Antibiotic Resistance Moving to the Top. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.01068

[2] Dadgostar, P. (2019). Antimicrobial Resistance: Implications and Costs. *Infection and Drug Resistance*, *Volume 12*, 3903–3910. https://doi.org/10.2147/idr.s234610

[3] Levy, S. B., & Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine*, *10*(S12), S122–S129. https://doi.org/10.1038/nm1145

[4] Wright, G. (2003). Mechanisms of resistance to antibiotics. *Current Opinion in Chemical Biology*, *7*(5), 563–569. https://doi.org/10.1016/j.cbpa.2003.08.004

[5] Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of Anaesthesiology Clinical Pharmacology*, *33*(3), 300. https://doi.org/10.4103/joacp.joacp_349_15

[6] Lima, L. M., Silva, B. N. M. D., Barbosa, G., & Barreiro, E. J. (2020). β-lactam antibiotics: An overview from a medicinal chemistry perspective. *European Journal of Medicinal Chemistry*, *208*, 112829. https://doi.org/10.1016/j.ejmech.2020.112829

[7] Bush, K. (2018). Past and Present Perspectives on β-Lactamases. *Antimicrobial Agents and Chemotherapy*, *62*(10). https://doi.org/10.1128/aac.01076-18

[8] Majiduddin, F. K., Materon, I. C., & Palzkill, T. G. (2002). Molecular analysis of beta-lactamase structure and function. *International Journal of Medical Microbiology*, *292*(2), 127–137. https://doi.org/10.1078/1438-4221-00198

[9] Salverda, M. L., de Visser, J. A. G., & Barlow, M. (2010). Natural evolution of TEM-1 β-lactamase: experimental reconstruction and clinical relevance. *FEMS Microbiology Reviews*, *34*(6), 1015–1036. https://doi.org/10.1111/j.1574-6976.2010.00222.x

[10] Pemberton, O. A., Noor, R. E., Kumar M. V., V., Sanishvili, R., Kemp, M. T., Kearns, F. L., Woodcock, H. L., Gelis, I., & Chen, Y. (2020). Mechanism of proton transfer in class A β-lactamase catalysis and inhibition by avibactam. *Proceedings of the National Academy of Sciences*, *117*(11), 5818–5825. https://doi.org/10.1073/pnas.1922203117

[11] Drawz, S. M., & Bonomo, R. A. (2010). Three Decades of β-Lactamase Inhibitors. *Clinical Microbiology Reviews*, *23*(1), 160–201. https://doi.org/10.1128/cmr.00037-09

[12] Carcione, D., Siracusa, C., Sulejmani, A., Leoni, V., & Intra, J. (2021). Old and New Beta-Lactamase Inhibitors: Molecular Structure, Mechanism of Action, and Clinical Use. *Antibiotics*, *10*(8), 995. https://doi.org/10.3390/antibiotics10080995

[13] Sulton, D., Pagan-Rodriguez, D., Zhou, X., Liu, Y., Hujer, A. M., Bethel, C. R., Helfand, M. S., Thomson, J. M., Anderson, V. E., Buynak, J. D., Ng, L. M., & Bonomo, R. A. (2005). Clavulanic Acid Inactivation of SHV-1 and the Inhibitor-

resistant S130G SHV-1 β-Lactamase. *Journal of Biological Chemistry*, *280*(42), 35528–35536. https://doi.org/10.1074/jbc.m501251200

[14] White, A. R. (2004). Augmentin(R) (amoxicillin/clavulanate) in the treatment of community-acquired respiratory tract infection: a review of the continuing development of an innovative antimicrobial agent. *Journal of Antimicrobial Chemotherapy*, *53*(90001), 3i–20. https://doi.org/10.1093/jac/dkh050

[15] Ehmann, D. E., Jahić, H., Ross, P. L., Gu, R. F., Hu, J., Kern, G., Walkup, G. K., & Fisher, S. L. (2012). Avibactam is a covalent, reversible, non–β-lactam β-lactamase inhibitor. *Proceedings of the National Academy of Sciences*, *109*(29), 11663–11668. https://doi.org/10.1073/pnas.1205073109

[16] Bonnefoy, A. (2004). In vitro activity of AVE1330A, an innovative broad-spectrum non- -lactam -lactamase inhibitor. *Journal of Antimicrobial Chemotherapy*, *54*(2), 410–417. https://doi.org/10.1093/jac/dkh358

[17] Boussoualim, N., Meziane-Cherif, D., & Baghiani, A. (2011). Kinetic study of different flavonoids as inhibitors of beta-lactamase enzyme. *African Journal of Biochemistry Research*, *5*(10), 321-327.

[18] Poyart, C., Mugnier, P., Quesne, G., Berche, P., & Trieu-Cuot, P. (1998). A Novel Extended-Spectrum TEM-Type β-Lactamase (TEM-52) Associated with Decreased Susceptibility to Moxalactam in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*, *42*(1), 108–113. https://doi.org/10.1128/aac.42.1.108

[19] Huang, N., Kalyanaraman, C., Bernacki, K., & Jacobson, M. P. (2006). Molecular mechanics methods for predicting protein–ligand binding. *Phys. Chem. Chem. Phys.*, *8*(44), 5166–5177. https://doi.org/10.1039/b608269f

[20] Morris G.M., Lim-Wilby M. (2008) Molecular Docking. In: Kukol A. (eds) Molecular Modeling of Proteins. Methods Molecular Biology™, vol 443. Humana Press. https://doi-org.ezproxy.its.uu.se/10.1007/978-1-59745-177-2_19

[21] Carcione, D., Siracusa, C., Sulejmani, A., Leoni, V., & Intra, J. (2021b). Old and New Beta-Lactamase Inhibitors: Molecular Structure, Mechanism of Action, and Clinical Use. *Antibiotics*, *10*(8), 995. https://doi.org/10.3390/antibiotics10080995

[22] Gelpi, J., Hospital, A., Goñi, R., & Orozco, M. (2015). Molecular dynamics simulations: advances and applications. *Advances and Applications in Bioinformatics and Chemistry*, 37. https://doi.org/10.2147/aabc.s70333

[23] King, G., & Warshel, A. (1989). A surface constrained all-atom solvent model for effective simulations of polar solutions. *The Journal of Chemical Physics*, *91*(6), 3647–3661. https://doi.org/10.1063/1.456845

[24] Hansson, T., Marelius, J. & Åqvist, J. (1998). Ligand binding affinity prediction by linear interaction energy methods. *J Comput Aided Mol Des* **12,** 27–35

[25] Jorgensen, W. L., & Thomas, L. L. (2008). Perspective on Free-Energy Perturbation Calculations for Chemical Equilibria. *Journal of Chemical Theory and Computation*, *4*(6), 869–876. https://doi.org/10.1021/ct800011m

[26] Literature Citation:
Jelsch, C., Mourey, L., Masson, J. M., & Samama, J. P. (1993). Crystal structure of Escherichia coli TEM1 β-lactamase at 1.8 Å resolution. *Proteins: Structure, Function, and Genetics*, *16*(4), 364–383. https://doi.org/10.1002/prot.340160406
DOI Citation:
Jelsch, C., Mourey, L., Masson, J.M., Samama, J.P. (1993). Crystal structure of Escherichia coli TEM1 β-lactamase at 1.8 Å resolution.
https://doi.org/10.2210/pdb1btl/pdb

[27] Literature Citation:
Stojanoski, V., Chow, D. C., Hu, L., Sankaran, B., Gilbert, H. F., Prasad, B. V. V., & Palzkill, T. (2015). A Triple Mutant in the Ω-loop of TEM-1 β-Lactamase Changes the Substrate Profile via a Large Conformational Change and an Altered General Base for Catalysis. *Journal of Biological Chemistry*, *290*(16), 10382–10394.
https://doi.org/10.1074/jbc.m114.633438
DOI Citation:
Stojanoski, V., Chow, D.-C., Hu, L., Sankaran, B., Gilbert, H., Prasad, B.V.V., Palzkill, T. (2014). A triple mutant in the omega-loop of TEM-1 beta-lactamase changes the substrate profile via a large conformational change and an altered general base for deacylation. https://doi.org/10.2210/pdb4rva/pdb

[28] Literature Citation:
Roose, B. W., Zemerov, S. D., Wang, Y., Kasimova, M. A., Carnevale, V., & Dmochowski, I. J. (2018). A Structural Basis for ^{129}Xe Hyper-CEST Signal in TEM-1 β-Lactamase. *ChemPhysChem*, *20*(2), 260–267.
https://doi.org/10.1002/cphc.201800624
DOI Citation:
Roose, B.W., Dmochowski, I.J. (2018). A Structural Basis for ^{129}Xe Hyper-CEST Signal in TEM-1 β-Lactamase. https://doi.org/10.2210/pdb6apa/pdb

[29] Literature Citation:
Gretes, M., Lim, D. C., de Castro, L., Jensen, S. E., Kang, S. G., Lee, K. J., & Strynadka, N. C. (2009). Insights into Positive and Negative Requirements for Protein–Protein Interactions by Crystallographic Analysis of the β-Lactamase Inhibitory Proteins BLIP, BLIP-I, and BLP. *Journal of Molecular Biology*, *389*(2), 289–305. https://doi.org/10.1016/j.jmb.2009.03.058
DOI Citation:
Lim, D.C., Gretes, M., Strynadka, N.C.J. (2009) Crystal Structure of Beta-Lactamse Inhibitory Protein-I (BLIP-I) in Complex with TEM-1 Beta-Lactamase
https://doi.org/10.2210/pdb3gmw/pdb